l'université nantes angers le mans
COMMUNAUTÉ D'UNIVERSITÉS ET ÉTABLISSEMENTS

UNIVERSITÉ DE NANTES

Thèse de Doctorat

Michelle YAP

Mémoire présenté en vue de l'obtention du
grade de Docteur de l'Université de Nantes
sous le label de L'Université Nantes Angers Le Mans

École doctorale : *Biologie-Santé*

Discipline : *Médicine*
Spécialité : *Immunologie*
Unité de recherche : *INSERM UMR1064*

Soutenue le *5 Novembre 2015*
Thèse N° : 22

The Characterization of CD8 T Lymphocyte Subset Differentiation and Their Immunometabolic Programming in Kidney Transplantation.

JURY

Rapporteurs :	**Thomas FEHR**, Professeur d'Université, University of Zurich, Zurich, Switzerland **Jacqueline MARVEL,** Directeur de Recherche, INSERM U1111, Lyon, France
Examinateurs :	**Antoine TOUBERT,** Professeur d'Université – Praticien Hospitalier, INSERM U1160, Paris, France
Directeur de Thèse :	**Sophie BROUARD**, Directeur de Recherche, INSERM UMR1064, Nantes, France
Co-directeur de Thèse :	**Nicolas DEGAUQUE**, Chargé de Recherche Titulaire d'une HDR, INSERM UMR1064, Nantes, France

Acknowledgements

I would like to thank Dr. Jacqueline Marvel and Dr. Thomas Fehr for agreeing to be my thesis evaluators and jury members. I would also like to thank Dr. Antoine Toubert for agreeing to serve as a jury member and for all the support and advice he has given as a part of my thesis committee these past three years.

I also want to thank my thesis supervisors, Dr. Nicolas Degauque and Dr. Sophie Brouard. Thank you for all your guidance these past few years and for giving me the opportunity to come to Nantes to be a part of your research team.

I would like to thank Dr. Nathalie Labarriere for her helpful advice and comments that she has given as a part of my thesis committee.

I would like to thank INSERM UMR1064 for allowing me the opportunity to pursue my thesis in Nantes. Thank you to everyone in the laboratory who have help and advised me throughout my thesis. It has been a grand adventure these past 3 years within this laboratory, and I have enjoyed getting to know everyone in the laboratory and exploring this beautiful city. Thank you to everyone for being patient with me when I arrived here with no knowledge of the French language and thank you for helping me learn and improve my (still not very good) French.

I would like to thank Gaëlle Tilly for her contributions to this thesis. Thank you for allowing me to join your immunometabolic project when my own project was not progressing well. Without Gaëlle, the second half of this thesis would have never advanced as quickly nor as successfully as it did.

I would also like to thank Dr. Claire Pecqueur for her collaboration and guidance in the immunometabolic project and review article.

Thank you to Nico and Fabie for opening your home to me when I first arrived here and for helping me get settled. And thank you for your continued help and support these past years.

Thank you to all the friends that I have made these past three years, from both within and outside the laboratory. Thank you to Séverine, who seemed to help me in everything. Thank you for all your help with the ARIA, with translating French, and with this thesis. And thank you for that first invitation to Friday night drinks, which has now turned into a weekly habit. Thank you to Jason, who has proofread and edited my poor French. To Sabrina, Lola, Sebastian, Roberto, Marie, Janina, Alex, Apolline, Gwen, Pierre-Jean, Sarah, Coraline, Julien, Lucile, Lucas, and all, thank you for all the help and support these past few years. Thank you to Gaëlle P., Elodie, Justine, Marion, Annie, Angelique, and Laetitia, who have all moved on to new positions but who were always friendly and helpful when they were a part of the lab. Thank you to Laura, for her help and friendship, and for her experiences here as a foreign thesis student, as her thesis was a model for my own.

Thank you to Jessica and Valerie, my oldest friends, for their friendship and support. Thank you for coming to see me here in France and for our fun trips across Europe.

Thank you my cousin Jade for giving me a small oasis in London when I needed a break.

Finally, thank you to my family for their constant love and support during these three years. Thank you to my father and mother, to Ee Ming and Shawn, and to Ee Seng, for your love and support.

The Characterization of CD8 T Lymphocyte Subset Differentiation and Their Immunometabolic Programming in Kidney Transplantation.

Table of Contents

Acknowledgements ... 2
Table of Contents ... 4
List of Figures ... 6
List of Tables ... 7
Abbreviations ... 8
1 Preface ... 9
2 Introduction ... 12
2.1 Transplantation ... 13
2.1.1 Hyperacute Rejection ... 15
2.1.1.1 Recipient-Donor Matching: ABO Incompatibility ... 15
2.1.1.2 Recipient-Donor Matching: HLA Matching & Crossreactivity ... 17
2.1.2 Acute Rejection ... 22
2.1.2.1 Acute Antibody-Mediated Rejection ... 22
2.1.2.2 Acute T Cell-Mediated Rejection ... 22
2.1.2.2.1 Direct Allorecognition ... 23
2.1.2.2.2 Indirect Allorecognition ... 24
2.1.2.2.3 Semi-direct Allorecognition ... 24
2.1.3 Chronic Rejection ... 25
2.1.3.1 Chronic Antibody-Mediated Rejection ... 26
2.1.3.2 Chronic T Cell-Mediated Rejection ... 26
2.1.4 Biomarkers of Kidney Allograft Injury ... 27
2.1.4.1 Current Established Clinical Biomarkers ... 28
2.1.4.1.1 Serum Creatinine ... 28
2.1.4.1.2 Proteinuria and Albuminuria ... 28
2.1.4.1.3 eGFR ... 29
2.1.4.1.4 Biopsy ... 29
2.1.4.1.5 Immunknow ... 30
2.1.4.2 Biomarker Research ... 31
2.1.4.2.1 Kidney Transplant Failure Score ... 31
2.1.4.2.2 Up and Coming Biomarkers of Kidney Injury ... 33
2.1.4.2.2.1 Transcriptional Biomarkers ... 35
2.1.4.2.2.2 Protein Biomarkers ... 36
2.1.4.2.2.3 Lymphocyte Biomarkers ... 37
2.1.4.2.2.4 Alloantibody Biomarkers ... 37
2.1.4.2.2.5 Urinary Biomarkers ... 38
2.2 CD8 T Lymphocytes ... 41
2.2.1 Naïve Cell Priming Gives Rise to Clonal Expansion of Effector Cells ... 41
2.2.2 Models of Memory Formation ... 43
2.2.3 CD8 Subset Nomenclature ... 45
2.2.4 Contrasting Portrayals of TEMRA Cells ... 46

2.3 Cellular Metabolism 50
2.3.1 Glucose Metabolism 51
2.3.1.1 Glycolysis 51
2.3.1.2 Lactic Acid Fermentation 52
2.3.1.3 The Citric Acid Cycle (TCA) 54
2.3.1.4 Oxidative Phosphorylation and the Electron Transport Chain 55
2.3.2 Alternative Energy Sources 57
2.3.2.1 Glutaminolysis 57
2.3.2.2 Fatty Acid Oxidation 58
2.3.3 CD8 T Cell Metabolic Programming 60
2.3.3.1 Metabolic Demands in Quiescent T Cells 60
2.3.3.2 Activation of CD8 T Cells Leads to Metabolic Reprogramming. 61
2.3.3.3 Metabolism and Immunomodulation 65
2.3.3.3.1 Targeting Bioenergetic Pathways 65
2.3.3.3.2 Targeting Transcriptional Regulators of Immunometabolism 66
3 Results 67
3.1 Thesis Overview 68
3.2 Article 1: Expansion of Highly Differentiated Cytotoxic Terminally Differentiated Effector Memory CD8+ T Cells in a Subset of Clinically Stable Kidney Transplant Recipients: A Potential Marker for Late Graft Dysfunction. 70
3.3 Article 2: The Benefits of Using CD45RA and CD28 to Investigate CD8 Subsets in Kidney Transplant Recipients 87
3.4 Article 3: Inclusion of CD8 Monitoring Improves the Prognostic Capacities of the Kidney Failure Transplant Score 118
3.5 Article 4: TEMRA CD8 T Cells Rapidly Engages a Sustained Glycolytic Switch to Sustain Their Potent Effector Functions 152
4 Discussion 193
4.1 CD8 T Cell Biomarker Research in Transplantation 194
4.2 Metabolic Pathways in CD8 Cell subsets 199
4.2.1 Metabolic Targets for Immunomodulating CD8 T Cells 200
4.2.1.1 Shutting down the Warburg effect in effector CD8 T cells 200
4.2.1.2 Targeting transcriptional regulators of immunometabolism 202
4.2.1.3 Boosting Treg populations with metabolic programming 204
5 References 206
6 Appendix 229
6.1 Summary of Thesis in French 230
6.2 Supplemental Review Article 236
6.3 Supplemental Article 245
6.4 Publications 281
6.5 Orals Presentations 281
6.6 Poster Presentations 282
6.7 Awards 283

List of Figures

Figure 1. Half-lives for adult kidney transplant recipients.

Figure 2. The relative timeline of hyper acute, acute, and chronic rejection in transplant patients.

Figure 3. Key protocol elements of ABOi transplantation.

Figure 4. Allo-HLA crossreactivity by viral-specific memory T cells.

Figure 5. The expansion and contraction of CD8 cells in response to antigen stimulation.

Figure 6. The three pathways of allorecognition.

Figure 7. The calculations for the KTFS.

Figure 8. Comparison of different medical approaches.

Figure 9. Kidney allograft analogous to an '*in vivo* flow cytometer'.

Figure 10. Lymphocytes receive three activation signals.

Figure 11. T cells differentiate into effector memory and central memory cells after an antigen-encounter.

Figure 12. Overview of the metabolic pathways available for energy production.

Figure 13. Overview of Glycolysis.

Figure 14. Overview of Lactic Acid Fermentation.

Figure 15. Overview of the Citric Acid Cycle.

Figure 16. Overview of the Electron Transport Chain.

Figure 17. The Stages of Fatty Acid Oxidation.

Figure 18. Metabolic pathways used by CD8 T cells in quiescent and active states.

Figure 19. Immunometabolic targets.

List of Tables

Table 1. HLA matching protocols for kidney transplantation.

Table 2. Morphology of specific chronic diseases involved in chronic allograft injury (non-rejection).

Table 3. Characteristics of an ideal biomarker.

Table 4. Overview of the equations used to estimate the glomerular filtration rate.

Table 5. The eight clinical parameters included into the calculation of the KTFS.

Table 6. Platforms for biomarker discovery and validation.

Table 7. Overview and characteristics of clinical studies investing the use of NGAL as a biomarker of acute kidney injury, renal replacement therapy, and in-hospital mortality.

Table 8. Phenotype of the different CD8 subsets.

Abbreviations

2-DG: 2-Deoxy-D-glucose
ABMR: Antibody-Mediated Rejection
ABO-i: ABO-incompatible
Acetyl-coA: Acetyl-coenzyme A
ADP: Adenosine Diphosphate
AKT: Protein Kinase B
AMP: Adenosine Monophosphate
AMPK: Adenosine Monophophate-activated Kinase
APC: Antigen Presenting Cell
ATP: Adenosine Triphosphate
BKV: BK Virus
CAMR: Chronic Antibody-Mediated Rejection
CAN: Chronic Allograft Nephropathy
CD: Cluster of differentiation
CD40L: CD40 Ligand
CM: Central Memory
CMV: Cytomegalovirus
CPT1: Carnitine Palmitoyltransferase 1
CTLA-4: Cytotoxic T Lymphocyte Associated Protein-4
DON: 6-diazo-5-oxo-l-norleucine
DSA: Donor Specific Antibody
EAE: Experimental Autoimmune Encephalomyelitis
eGFR: Estimated Glomerular Filtration Rate
EM: Effector Memory
ETC: Electron Transport Chain
$FADH_2$: Flavin Adenine Dinucleotide
GZM-A/B: Granzyme-A/B
HLA: Human Leukocyte Antigen
IFNγ: Interferon-γ
Ig : Immunoglobulin
IL-2/7/10: Interleukin-2/7/10
IL-7R: Interleukin-7 Receptor
ILT3/4: Immunoglobulin-Like Transcript 3/4
JAK1/3: Janus kinase 1/3
KTFS: Kidney Transplant Failure Score
MHC: Major Histocompatibility Complex
MIP-1β: Macrophage Inflammatory Protein-1β
mTOR: Mammalian Target of Rapamycin
mTORC1: Mammalian Target of Rapamycin Complex 1
mTORC2: Mammalian Target of Rapamycin Complex 2
NADH: Nicotinamide Adenine Dinucleotide
NGAL: Neutrophil Gelatinase-Associated Lipocalin
OXPHOS: Oxidative Phosphorylation
PBMC: Peripheral Blood Mononuclear Cells
PERF: Perforin
PI3K: Phosphoinositide 3-Kinase
RANTES: Regulated on Activation, Normal T cell Expressed and Secreted
SLC: Solute Carrier
SLE: Systemic Lupus Erythematosus
STAT3/5: Signal Transducer and Activator of Transcription 3/5
TCA: Tricarboxylic Acid Cycle/The Citric Acid Cycle
TCR: T Cell Receptor
TEMRA: Terminally-differentiated effector memory
TNFα: Tumor Necrosis Factor-α
TRAF6: Tumor Necrosis Receptor-Associated Factor 6
Treg: Regulatory T cell

1 Preface

The purpose of this thesis is to document and discuss the various research projects from the past 3 years at that have my participation and contribution. The topic of my thesis is 'the characterization of CD8 T lymphocyte subsets differentiation and their immunometabolic programming in kidney transplantation'. Before presenting the results of my thesis, I discuss the following topics in my introduction: kidney allograft transplantation, CD8 T lymphocytes, and metabolic pathways and how they pertain to the lymphocytes.

The primary role of the immune system is to protect against foreign antigens. Consequently, the immune system plays a large role in the treatment and care of renal transplant patients. Lymphocytes are involved in the cell-mediated immune response and can be categorized as either T or B lymphocytes, depending on their function and place of maturation. T lymphocytes, in turn, mainly consist of either CD4 or CD8 cells, and both types of cells have been shown to play important roles in transplantation. End-stage renal disease and kidney failure can be treated by two methods: dialysis or renal transplantation. Kidney transplantation is the preferred treatment because it results in a longer life and a higher quality of life for the patient (Tonelli et al. 2011). However, it is restricted by several logistical and immunological factors. The main logistical obstacle is the lack of available organs. In 2012, 57,903 patients were registered on the United States kidney transplant waitlist while only 17,287 kidney transplants were performed that year, showing that the need greatly outstrips organ availability (Department of Health and Human Services 2014). Allograft rejection is the main immunological barrier that transplant patients face, and immunosuppression therapies have emerged to treat this problem. CD8 T cells have been shown to play an important role in cell-mediated injury to renal allografts, and while calcineurin immunosuppressive drugs are meant to inhibit their alloresponse, CD8 T cells have still been shown to be involved in long-term graft dysfunction. In the recent years, there has been a renewed interest into investigating the roles of metabolic pathway into the immune function. Immunometabolic research has

shown that lymphocytes use their metabolic pathways for much more beyond energy production. Lymphocyte differentiation and proliferation are tied to the changes made to the immunometabolic programming.

The aim of my thesis was to investigate the role of CD8 T cell subsets and the involvement of their immunometabolic programming in kidney transplant recipients and evaluate the possibility of using CD8 T cells as biomarkers of allograft rejection.

The appendix contains a summary of my thesis in French, copies of two article manuscripts, and an overview of the publications, abstracts, and awards that were achieved throughout this thesis. The first manuscript, entitled “Targeting CD8 T Cell Metabolism in Transplantation”, is a mini-review article on the topic of immunometabolics in CD8 T in transplantation. It has been placed in the appendix because the majority of its contents can be found within the body of this thesis and is therefore redundant. The second manuscript, entitled “B Cell depletion therapy impact CD8 T cells in ANCA-associated vasculitis” covers a project in which I participated in the immunophenotyping portion. However, as the main elements of this project are not related to my thesis, this manuscript has been placed in the appendix.

2 Introduction

2.1 Transplantation

Transplantation is the best treatment for end stage organ failure and kidney transplants are the most commonly perform solid organ transplant procedure performed. Transplantation has been shown to not only increase the length of survival time of transplant recipients over dialysis patients but also improve the quality of life of transplant recipients (Burra and De Bona 2007; Russell et al. 1992).

The surgical procedure for organ transplantation in humans was first attempted by French surgeons Jaboulay and Carrel in 1906, who attempted to graft a sheep and a pig kidney into two patients with end-stage renal failure. While their attempts were not successful, they pioneer the vascular anastomosis technique which is still used in kidney transplantation today. The first successful human transplant occurred in 1954 when a surgical team lead by Joseph Murray transplanted a kidney from a healthy individual to his identical twin (Morris 2004; Merrill et al. 1956).

English scientist Sir Peter Medawar's work on skin grafts during and following World War II was pivotal for advancing the scientific community's knowledge and understanding concerning the role of the immune system in transplantation by showing that allograft rejection was caused by an immunological component (Watson and Dark 2012; Starzl 1995; Billingham and Medawar 1951; Gibson and Medawar 1943). Additionally, Medawar, along with his colleagues Billingham and Brent, show that it was possible to induce tolerance in murine skin graft transplant model (Billingham, Brent, and Medawar 1953). Due to his contributions to the transplantation field, Medawar is now commonly regarded as the "Father of Transplantation".

The most commonly solid organ transplanted is kidney, but the solid organ transplant field encompasses heart, liver, lung, pancreas, and intestine transplants (Department of Health and

Human Services 2014). The main hurdle that needs to be overcome for a successful kidney transplantation is rejection. There are three types of rejection: hyperacute rejection, acute rejection, and chronic rejection. Due to advancements in medical testing, hyperacute rejection is no longer a concern in modern day transplantation. The current repertoire of immunosuppressive drugs available on the market has also greatly lowered the occurrence of acute rejection and as a result, the current 1-year conditional half-life of kidney transplant for deceased donor transplant is 12.5 years and for living donor transplant is 15.3 years (**Figure 1**) (Department of Health and Human Services 2014). On the other hand, chronic rejection is still a major concern for transplant recipients because the cause of chronic kidney rejection is still unclear and there is currently a lack of proper biomarkers that can accurately predict and diagnose chronic graft failure (Colvin and Smith 2005).

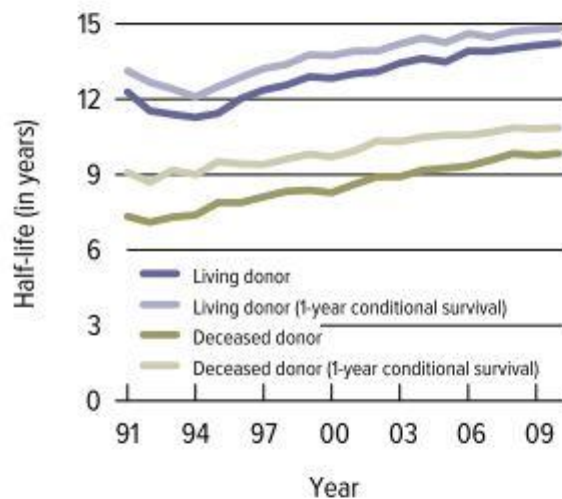


Figure 1. Half-lives for adult kidney transplant recipients (Department of Health and Human Services 2014).

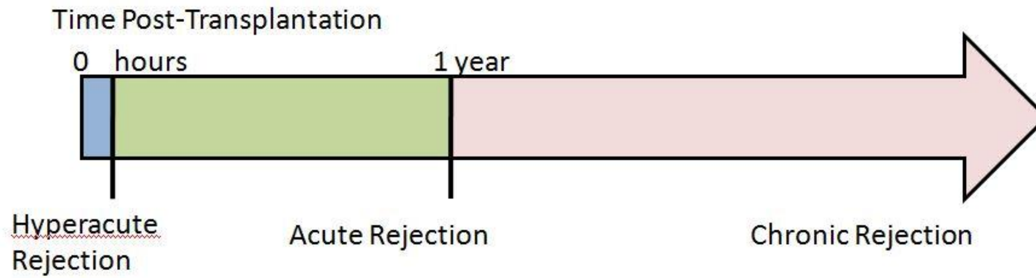


Figure 2. The relative timeline of hyperacute, acute, and chronic rejection in transplant patients.

2.1.1 Hyperacute Rejection

Hyperacute rejection occurs within the first few hours post-transplantation (**Figure 2**). The cause is due either to ABO blood type mismatch between the donor and recipient or due to pre-existing anti-human leukocyte antigens (HLA) donor specific antibodies (Cornell, Smith, and Colvin 2008; Smith and Colvin 2012; Colvin and Smith 2005). However, hyperacute rejection is no longer a major concern for current transplant patients because of the HLA testing and cross-matching that occurs before transplantation. Furthermore, due to organs shortages and thanks to advancements in induction and immunosuppressive therapy, it is now possible to successfully carry out ABO incompatible transplants. Additionally, progress in HLA research has shown that successful long-term allografts depend on more than counting the number of mismatched antigens.

2.1.1.1 Recipient-Donor Matching: ABO Incompatibility

Blood incompatibility originates from A, B, and H blood group antigens which are expressed on a variety of different cell types. The H antigen, which is ubiquitously expressed in all humans, determines blood type O. Individuals of the O blood group possess agglutinins which are anti-A and anti-B, while individuals with blood type A or B have anti-B or A,

respectively, agglutinins. In the case of a poorly matched blood transfusion, these antibodies cause a clumping reacting, termed hemagglutination; in transplantation, an ABO-incompatible (ABOi) transplants without treatment causes allograft thrombosis and hyperacute rejection (Murphy, Travers, and Walport 2008; Böhmig et al. 2015; Wongsaroj et al. 2015). While the convention in transplantation is to match recipient-donor blood types in order to eliminate hyperacute rejection due to ABO-incompatibility, organ shortages have lead to the implementation of protocols for ABO-incompatible transplants (**Figure 3**).

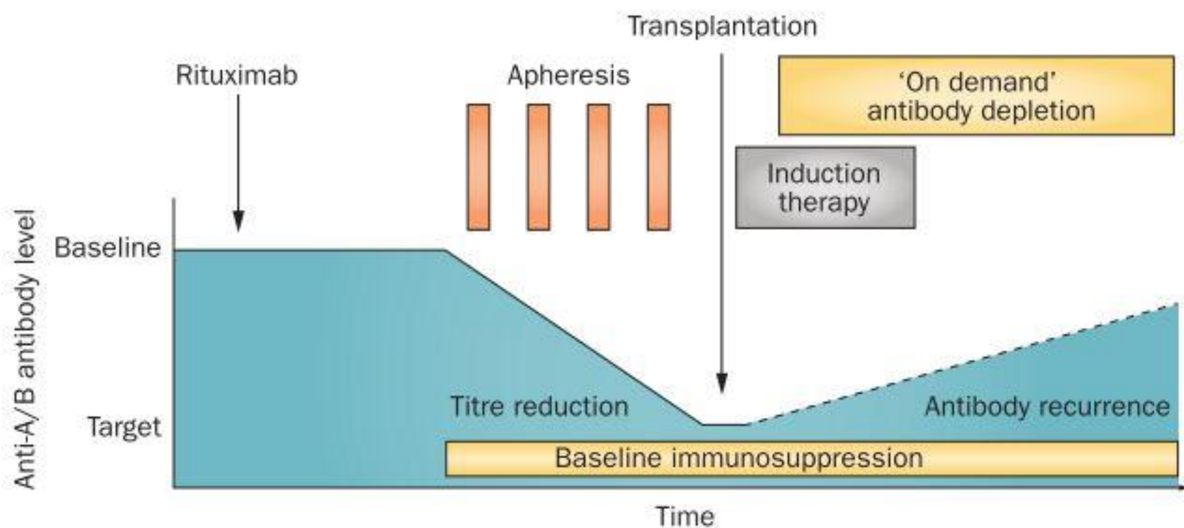


Figure 3. Key protocol elements of ABOi transplantation (Böhmig et al. 2015)**.**

Current protocols for ABOi transplants include apheresis techniques where anti-A/B antibodies are removed from whole blood by filtration or immunoadsorption before being reinfused. Unfortunately, apheresis alone was not enough to prevent allograft rejection, and ABOi transplant recipients were at risk of severe antibody-mediated rejection. A splenectomy, which would have removed a large pool of B cells, was found to be a successful treatment for this problem. However, a splenectomy was not a palatable procedure to transplant patients and the introduction of rituximab, an anti-CD20 B cell depleting therapy, eliminated the need for a splenectomy, except in severe cases of antibody-mediated rejection,

and thus was adopted into ABOi transplant protocols. Consequently, ABOi transplant programs have successful short- and long-term outcomes, in part thanks to the new advancements in immunosuppressive therapy (Böhmig et al. 2015; Wongsaroj et al. 2015).

2.1.1.2 Recipient-Donor Matching: HLA Matching & Crossreactivity

In addition to ABO incompatibility, kidney allografts are at risk of alloreactive immune responses. Even though many transplant recipients have not been previously exposed to alloantigens, they still have a portion of their pre-existing memory T cell repertoire that is alloreactive due to the crossreactivity of viral specific memory T cells (**Figure 4**) (Murphy, Travers, and Walport 2008; D'Orsogna et al. 2010).

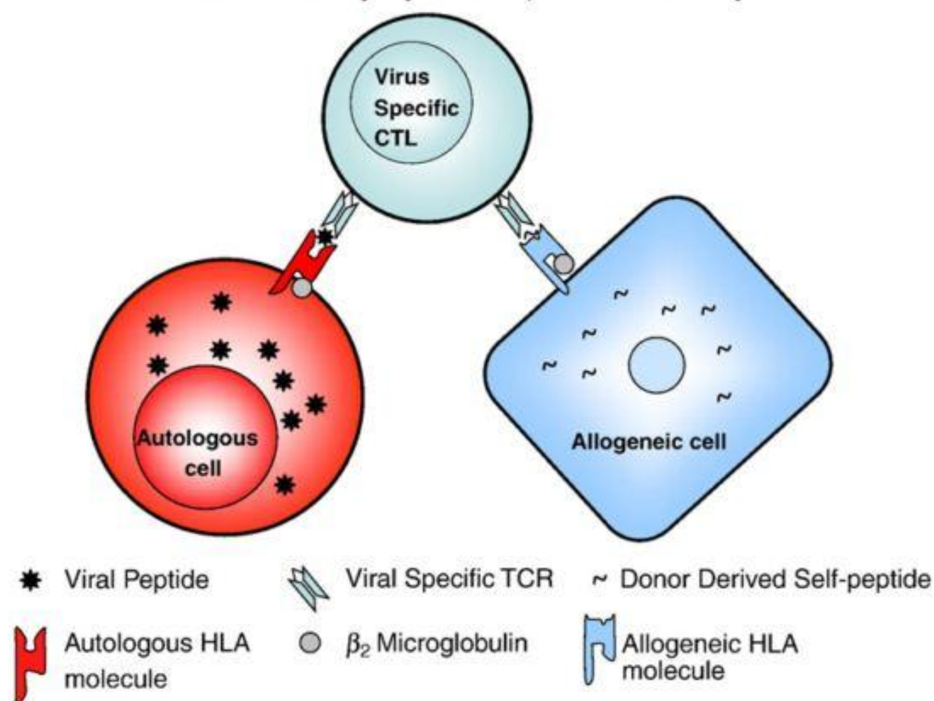


Figure 4. Allo-HLA crossreactivity by viral-specific memory T cells. Viral specific cytotoxic T lymphocytes (CTL) can target autologous cells that are infected or can crossreact against self-peptide presenting allogeneic HLA molecules (D'Orsogna et al. 2010).

The main purpose of the immune system is to the body from against diseased cause by foreign antigens and viruses. In order to fulfill this role, the immune system needs to have mechanisms for detecting the antigen, and a large part of this immunological recognition is based on the distinction of 'self' from 'non-self'. Immunological recognition via the engagement of the Major Histocompatibility Complex (MHC) is part of the process of selection and maturation of thymocytes into mature peripheral T cells.

The MHC is a set of glycoproteins expressed on antigen-presenting cells (APC) which are involved in antigen processing and presentation. In humans, the MHC is also called the Human Leukocyte Antigen because it is encoded by a locus of genes located on chromosome 6 called the Human Leukocyte Antigen (HLA) genes. CD8 T cell receptors recognize MHC class I molecules, which is widely expressed on most cell types, while CD4 T cells bind to MHC class II molecules, which is only expressed on B lymphocytes, macrophages, monocytes, and dendritic cells. It is the expression of the self-antigens by the MHC on the donor allograft that is a key cause of allograft rejection (Murphy, Travers, and Walport 2008; Trivedi 2007; Tan et al. 1993; Mehra 2001; Berg, Tymoczko, and Stryer 2002).

Thymocytes are first positively selected for depending on their ability to engage the peptide:MHC complexes on the thymic epithelium. Thymocytes with poor affinity are deleted. This is done to ensure that T cells can bind to MHC molecules and thus can fulfill their immunological function. The thymocytes that survive positive selection continue onto negative selection, where thymocytes that react strongly to a self-peptide:MHC complex are deleted. The aim of negative selection is to promote self-tolerance and prevent the maturation of T cells that could potentially cause harm to the body should they encounter self-antigen peptides (Murphy, Travers, and Walport 2008; Berg, Tymoczko, and Stryer 2002).

CD8 T cell are largely responsible for the human immune response against viral infections. When naïve T cells are stimulated by viral peptides, they differentiate and expand into effector and memory T cells in order to contain the infection (**Figure 5**). These viral specific memory T cells have been shown to be alloreactive against allogeneic MHC class I molecules (Murphy, Travers, and Walport 2008; Kaech and Cui 2012). Due to the crossreactivity of substantial portion of viral specific T cells, where these cells react to allogeneic HLA molecules, kidney allografts are at risk of hyperacute and acute cell-mediated rejection. Therefore, in order to minimize the effects of crossreactivity and to improve allograft survival, HLA testing and matching protocols were established (Murphy, Travers, and Walport 2008; D'Orsogna et al. 2010; Burrows et al. 1999).

HLA-A, -B, and -C genes encode for MHC Class I α chains, while MHC Class II α- and β-chain genes are HLA-DR, -DP, and -DQ(Murphy, Travers, and Walport 2008). Historically, the clinical goal of HLA matching in kidney transplantation has been to reduce the number of mismatched HLA-A, -B, and -DR antigens of the donor because increased numbers of mismatched antigens leads to reduced allograft survival (Opelz 1988). However, as more immunological research emerges, new levels of HLA matching are added to existing clinical protocols (**Table 1**). Furthermore, HLA typing methods have evolved from serologic alloantibody typing methods to more precise DNA-based typing technology which can distinguish allelic differences (S. Takemoto et al. 2004). HLA-matching improves kidney allograft survival and reduces acute rejection, thus benefiting the patients clinically and socioeconomically (S. K. Takemoto et al. 2000; Pirsch et al. 1996; Taylor et al. 1993; S. Takemoto et al. 2004; Mehra et al. 2013).

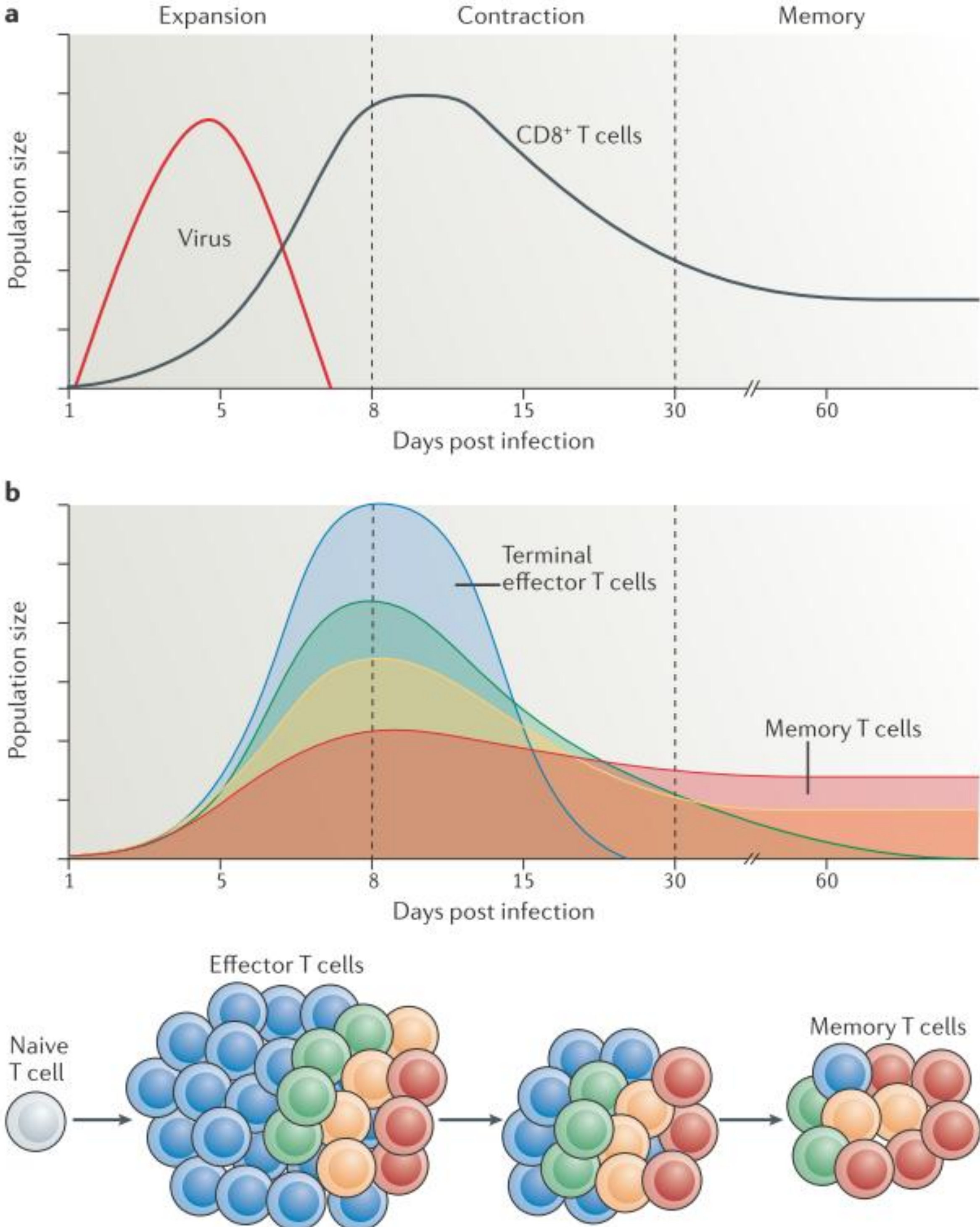


Figure 5. The expansion and contraction of CD8 cells in response to antigen stimulation (Kaech and Cui 2012).

- Matching for HLA-A, -B, and -DR antigens
- Mismatching for HLA-A, -B, and -DR antigens
  - Broad vs split antigens
  - Acceptable and unacceptable mismatches for highly sensitized candidates
  - Permissible mismatches with graft outcome similar to nonmismatched transplants
- DR matching
- CREG matching
  - Public and private class I epitopes
- Structurally based matching
  - Amino acid residue mismatching
  - HLAMatchmaker

Abbreviations: HLA = human leukocyte antigen; CREG = cross-reacting groups of antigens.

Table 1. HLA matching protocols for kidney transplantation (S. Takemoto et al. 2004).

While HLA matching provides many benefits to the transplant recipients, there are also some instances where HLA mismatching is important. This mainly concerns cases of viral serostatus, specifically cytomegalovirus (CMV). CMV is a ubiquitous virus where the majority of people have contracted it by the time they reach maturity. The virus usually remains in a dormant state in healthy individuals; however, transplant patients who are under immunosuppressive treatments are prone to reactivation or de novo infection of the virus, which can cause allograft rejection and loss (Razonable, Humar, and Practice 2013; van der Bij and Speich 2001). Interestingly, patients who have matched HLA-DR are more likely to face CMV diseases as opposed to patients who have mismatched HLA-DR (Blancho et al. 1992; Schnitzler et al. 1997; Kraat et al. 1994). Fortunately, prophylactic treatment with ganciclovir and minimizing transplantation of seropositive donors into seronegative recipients has improve long-term graft survival (Razonable, Humar, and Practice 2013; D’Orsogna et al. 2010).

2.1.2 Acute Rejection

Rejection episodes that occur within the first year post-transplant are classified as acute rejection. Modern immunosuppression therapy, beginning with the use of cyclosporine, has drastically reduced the occurrence of acute rejection episodes (Dharnidharka 2005).

2.1.2.1 Acute Antibody-Mediated Rejection

C4d is the inactive fragment of C4b in the complement cascade and the detection of C4d depositions is one of the hallmarks of antibody-mediated rejection. In kidney transplants, C4d deposits occur largely in the peritubular capillaries and strongly correlate with the presence of circulating donor-specific alloantibodies (Kato et al. 2003; Nickeleit et al. 2002; Colvin and Smith 2005). Patients with anti-class I HLA antibodies have severe rejection episodes and sustain injury to the allograft microvasculature, which leads to rapid allograft deterioration (P F Halloran et al. 1990; P F Halloran et al. 1992).

2.1.2.2 Acute T Cell-Mediated Rejection

T cell-mediated rejection is responsible for a large percentage of acute kidney allograft loss, and as a result, the majority of current immunosuppressive treatments available target T cells (Philip F Halloran 2004). T cell-mediated rejection is usually identified by tubulitis, where infiltrating T cells damage tubular epithelium. These infiltrating T cells cause cellar damage by releasing cytolytic granules containing granzymes and perforin, as well as various cytokines and chemokines, such as interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) (Robertson et al. 1996). T cell-mediated rejection is caused by allorecognition, where the recipient T cells are primed against mismatched histocompatibility alloantigens. There are three pathways to allorecognition: Direct, Indirect, and Semi-direct (**Figure 6**) (Issa, Schiopu, and Wood 2010; Benichou et al. 2011).

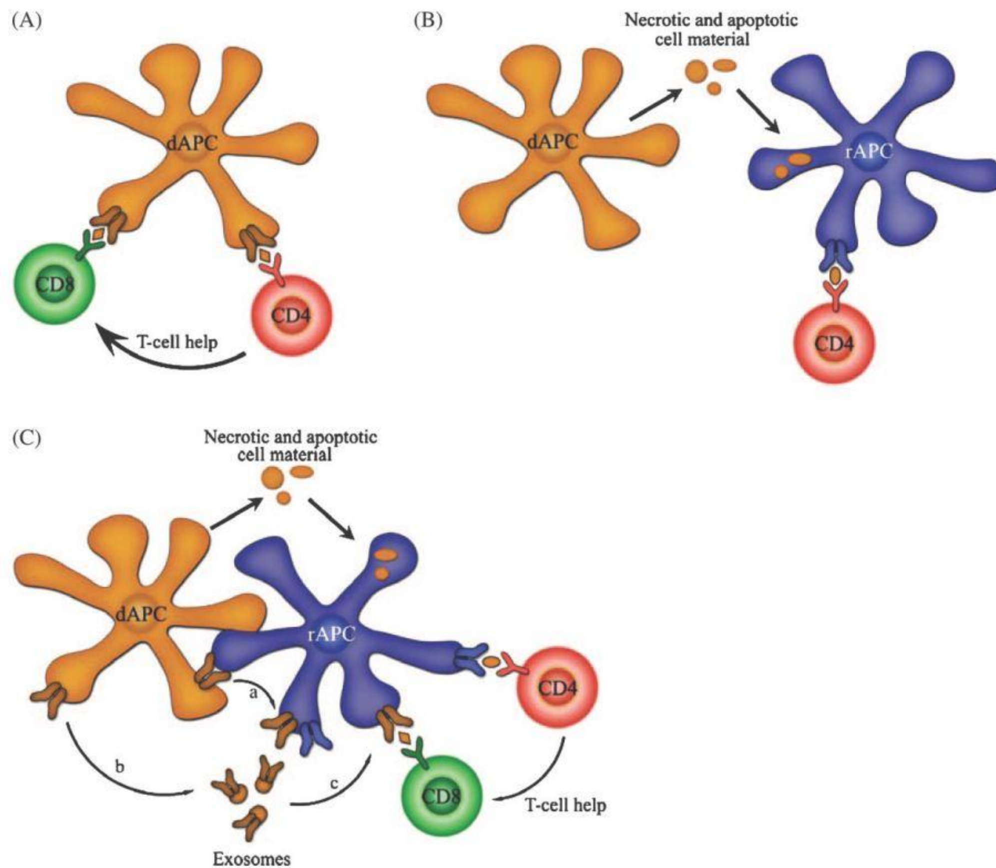


Figure 6. The three pathways of allorecognition (Afzali, Lechler, and Hernandez-Fuentes 2007). (A) The direct pathway where donor APC presents donor MHC to recipient T cells. (B) The indirect pathway of allorecognition, where recipient APC internalize and process donor alloantigens before presenting them to recipient lymphocytes. (C) The semi-direct allorecognition pathway where the recipient APC receives donor MHC through cell-to-cell contact or through exosome uptake and activates the recipient T cells.

2.1.2.2.1 Direct Allorecognition

When an organ is transplanted, infiltrating T cells can recognize foreign MHC on the donor organ. Additionally, donor APC are carried as passengers into the organ recipient. These donor APC travel to the host lymphoid organs where they interact and stimulate recipient naive T cells into action (**Figure 6A**) (Larsen, Morris, and Austyn 1990; Lechler and Batchelor 1982). Direct allorecognition is usually associated with acute rejection as passenger

donor APC are present the first few months post-transplantation (Ballet et al. 2009). However, Bestard et al. showed that patients who were at least two years post-transplant still have positive ELISPOT test for the direct pathway, indicating that T cells that were primed by donor APC can still maintain the direct allorecognition pathway long term (Bestard et al. 2008).

2.1.2.2.2 Indirect Allorecognition

Over time, passenger APC are lost and the direct pathway of allorecognition plays a less important role in allograft rejection. Instead, the non-self MHC antigens of the donor organ are presented to T cells by the recipient's own APC. This pathway is predominantly used by CD4 T cells as most of the alloantigens are presented by MHC class II (**Figure 6B**). However, cross-priming between MHC class I and class II molecules can occur, thereby stimulating CD8 T cells as well. Furthermore, the indirect allorecognition pathway has been shown to play a role in both acute and chronic rejection (Auchincloss et al. 1993; Morón, Dadaglio, and Leclerc 2004; Fluck et al. 1999; Dalchau, Fangmann, and Fabre 1992).

2.1.2.2.3 Semi-direct Allorecognition

In semi-direct allorecgnition, recipient APC acquire MHC molecules through cell-to-cell contact or through secreted exosomes and then prime naive CD4 and CD8 T cells. One the donor MHC molecules are internalized and processed, the APC can present the donor MHC molecules directly to CD8 cells or indirectly to CD4 cells (**Figure 6C**) (Issa, Schiopu, and Wood 2010; Herrera et al. 2004; Jiang, Herrera, and Lechler 2004).

2.1.3 Chronic Rejection

Because of modern immunosuppression therapies, acute rejection is no longer a pressing concern within the transplant community. Instead, focus has begun to shift to chronic, long term allograft rejection, which seemingly is unaffected by immunosuppression. For a long time, chronic rejection was not well understood and not very well defined. As a result, the general term of chronic allograft nephropathy (CAN) was used to describe all pathologies related to the progressive slide of a renal allograft into dysfunction that occurs over time. The term CAN was first introduced by the pathologists, surgeons, and nephrologists at the first Banff conference, where a schema was determined in order to standardize the nomenclature and classification of renal allograft pathology. At the time, CAN was graded on three levels (mild, moderate, and severe) depending on the severity of pathological damage observed in the glomerular, interstitial, tubular, and vascular regions in the kidney biopsies (Solez et al. 1993).

However, as more research into chronic allograft dysfunction emerged and knowledge into the pathology expanded, researchers and clinicians felt that the term CAN, as it was being used to diagnose any and all chronic pathologies, had become too widely used and undermined the effort to identify the underlying pathological disease causing the allograft rejection (Mengel, Sis, and Halloran 2007; Bhowmik et al. 2010; Solez and Racusen 2013). As a result, the 8th Banff conference decided to abolish the term and replaced it with a more detailed schema that first divided chronic allograft injury into rejection and non-rejection. Non-rejection was described as allograft injury due to interstitial fibrosis and tubular atrophy cause by diseases (**Table 2**). The rejection arm was subdivided into chronic antibody-mediated rejection (CAMR) and chronic T cell-mediated rejection (Solez et al. 2007). While the term CAN is still be found in contemporary literature, it is sparingly used and is usually accompanied with a more detailed description of the pathology.

Causes of IF/TA (non-rejection)	
Etiology	Morphology
Chronic hypertension	Arterial/fibrointimal thickening with reduplication of elastica, usually with small artery and arteriolar hyaline changes.
CNI[1] toxicity	Arteriolar hyalinosis with peripheral hyaline nodules and/or progressive increase in the absence of hypertension or diabetes. Tubular cell injury with isometric vacuolization.
Chronic obstruction	Marked tubular dilation. Large Tamm–Horsfall protein casts with extravasation into interstitium, and/or lymphatics.
Bacterial pyelonephritis	Intratubular and peritubular neutrophils, lymphoid follicle formation.
Viral infection	Viral inclusions on histology and immunohistology and/or electron microscopy.

[1]CNI, calcineurin inhibitor toxicity.

Table 2. Morphology of specific chronic diseases involved in chronic allograft injury (non-rejection) (Solez et al. 2007)**.**

2.1.3.1 Chronic Antibody-Mediated Rejection

CAMR is defined by transplant glomerulopathy and inflammation and lesions in the peritubular capillary basement membrane. While C4d deposition in the peritubular capillaries is also found in a large percentage of patients with chronic rejection, it is not a requirement for CAMR (Mauiyyedi et al. 2001). Allograft fibrosis and circulating donor specific antibodies to MHC class II antigens are also associated with chronic allograft rejection (Sis et al. 2007; Solez et al. 2007; Hara 2015).

2.1.3.2 Chronic T Cell-Mediated Rejection

Interstitial fibrosis and tubular atrophy are also signs of chronic T cell-mediated rejection, however the key characteristic of chronic T cell-mediated rejection is infiltrating mononuclear lymphocytes at the areas of allograft injury. Fibrosis, transplant glomerulitis, intima thickening, and the epithelial-mesenchymal transition to tubular cells have all been associated

with the presence of infiltrating T cells (Cornell, Smith, and Colvin 2008; Bueno and Pestana 2002; Robertson et al. 2004; Solez et al. 2007).

2.1.4 Biomarkers of Kidney Allograft Injury

Biomarker research plays an important role in the effort to prolong kidney allograft survival. While the current clinical parameters used to measure kidney heath have been in use for years, it is commonly accept that many of these practices are not very accurate and that there is much room for improvement. As a result, there has been a great deal of effort in searching for new biomarkers that are highly sensitive to acute changes in kidney health and function and can act as predictive prognostic factors to help improve patient care and extend allograft half-life (Urbschat, Obermüller, and Haferkamp 2011; Wasung, Chawla, and Madero 2015). Ideally, biomarkers should be highly sensitive and specific to the condition of the kidney and should be able to act as diagnostic markers of kidney injury and prognostic marker of disease risk (**Table 3**).

Characteristics of an ideal biomarker.

1. Noninvasive
2. Highly sensitive and specific
3. Increases rapidly and reliably in response to kidney disease.
4. Correlates with the amount of kidney injury
5. Provides risk stratification and prognostic information
6. Is site specific
7. Applicable across different populations
8. Identifies possible mechanisms of injury (prerenal, intrarenal, postrenal)
9. Highly stable over time and across different temperatures and PH
10. Does no interfere with drugs

Table 3. Characteristics of an ideal biomarker(Wasung, Chawla, and Madero 2015).

2.1.4.1 Current Established Clinical Biomarkers

2.1.4.1.1 Serum Creatinine

The most commonly used clinical biomarker used by nephrologists to assess kidney function and health is serum creatinine. However, serum creatinine is also widely criticized as being unreliable as it is easily influenced by multiple external factors, such as diet, exercise and hydration. Additionally, it does not reflect acute changes in kidney function and increases of serum creatinine are usually not observed until a new steady-state equilibrium has been achieved. By this time sustain damage and injury to renal tissue has already occurred (Nickolas, Barasch, and Devarajan 2008).

2.1.4.1.2 Proteinuria and Albuminuria

Proteinuria is another common biomarker used in clinical setting for evaluating kidney function, but like serum creatinine, proteinuria also suffers from low sensitivity and specificity. There is also a lack of standardization regarding the clinical use and assessment of proteinuria. In fact, there is still confusion as to whether the term proteinuria refers to total protein concentration (encompassing albumin and non-albumin proteins) or to albuminuria, as the two terms are often used interchangeably. Proteinuria and albuminuria can be measured by a variety of means, ranging from a dip-stick during a spot collection to a 24-hour timed urine collection, and all technique have advantages and disadvantages (Guh 2010; Viswanathan and Upadhyay 2011; Urbschat, Obermüller, and Haferkamp 2011). While proteinuria has been an important clinical tool for evaluating chronic kidney dysfunction, there is still a persistent desire in to find new biomarkers to replace this clinical biomarker.

2.1.4.1.3 eGFR

Estimated Glomerular Filtration Rate (eGFR) calculations use serum creatinine or serum cystatin-c measurements to give an approximate measure of the number of functioning nephrons (**Table 4**). While eGFR is considered a good tool for measuring chronic kidney failure, it is a poor measure of acute kidney dysfunction. Because most equations are based on serum creatinine, it is also plagued by the same problems of being unable to accurately reflect the degree of renal dysfunction (Urbschat, Obermüller, and Haferkamp 2011; Wasung, Chawla, and Madero 2015).

Estimating equations for glomerular filtration rate. MDRD and CKD-EPI equations incorporating creatinine, cystatin C or both.

Equation
MDRD
$GFR\ (ml/min/1.73\ m^2) = 175 \times (SCr)^{-1.154} \times (age)^{-0.203} \times (0.742$ if female$) \times (1.212$ if African American$)$
2009 CKD-EPI creatinine
$GFR = 141 \times \min(SCr / \kappa, 1)^{\alpha} \times \max(SCr / \kappa, 1)^{-1.209} \times 0.993^{age} \times 1.018$ [if female] $\times 1.159$ [if Black]
2012 CKD-EPI cystatin C
$eGFR = 133 \times \min(Scys / 0.8, 1)^{-0.499} \times \max(Scys / 0.8, 1)^{-1.328} \times 0.996^{age}$ [$\times 0.932$ if female]
2012 CKD-EPI creatinine and cystatin C
$eGFR = 135 \times \min(SCr / k, 1)^{-a} \times \max(SCr / k, 1)^{-0.601} \times \min(Scys / 0.8, 1)^{-0.375} \times \max(Scys / 0.8, 1)^{-0.711} \times 0.995^{age}$ [$\times 0.969$ if female] [$\times 1.08$ if Black]

SCr is serum creatinine and Scys is serum cystatin C; k is 0.7 for females and 0.9 for males; a is −0.248 for females and −0.207 for males.
MDRD, modification of diet in renal disease equation.
CKD-EPI, chronic kidney disease epidemiology collaboration equation.

Table 4. Overview of the equations used to estimate the glomerular filtration rate (Wasung, Chawla, and Madero 2015).

2.1.4.1.4 Biopsy

In contrast to the previous clinical biomarkers discussed in this section, biopsies are considered the golden standard for kidney injury diagnosis. Biopsies play a critical role for diagnosis acute and chronic humoral rejection because detecting C4d deposition kidney

allografts is mainly done through biopsies. Due to their vast benefits and advantages for evaluating kidney function, many transplant centers have adopted 1-year protocol biopsies as a part of their standard of care. However, biopsies are not without their disadvantages. Primarily, biopsies are a much more invasive procedure compared to urine or blood collection and have the risk of bleeding complications and hematuria (Brachemi 2014). Also, biopsies are limited as a diagnostic tool as they offer only a cross-sectional image of the kidney which does not represent the whole kidney. Additionally, diagnosis of kidney dysfunction from a biopsy relies heavily on the pathologist, and there is major concern regarding the reproducibility and consistency of pathologists (Furness et al. 2003; Dhaun et al. 2014)

2.1.4.1.5 Immunknow

The Immunknow assay (Viracor-IBT) is a T cell immune function assay which measures the amount of adenosine triphosphate (ATP) in CD4 T cells as a biomarker of the immune response in transplant recipients. It was approved in 2002 by the United States Food and Drug Administration as a clinical tool to evaluate the immunoreactivity in immunocompromised patients. Renal transplant patients with an Immunknow value ranging from 225 to 524 ng/mL are considered to be at moderate levels of immune response and immunosuppression and at low risk for viral infections or rejection episodes. Patients with low Immunknow values (below 225 ng/mL) are categorized as having a low immune response and therefore over immunosuppressed. Consequently, these patients are at risk of viral infection. Patients on the other end of the spectrum, with Immunknow values of 525 ng/mL and above, are under immunosuppressed and have a high immune response, which puts them at risk of acute rejection episodes (Kowalski et al. 2006).

Despite its approval as a new diagnostic tool, Immunknow has not been universally adopted as the new clinical biomarker to replace serum creatinine. While most centers who tested the

assay do affirm that Immunknow assay is predictive for some transplant pathologies and is correlated with fluctuations in serum creatinine, there is no consensus nor standards regarding which clinical settings benefit from the Immunknow assay. For example, there are several centers that report that low Immunoknow values in their transplant patients are indeed associated with viral (BK or CMV) infections, but high Immunknow values are not associated with acute rejection (De Paolis et al. 2011; Moon et al. 2012; Quaglia et al. 2014). On the other hand, other centers report that they find Immunknow values correlate with acute rejection episodes, but this does not seem to be associated with immunosuppression drug trough levels (Cadillo-Chávez et al. 2006; Pérez-Flores et al. 2009). Overall, the wide range of reports from multiple centers indicates that the Immunknow assay has a promising future as a clinical biomarker once standards and consensus have been established.

2.1.4.2 Biomarker Research

2.1.4.2.1 Kidney Transplant Failure Score

While there are many new promising biomarkers being evaluated, it will still be a while before they can be adopting into clinical use as they still need to undergo a long and thorough validation process. Therefore, there has been interest in finding a way to use current clinical parameters as predictive biomarkers of allograft survival. Y. Foucher et al. evaluated several clinical parameters that are collected during the first year post-transplantation before selecting 8 parameters (**Table 5**) to be included into a clinical composite score called the Kidney Transplant Failure Score (KTFS) (**Figure 7**). The KTFS is calculated using a multivariate Cox model combined with time-dependent receiver-operator characteristics and is predictive of allograft failure 8 years post-transplant. Patients with a KTFS below 4.17 are considered to be at low-risk for allograft failure while patients above this threshold are considered high-risk for long-term graft failure. In their validation group, Foucher et al. found that 8% of the low-

risk recipient group and 25% of the high-risk group went on to lose their allograft, showing that the KTFS is in need of some improvements and more validation (Foucher et al. 2010).

Abbreviation	Value
Gender	1 for male recipients and 0 for females
Cr_D	1 if the blood creatinine of the donor is >190 μmol/l, and 0 otherwise
Age_R	1 if the recipient age is >25 years, and 0 otherwise
Ntrans	1 if the number of previous transplantations is >2, and 0 otherwise
Cr_{3m}	Blood creatinine level measured 3-months post-transplantation in μmol/dl
Cr_{12m}	Blood creatinine level measured 1-year post-transplantation in μmol/l
Pr_{12m}	Proteinuria measured 1-year post-transplantation in g/day
AR	1 if an acute rejection occurs in the first year, and 0 otherwise

Abbreviation: KTFS, Kidney Transplant Failure Score; ROC, receiver-operator characteristic. These definitions are chosen in order to maximize the quality of adjustment to the Cox model and to maximize the area under the time-dependent ROC curve at 8 years. To calculate the KTFS for a subject, the abbreviations in the KTFS formula (page 2) are simply replaced by the corresponding value. If at least one variable is missing, the KTFS cannot be calculated.

Table 5. The eight clinical parameters included into the calculation of the KTFS (Foucher et al. 2010).

$$
\begin{aligned}
\mathrm{KTFS} = & -0.75072 * \mathrm{Cr_D} - 1.02316 * \mathrm{Age_R} \\
& +1.17295 * \mathrm{Ntrans} + 0.22288 * \mathrm{AR} \\
& +0.01881 * \mathrm{Cr_{3m}} + 0.41551 * \sqrt{(\mathrm{Cr_{12m}})} \\
& -0.88001 * \mathrm{Gender} + 0.61121 * \mathrm{Pr_{12m}} \\
& +0.04077 * (\mathrm{Pr_{12m}})^2 + 0.48601 * \mathrm{Gender} * \mathrm{Pr_{12m}} \\
& -0.06115 * \mathrm{Gender} * (\mathrm{Pr_{12m}})^2.
\end{aligned}
$$

Figure 7. The calculations for the KTFS (Foucher et al. 2010).

2.1.4.2.2 Up and Coming Biomarkers of Kidney Injury

The task of discovering and moving a biomarker from bench to bedside is an uphill process and faces many hurdles. However, the enthusiasm for biomarker research has not been dampened; instead, different approaches for biomarker discovery have emerged in the past decades (**Figure 8**) (Willis and Lord 2015).

The traditional approach for clinical diagnosis is an empirical one, where one treatment fits all. While this is how medicine is largely conducted, breakthroughs in medical research have shown that patients are not homogenous and therefore a ‘one size fits all’ approach is not always in the best interest of the patient. Since the empirical method was too broad, a stratified approach to medical diagnostics was developed. In this methodology, patients would be grouped into different categories depending on the pathology and mechanism of disease and on possible treatment therapies. Stratified medicine is now commonly used in the transplant field. For example, patients receive therapy depending on the type and grade of the rejection episode. Evolving from the stratified medicine strategy is personalized medicine, where therapies are tailored specifically for each individual. While there are several advantages to this approach, it is also very costly and time consuming. However, the evolution of new techniques and technologies has help alleviate some of these problems, and as they continue to advance, the cost of personalized medicine will be reduced. The ONE Study is a clinical study that embraces the personalized medicine approach in transplant patients. The aim of this study is to expand a patient's immunoregulatory cells from whole blood before reinfusing them into the patient as a means to induce allograft tolerance (Willis and Lord 2015; Geissler 2012).

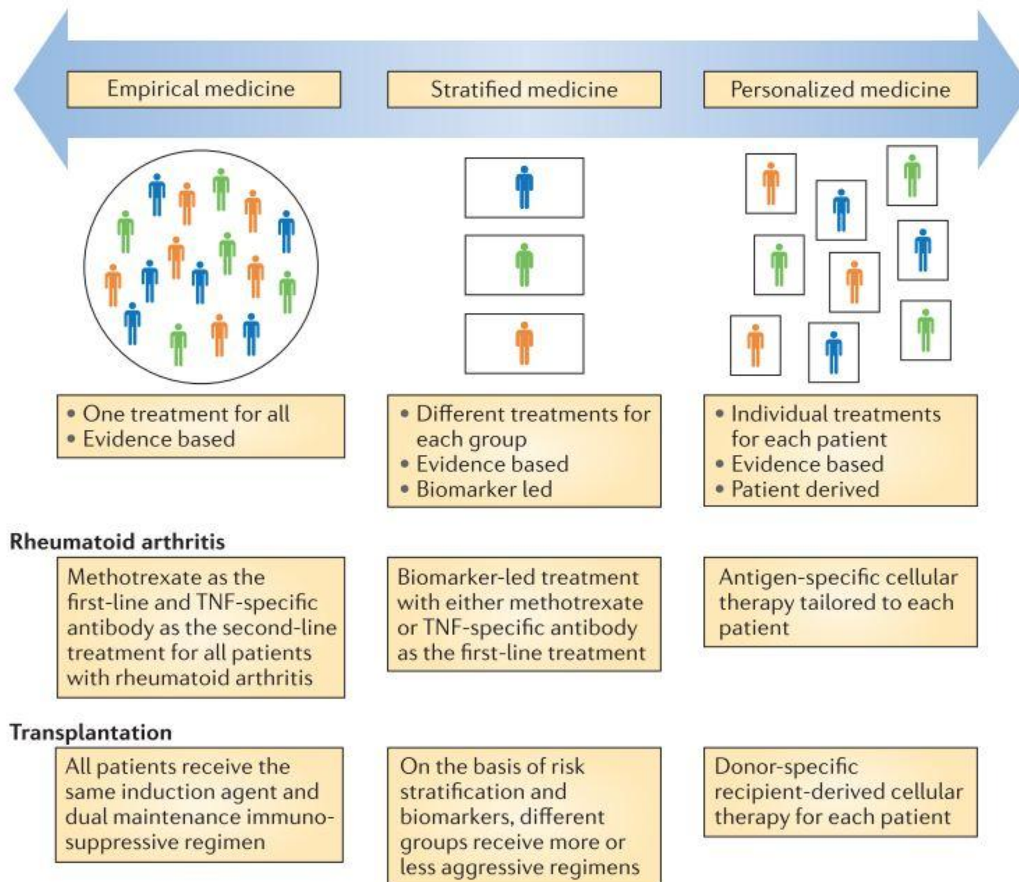


Figure 8. Comparison of different medical approaches. The figure compares features of the empirical, stratified, and personalized medicine approaches. It also includes examples of each approach in the context of rheumatoid arthritis and solid organ transplantation (Willis and Lord 2015).

Because of the various problems and disadvantages of the current clinical biomarkers, the research community has been using the stratified and personalized approaches to find new biomarkers to replace serum creatinine and proteinuria measurements. Additionally, biomarker research has expanded across several platforms and into various target types (**Table 6**).

TEST		PLATFORM	EXAMPLES OF POTENTIAL BIOMARKERS
Gene Transcripts	Single gene	RT-PCR	mRNA: Granzyme B, Perforin, FoxP3; miRNA: miR155, miR223
	Multiple Genes	DNA Microarray	
Proteins	Single Protein	Enzyme-linked immunosorbent assay (ELISA)	Fractalkine, Amyloid A, β2 microglobulin
	Multiple Proteins	Protein Microarray	
Lymphocyte Function	Cytokine Producing Cells	Enzyme-linked immunosorbent spot assay (ELISPOT)	IFN-γ
	ATP levels in Activated T cells	Immuknow®	ATP
Alloantibodies	Single or Multiple Antibodies	Luminex xMAP®	Anti-HLA Antibody, Anti-MICA Antibody

Table 6. Platforms for biomarker discovery and validation (Hartono, Muthukumar, and Suthanthiran 2010).

2.1.4.2.2.1 Transcriptional Biomarkers

Transcriptomics have been a corner stone of transplant biomarker research. Approaches to transcriptomics have ranged from single gene studies to large microarray studies. Now, with the implementation and establishment of online data repositories, data mining and bioformatics have helped identify potential genes transcripts which are involved in the pathology of allograft rejection and could serve as predictive biomarkers (Fehr and Cohen 2011).

The research team lead by Minnie Sarwal has been one of the key players in developing transcriptional biomarker assay for organ failure. They have developed gene sets which are predictive of acute rejection and of allograft inflammation, which in turn can be an indicator of chronic dysfunction. They have established a common rejection module which consists of 11 genes that are overexpressed in acute rejection in all solid organ transplants. The score calculated from this model is predictive of interstitial fibrosis and tubular atrophy at 2 years post-transplant (Naesens et al. 2011; Sigdel et al. 2015; Khatri et al. 2013) .

2.1.4.2.2.2 Protein Biomarkers

There are several proposed protein markers of acute kidney injury that are currently under evaluation. Neutrophil gelatinase-associated lipocalin (NGAL) has long been a lead contender as a new biomarker (**Table 7**) (Devarajan 2011; Haase et al. 2009). In fact, a simple term search for 'NGAL' on the United States National Institutes of Health online website ClinicalTrials.gov yielded 148 registered clinical trials at various stages of completion for various pathologies which involved NGAL. It is a protein expressed by neutrophils and by renal proximal tubular epithelial cells and has been shown to be predictive of kidney injury and delayed graft function (Haase et al. 2009; Mamatov et al. 2015).

Reference	Sample Size	Population Type	Age (y)	Women (%)	Mean Baseline Serum Creatinine (mg/dL)	Impaired Renal Function (%)	Setting	NGAL Measurement	Country
Mishra et al, 2005[13]	71	Children	3.0	36.6	0.45	0	CS	Plasma + urine	United States
Wagener et al, 2006[15]	81	Adults	64.7	34.6	1.10	32.1	CS	Urine	United States
Dent et al, 2007[25]	123	Children	4.2	48.8	0.50	0	CS	Plasma	United States
Zappitelli et al, 2007[18]	39	Children	7.1	48.7	0.44	0	ICU	Urine	United States
Hirsch et al, 2007[23]	91	Children	6.9	44.0	0.73	0	CIN	Plasma + urine	United States
Wagener et al, 2008[26]	426	Adults	63.2	33.8	1.08	27.2	CS	Urine	United States
Bennett et al, 2008[16]	196	Children	4.0	46.4	0.39	0	CS	Urine	United States
Ling et al, 2008[20]	40	Adults	67.9	40.0	0.83	0	CIN	Urine	China
Koyner et al, 2008[22]	72	Adults	61.3	29.2	1.24	26.4	CS	Plasma + urine	United States
Nickolas et al, 2008[14]	541	Adults	59.2	48.4	1.20	26.8	ED	Urine	United States
Lima et al, 2008[27]	52	Adults	54.7	42.3	1.20	53.8	CS	Urine	Brazil
Wheeler et al, 2008[19]	143	Children	2.2	28.0	0.76	—	ICU	Plasma	United States
Xin et al, 2008[28]	33	Children + adults	38.0	42.4	0.77	0	CS	Urine	China
Cruz et al, 2009[29]	301	Adults	58.6	31.2	0.97	6.7	ICU	Plasma	Italy
Makris et al, 2009 (CIN)[30]	60	Adults	62.8	18.3	0.86	13.3	CIN	Urine	Greece
Makris et al, 2009 (ICU)[31]	31	Adults	41.9	19.4	0.97	—	ICU	Urine	Greece
Tuladhar et al, 2009[24]	50	Adults	66.7	30.0	1.10	42.0	CS	Plasma + urine	United Kingdom
Constantin et al, 2009[32]	88	Adults	57.0	45.5	0.81	—	ICU	Plasma	France
Haase-Fielitz et al, 2009[17]	100	Adults	69.5	39.0	1.04	27.0	CS	Plasma	Australia

Note: Conversion factor for serum creatinine in mg/dL to μmol/L, ×88.4.
Abbreviations and definitions: CIN, contrast-induced nephropathy; CS, cardiac surgery–associated acute kidney injury; ED, emergency department; ICU, intensive care unit; NGAL, neutrophil gelatinase-associated lipocalin.

Table 7. Overview and characteristics of clinical studies investing the use of NGAL as a biomarker of acute kidney injury, renal replacement therapy, and in-hospital mortality. Studies were published from 2005 to 2009. Interventional studies involving patients were excluded (Haase et al. 2009).

2.1.4.2.2.3 Lymphocyte Biomarkers

Lymphocytes are also being evaluated as potential biomarkers in transplant recipients. Gene signatures of B lymphocytes have been shown to be biomarkers of allograft tolerance in renal transplant patients (Sagoo et al. 2010; Brouard et al. 2007). Furthermore, a high expression of $CD4^+CD25^+FOXP3^+$ suppressor regulatory T cells (Treg) in the first year post-transplantation are a predictive biomarker of long-term graft survival (D. San Segundo et al. 2012) whereas high levels of activated Treg ($CD4^+CD25^+CD62L^+CD45RO^+$) within the first year post-transplantation is a predictive biomarker of acute rejection (David San Segundo et al. 2014). Additionally, an increase in the TEMRA CD8 T cell population pre-transplantation has been shown to be predictive of acute renal rejection (Betjes et al. 2012).

2.1.4.2.2.4 Alloantibody Biomarkers

The presence of donor-specific anti-HLA antibodies (DSA) plays a key role in antibody-mediated renal allograft failure. Pre-transplant serotyping is performed on patients who have been sensitized, through pregnancy or previous transplants, and appropriate measures are taken in order to minimize their potency. Consequently, detection of DSA in the blood as wells as deposition of the complement component C4d are considered signs of antibody-mediated rejection (ABMR). However, Loupy et al. posited that concentration of complement binding donor-specific anti-HLA antibodies are a predictive biomarker of allograft failure. Their study shows that patients with C1q-binding DSA in the first year post-transplantation had more severe transplant glomerulopathy and higher scores for peritubular capillary C4d deposition compared to patients with non-C1q-binding DSA. Furthermore, these patients were more than 4 times likely to lose their allograft five years post-transplant (Loupy et al. 2013).

Alloantibody biomarker research is not only confined to the periphery blood. A molecular microscope system, which has been developed by the research team of Phillip Halloran,

combines microarray gene expression with biopsies to act as a diagnostic tool to assess ABMR. This new diagnostic system aims to eliminate misdiagnosis of ABMR, including C4d-negative ABMR, which are often misdiagnosed by pathologist with the current clinical biopsy system. With this system, biopsies are awarded an ABMR score based on a classifier set of gene transcripts. This score correlates with the presence of histologic lesions and DSA and is predictive of allograft loss. Overall, the goal of the molecular microscope system and the ABMR score is to assist pathologist in diagnosing difficult cases of ABMR and to help stratify patients at risk of allograft failure (Sellarés et al. 2013; Loupy et al. 2014; P. F. Halloran et al. 2013).

2.1.4.2.2.5 Urinary Biomarkers

While a great deal of the biomarker research has been performed in samples of peripheral blood or biopsies, there has been a significant drive in urinary biomarker discovery in the renal transplant field. As urine is a direct byproduct of the kidney, it was reasoned that the allograft would filter into the urine any cells and molecules involved in kidney damage and rejection. This hypothesis was described by Lee et al. by comparing the kidney allograft to an '*in vivo* flow cytometer' which could be used to detect acute cellular rejection (**Figure 9**) (Lee et al. 2014).

Many of the biomarkers that are evaluated in the serum are also assessed as urinary biomarkers. For example, NGAL as a biomarker of allograft injury has been widely investigated in both serum and urine, and researchers have found both to be justifiable sources for NGAL research (Hollmen et al. 2011). However, detection of urinary biomarkers is not as straightforward as in serum. Due to fluctuations in flow rate and hydration level, it is argued that in order for urinary biomarkers to be accurate and reliable, they must be normalized. Urinary creatinine is the most common parameter used to normalize protein biomarkers

(Waikar, Sabbisetti, and Bonventre 2010). Gene transcripts are normalized against total RNA concentrations or against endogenous controls (B. Li et al. 2001; Muthukumar et al. 2005).

The Cornell team lead by M. Suthanthiran has been at the forefront of investing urinary gene transcripts as biomarkers of acute and chronic renal allograft rejection. They have proposed perforin and granzyme-b, markers traditionally associated with effector T lymphocytes, and FOXP3, as biomarkers of renal allograft failure (B. Li et al. 2001; Muthukumar et al. 2005).

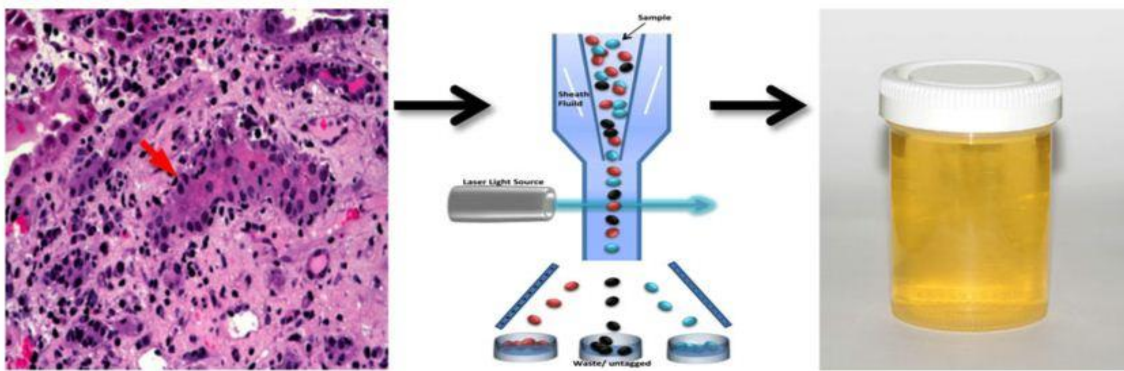


Figure 9. Kidney allograft analogous to an '*in vivo* flow cytometer'. The proposed rationale on why the urine is a promising resource for biomarker discovery is that allografts undergoing acute cellular rejection (left panel) will act similarly to a flow cytometer (center panel) and filter the cells and molecules involved in the rejection episode into the urine (right panel) (Lee et al. 2014).

While renal transplantation is considered the best treatment for end-stage renal disease, it is not a permanent cure because allograft rejection is always an impending fate. Clinical serotyping and immunosuppression regiments have now minimize early graft loss and extend allograft survival, but chronic allograft dysfunction is still a problem. Lymphocyte alloresponse has been shown to a key barrier to long-term allograft survival. Furthermore, despite the conventional belief that immunosuppressive regimens have dealt with the CD8 component of allograft loss, evidence has shown that there are CD8 T cells that escape immunosuppression and can cause allograft dysfunction and rejection. Therefore in order to gain insight in the role of CD8 cells in renal transplant recipients, it is important to examine the immunological mechanisms and functions of CD8 T cells.

2.2 CD8 T Lymphocytes

Commonly recognized as lymphocytes with mainly a cytotoxic function, CD8 cells have been shown to have an integral part in the immune response, especially against viral infections. Lymphocyte response begins when naïve CD8 cells encounter an antigen. This is followed by a clonal expansion of effector cells. Once the antigen has been cleared, the effector CD8 population will contract and long-lived memory cells are formed (**Figure 5**) (Murphy, Travers, and Walport 2008).

2.2.1 Naïve Cell Priming Gives Rise to Clonal Expansion of Effector Cells

Naïve CD8 cells circulate freely in the peripheral bloodstream in order to come into contact with numerous peptide:MHC complexes on APC. When a naïve cell recognizes the presented peptide, the cell is activated via three different types of signals (**Figure 10**). Signal 1 comes from the T cell receptor upon contact to the antigen peptide:MHC complex. Signal 1 is often not sufficient for CD8 T cells to mount a sustain immune response and thus, the cells require additional signaling. Costimulation from CD28-CD80/86 complex deliver signal 2, which is necessary for optimal clonal expansion. CD28 costimulation can be modulated by other costimulatory factors. CTLA-4 has a similar molecular configuration to CD28 and competes for binding with CD80/86, thereby regulating the strength of the T cell response. Contrarily, costimulation from CD40-CD40L complex has a promoter effect. When CD40L (CD40 Ligand), which is expressed on T cells, binds to CD40 on dendritic cells, it causes an upregulation in the expression of CD80/86, which in turn, binds to CD28 and strengthen to costimulation of CD8 T cells. While CD8 T cells do express CD40L, the upregulatory effect of the CD40-CD40L bond usually occurs through the assistance of helper CD4 T lymphocytes expression CD40L. Signal 3 is provided by cytokine stimulation, such as interleukin-2 (IL-2), interleukin-15 (IL-15), or the family of tumor necrosis factors (TNF)

which helps maintain T cell growth and proliferation and direct T cell differentiation (Murphy, Travers, and Walport 2008; Philip F Halloran 2004).

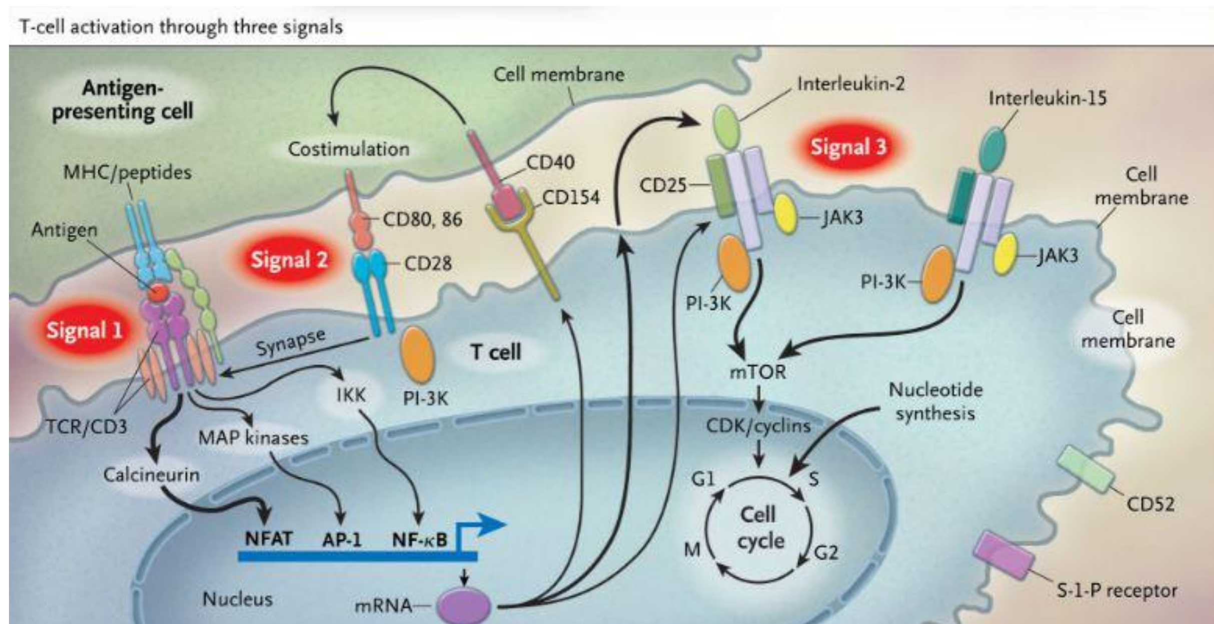


Figure 10. Lymphocytes receive three activation signals. Modified from Halloran 2004. (Philip F Halloran 2004)**.**

After a few days of proliferation, activated CD8 T cells differentiate into effector cells with cytotoxic functions. Once naïve cells have differentiated into effector cells, they no longer need costimulation to fulfill their effector functions, and as a result they begin to downregulate the expression of costimulatory molecules CD27 and CD28 (Murphy, Travers, and Walport 2008; Appay et al. 2002). Effector CD8 cells are also characterized by a loss of the surface marker CD45RA, an isoform of CD45, and an increased capacity to secrete cytotoxic effector molecules and proinflammatory cytokines. The cytotoxic molecules perforin, granulysin, and granzyme are stored within granules, which are released in a direct fashion at the target cell. Perforin form pores in the membrane of the target cell and is important for ensuring the passage of the other cytotoxic molecules into the target cell. Granulysin has antibacterial effects and can induce apoptosis in high concentrations.

Granzyme is family of potent cytotoxic molecules which induces apoptosis through various pathways. The most potent granzyme, Granzyme-B (GZMB), cleaves cellular substrates and induces apoptosis (Murphy, Travers, and Walport 2008; Hersperger, Makedonas, and Betts 2008; Voskoboinik, Whisstock, and Trapani 2015).

2.2.2 Models of Memory Formation

Once the infection is cleared, there is no longer a need for the large effector CD8 population. There is a contraction in the effector population and memory T cells are formed in order to retain immunological memory of the antigen (**Figure 11**). Memory T cell allow for a rapid immune response in cases of reinfection, as they require less stimulation compared naïve cells in order to activate and thus can be mobilize quickly. It has been demonstrated that naïve CD8 cells are incredibly plastic and can develop into all major T cell subsets *in vivo* (Stemberger, Huster, et al. 2007). However, there is still a lot unknown about the mechanisms which drive the memory formation process.

The CD8 memory compartment mainly consists of effector memory (EM) and central memory (CM) CD8 cells. Effector memory cells circulate in the peripheral blood stream and can rapidly mature into effector T cells when activated. Central memory cells remain mainly in the lymphoid regions and are sensitive to T cell receptor (TCR) cross-linking, but they are slower at differentiating into effector cells (Murphy, Travers, and Walport 2008; Kaech and Cui 2012).

There are several proposed mechanisms to explain how memory cells are differentiated and formed after an infection. The most commonly accepted model is a linear or unidirectional model where naïve cells give rise to effector cells, which in turn differentiate into EM and CM CD8 cells. Within this model there is also debate as to how EM and CM differentiate from effector cells. In the "signal-strength model", the strength of the activation signal (from

signals 1, 2 and 3) received by the naïve T cell will determine the specific effector phenotype, which will in turn affect their progeny. A strong signal will give rise to effector CD8 cells which will undergo cell death during the contraction phase; on the other hand, a weaker signal will give rise to EM or CM cells. Another potential linear differentiation model is where the heterogeneous memory population is formed due to the cumulative history of signals received during an infection, and that the repetitive stimulation from the antigen and various cytokines will give rise to different CD8 cell subsets.

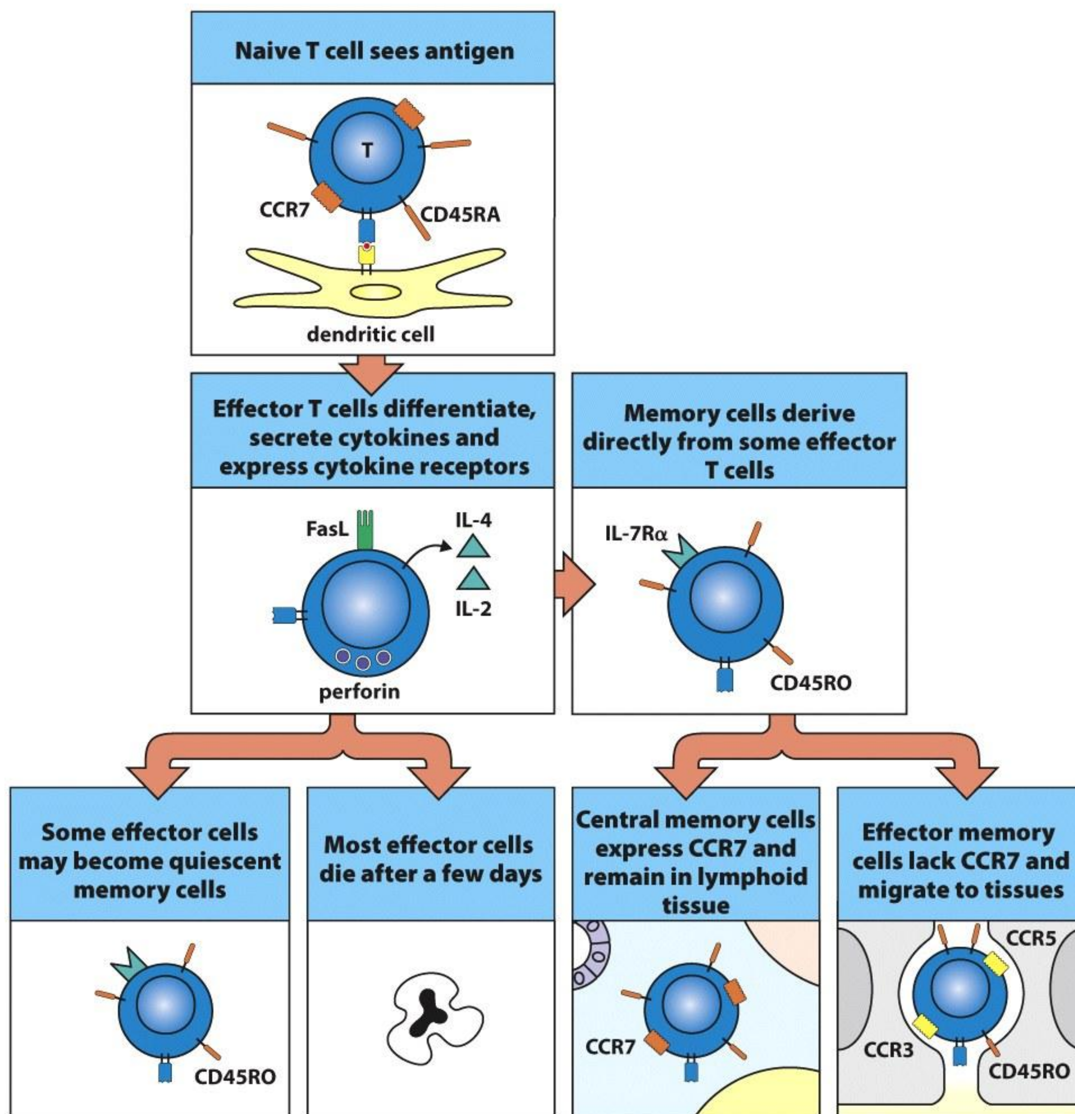


Figure 11. T cells differentiate into effector memory and central memory cells after an antigen-encounter (Murphy, Travers, and Walport 2008)**.**

The asymmetric model differs from the linear fate model in that it proposes that naïve CD8 T cells have a bi-directional fate that occurs at the first cell division after antigen stimulation. The naïve cell will divide unevenly and unequally distribute cellular components in the daughter cells based on proximity to the APC. The daughter cell that is distal from the APC will become a memory cell whereas the proximal cell is fated to becoming an effector CD8 cell (Parish and Kaech 2009; Kaech and Cui 2012; Stemberger, Neuenhahn, et al. 2007).

2.2.3 CD8 Subset Nomenclature

Distinguishing CD8 subset relies mainly on the expression of isoforms of CD45. Sallusto et al. proposed using a combination of CD45RA and lymph node homing marker CCR7 (also known as CD197) as a means to identify naïve, effector memory, central memory, and TEMRA CD8 cell subset (Sallusto et al. 1999) The combination of CD45RA and CCR7 is also widely used as a means of identify the different CD8 subsets (Appay et al. 2008; Sallusto et al. 1999). Furthermore, other groups have proposed other alternative nomenclatures. Costimulatory molecules CD27 and CD28 were described as being able to identify the different stages of CD8 effector memory differentiation in response to viral stimulation (Appay et al. 2002). Additionally, Hamann and colleagues used CD45RA and CD27 to identify naive, memory and effector-type cells (D Hamann et al. 1997; Dörte Hamann, Roos, and van Lier 1999). The CD45RA and CD28 nomenclature was also used in a similar fashion as the CD45RA and CD27 nomenclature as a means to identify effector CD8 cells from the memory and naive subsets (**Table 8**) (Tomiyama, Matsuda, and Takiguchi 2002; Tomiyama et al. 2004; Dörte Hamann, Roos, and van Lier 1999; Sobao et al. 2001). As a result of the extensive research done by many groups, several phenotyping data using different protein and transcription factor markers to identify unique $CD8^+$ cell subsets thus exist (Appay et al. 2008; Hersperger, Makedonas, and Betts 2008; Kaech and Cui 2012; Tomiyama et al. 2004; Dolfi et al. 2013).

Naïve	CM	EM	TEMRA
$CD45RA^{+}$	$CD45RA^{-}$	CD45RA-	$CD45RA^{+}$
$CCR7^{+}$	$CCR7^{+}$	$CCR7^{-}$	$CCR7^{-}$
$CD27^{+}$	$CD27^{+}$	$CD27^{+/-}$	$CD27^{-}$
$CD28^{+}$	$CD28^{+}$	$CD28^{+/-}$	$CD28^{-}$
$CD127^{+}$	$CD127^{+}$	$CD127^{low}$	$CD127^{-}$
$PERF^{-}$	$PERF^{-}$	$PERF^{+}$	$PERF^{+}$
$GZMb^{-}$	$GZMb^{-}$	$GZMb^{+}$	$GZMb^{+}$

Table 8. Phenotype of the different CD8 subsets.

2.2.4 Contrasting Portrayals of TEMRA Cells

Terminally-differentiated effector memory (TEMRA) CD8 T cells are a class of effector memory CD8 T cells who have a lower capacity for proliferation but still retain strong cytotoxic properties. These cells are characterized by their re-expression of CD45RA and their lack of expression of costimulatory molecules CD27 and CD28. The prevalence of TEMRA CD8 cells is associated with age and chronic viral infections; as a result, they are commonly labeled as replicatively senescent or clonally exhausted cells (Arosa 2002; Brenchley et al. 2003; G. C. Wang and Casolaro 2014; Shen et al. 2010). They have also have shortened telomere and reduced telomerase activity compared to CD8 T cells who express CD28 (Monteiro et al. 1996; Pawelec 2001; Pawelec et al. 2000). Furthermore, there is evidence that they play a role in various diseases and pathologies, such as systemic lupus erythematosus and multiple sclerosis (Kaneko et al. 1996; Salou et al. 2015) and that their presence is a barrier to bone regeneration and allograft tolerance (Reinke et al. 2013; Koyama et al. 2007; Yamada et al. 2012).

However, there is a branch of researchers who have contested the senescent label given to TEMRA CD8 cells. While the association of TEMRA CD8 prevalence and aging is not contested, there is growing evidence that the reduced telomerase activity and proliferative capacity in TEMRA CD8 cells may not be as straightforward as previously believed (Strioga, Pasukoniene, and Characiejus 2011). Chong et al. showed the OKT3 and IL-2 could stimulate

proliferation in $CD8^+CD57^+$ TEMRA cells and proposed that CFSE, a common tool used to track cellular proliferation, was the reason that other researchers did not observe proliferation in their TEMRA populations. To support this claim, Chong et al. used both CFSE and [^{3}H]thymidine to track the proliferation of their cells. They found that in proliferative assay that used [^{3}H]thymidine, the $CD8^+CD57^+$ cells proliferated very successfully in response to OKT3 or IL-2. On the other hand, in the assays which used CFSE, there were large increases in the percentage of $CD8^+CD57^+$ cells that were apoptotic or dead. Additionally, CFSE toxicity did not affect $CD8^+CD57^-$ cells, and this discrepancy in the affects of CFSE toxicity may have lead to the hypothesis that TEMRA CD8 cells are clonally exhausted. Chong et al. also suggested that the culture medium also played a key role to proliferative success of the TEMRA subset, as previous experiments which showed the reduced proliferative capacities of TEMRA cells involved culture in fetal calf serum, whereas Chong et al. used human AB serum for their experiments (Chong et al. 2008). Also, it has been put forward that type of stimulation play a role in TEMRA CD8 cell proliferation. Chiu et al. showed that IL-15 induced proliferation in $CD8^+CD28^-$ TEMRA cells that was comparable to $CD8^+CD28^+$ cells. Additionally, they showed that IL-15 was important to the generation and regulation of the $CD8^+CD28^-$ population, in that IL-15 caused increase in tumor necrosis factor-α (TNFα), which in turn, caused a downregulation in the expression of CD28. Also, IL-15 induced the expression of macrophage inflammatory protein-1β (MIP-1β), a chemokine which has an inhibitory affect on $CD8^+CD28^-$ cells, but not $CD8^+CD28^+$ cells, proliferation. Furthermore, Plunkett et al. showed that irradiated APC could induce telomerase activity and proliferation in $CD8^+CD28^-$ cells (Plunkett et al. 2007). CD134, CD137, and CD278 costimulatory molecules were all upregulated on $CD8^+CD28^-$ and that these molecules could induce telomerase activity in CD8 T cells (Plunkett et al. 2007; Kober et al. 2008).

The increased interest in CD8 regulatory T cells (Treg) in the recent years has further added to the doubt surrounding the proper characterization of TEMRA CD8. While the term Treg are normally associated with $CD4^{+}FOXP3^{+}$ cells, there has been evidence of CD8 lymphocyte populations which also possessed immunosuppressive features. However, there is still disagreement to the phenotypic characteristics of this subset of CD8 cells, and among the various descriptions used to identify these cells ($CD8^{+}FOXP3^{+}$, $CD8^{+}CD45RC^{low}$, etc.), one of the popular phenotypes used is $CD8^{+}CD28^{-}$ (Ligocki and Niederkorn 2015; Xystrakis et al. 2009; Suzuki et al. 2012; Liu et al. 2007). Unfortunately, this is also a description commonly used for TEMRA CD8 cells. $CD8^{+}CD28^{-}$ $FOXP3^{+}$ Treg activity is MHC class I restricted and they cause APC to become tolerogenic by inducing the increased expression of immunoglobulin-like transcript 3 (ILT3) and 4 (ILT4). These tolerogenic APC, in turn, induce naïve CD4 T cells to differentiate into CD4 Treg cells, whose regulatory activities suppress the immune response. On the other hand, $CD8^{+}CD28^{-}FOXP3^{-}$ Treg cells inhibit APC activity and T cell proliferation, and secrete IL-10, a cytokine that suppresses proinflammatory responses (Suciu-Foca et al. 2005; Strioga, Pasukoniene, and Characiejus 2011). As it is widely accepted that TEMRA CD8 cells are cytotoxic in function, the emergence of these suppressive cells indicates that the immunological role of TEMRA is not as simple as formerly believed (Strioga, Pasukoniene, and Characiejus 2011).

The role of CD8 T cells in the immune response to viral infections has long been established. However, our understanding into the role they play in various pathologies, including transplantation, continues to expand and places emphasis on the importance of investigating the differentiation of the CD8 subsets. TEMRA CD8 cells have gain significant attention because of their strong cytotoxic properties and senescent characteristics, which are both associated with a variety of pathologies and conditions. However, emerging research suggests that their immunological function extends beyond cytotoxicity and that, despite their name, they are not terminally-differentiated.

Therefore, in order to fully understand the functionality and mechanisms of CD8 cells, there has been a resurgence of interest in examining the metabolic pathways involved in CD8 cell differentiation and immunological function.

2.3 Cellular Metabolism

The purpose of cellular metabolism is to provide energy for cellular functions. Additionally, metabolic intermediates are used for biosynthesis. Glycolysis and oxidative phosphorylation are the two main energetic pathways that cells rely on for most metabolic functions. Cells can also catabolize other molecules, such as glutamine or fatty acids, for energy production and macromolecule biosynthesis (**Figure 12**) (Nelson and Cox 2005; MacIver, Michalek, and Rathmell 2013).

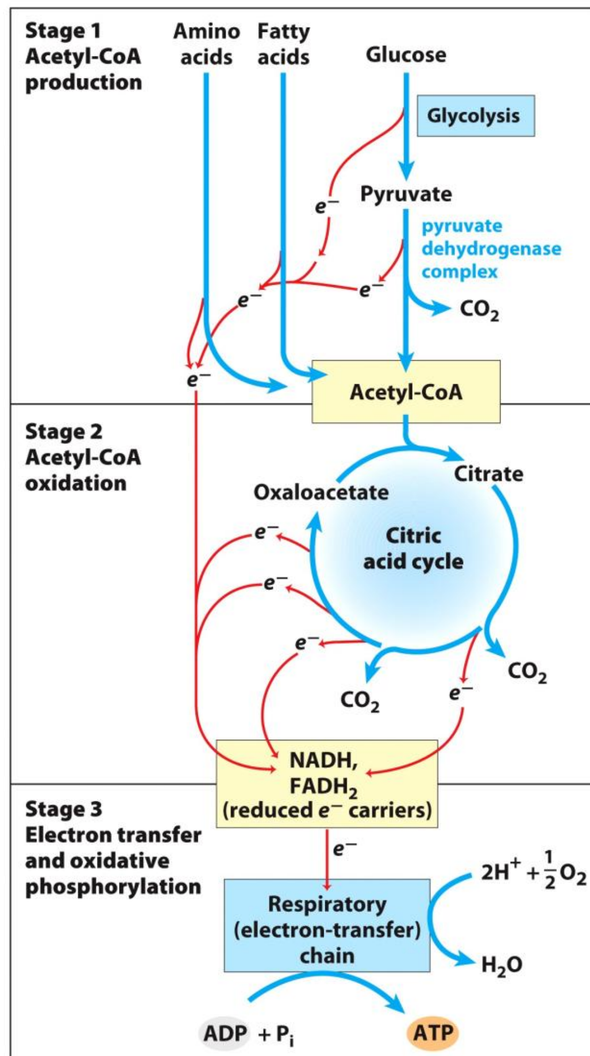


Figure 12. Overview of the metabolic pathways available for energy production (Nelson and Cox 2005).

2.3.1 Glucose Metabolism

Glucose is the main energy source that most organisms utilize for energy production, and its catabolic pathways are widely conserved in all cells types. Glucose can be metabolized in both aerobic and anaerobic conditions, though the resulting byproducts from these two different conditions differ (Nelson and Cox 2005). Glycolysis is one of the main catabolic pathways cells use to break down glucose. In aerobic conditions, after a glucose molecule is metabolized, the resulting pyruvate molecules are transported into the mitochondria for oxidative phosphorylation. However, in oxygen low environments, pyruvate usually undergoes lactic acid fermentation (Nelson and Cox 2005).

2.3.1.1 Glycolysis

Glycolysis is a six step metabolic pathway used by cells to convert the six carbon glucose molecule into two three carbon pyruvate molecules and to produce a net of two adenosine 5'-triphosphate (ATP) and two nicotinamide adenine dinucleotide (NADH) molecules. Glycolysis consists of two phases: a preparatory phase and a payoff phase (**Figure 13**). In the preparatory phase, glucose is first phosphorylated by hexokinase into glucose-6-phosphate, which undergoes a conversion into fructose 6-phosphate by phosphohexose isomerase. The fructose 6-phosphate is phosphorylated again by phosphofructokinase-1 before being cleaved by aldolase into two 3-carbon sugar molecules (glyceraldehydes 3-phosphate and dihydroxyacetone phosphate). The dihydroxyacetone molecule is isomerized into a second glyceraldehydes 3-phosphate molecule, which is oxidized and phosphorylated into 1,3-bisphosphoglycerate in the next step. This oxidation and phosphorylation step results in NADH formation, with two molecules of NADH forming for each molecule of glucose. The final few steps of glycolysis augment the energy production as four molecules of ATP are produced. Two ATP molecules are produced from the conversion of 1,2-bisphosphoglycerate

into 3-phosphoglycerate, which is converted into 2-phosphoglycerate and then into phosphoenolpyruvate. The final enzyme, pyruvate kinase, converts phosphoenolpyruvate into pyruvate and also generates two ATP per glucose. By the end of glycolysis, one glucose molecules produces a net of two ATP (while four ATP were generated, two ATP are required for the priming of fructose 1,6-bisphosphate), two NADH, and two pyruvate molecules (**Figure 13**) (Nelson and Cox 2005).

This process occurs in the cellular cytoplasm and the final product pyruvate is either further processed into lactate or otherwise transported into the mitochondria where it is converted into acetyl-coenzyme A (acetyl-CoA). Acetyl-CoA enters the tricarboxylic acid (TCA) cycle, also known as the Krebs cycle, where it is broken down into CO_2 (MacIver, Michalek, and Rathmell 2013; van der Windt and Pearce 2012; Nelson and Cox 2005).

2.3.1.2 Lactic Acid Fermentation

If pyruvate is not shuttled into the mitochondria for oxidative phosphorylation (OXPHOS), then it is converted into lactate. This procedure usually occurs in anaerobic conditions; however, Otto Warburg has observed that cancer cells also use this metabolic pathway in oxygen rich conditions and since his initial observation, other researchers have made similar observation in other cell types (Warburg 1928; MacIver, Michalek, and Rathmell 2013).

In lactic acid fermentation, lactate dehydrogenase converts pyruvate into lactate (**Figure 14**). During this process, NAD^+ is regenerated from NADH. The replenishment of the NAD^+ pool is the main goal of lactic acid fermentation. This is because during the glycolytic process, NAD^+ generates NADH, but if pyruvate does not undergo OXPHOS, the NADH will not be re-converted back into NAD^+, thus drastically diminishing the overall pool of NAD^+ (Nelson and Cox 2005).

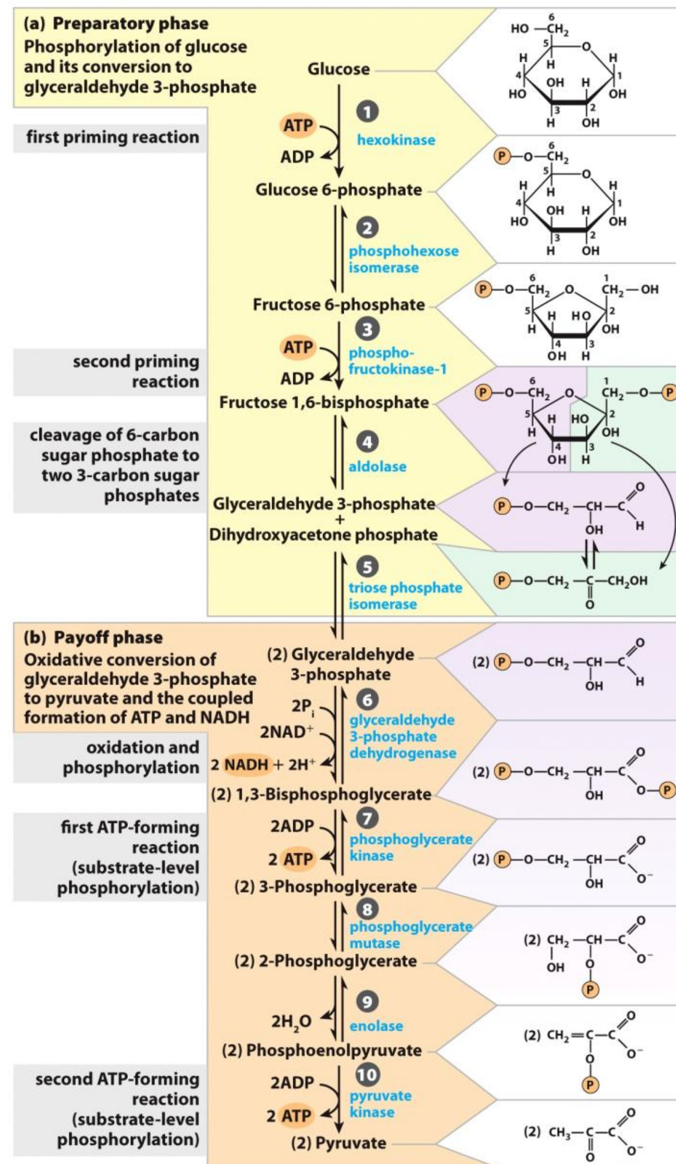


Figure 13. Overview of Glycolysis (Nelson and Cox 2005).

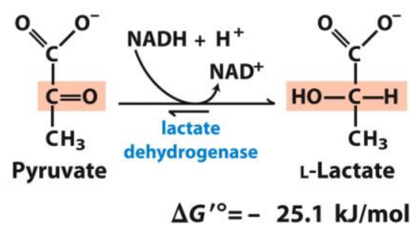


Figure 14. Overview of Lactic Acid Fermentation (Nelson and Cox 2005).

2.3.1.3 The Citric Acid Cycle (TCA)

The main purpose of the citric acid cycle, also known as the Krebs cycle or the tricarboxylic acid cycle, is to take the fragments of glucose, fatty acids, and amino acid molecules and enzymatically oxidize them (**Figure 12**). This process releases energy in the form of energy-rich electrons and protons, which are bond to carriers NAD^+ and FAD to form NADH and $FADH_2$, respectively (Lunt and Vander Heiden 2011; Nelson and Cox 2005).

Before the TCA can continue the metabolism of a glucose molecule, the resulting pyruvate from glycolysis must be converted into acetyl-coenzyme A (acetyl-CoA). Pyruvate dehydrogenase converts pyruvate into acetyl-CoA, which is then shuttled into the mitochondria (Schell and Rutter 2013; Nelson and Cox 2005).

Once in the mitochondrial matrix, acetyl-CoA enters into the TCA by giving its acetyl group to oxaloacetate to form citrate. Citrate becomes isocitrate which becomes α-ketoglutarate. A-ketoglurate is a common biosynthetic precursor and can be removed from the TCA at this point if the cell requires (Wasinski et al. 2014; van der Windt and Pearce 2012). If not, α-ketoglutarate becomes succinate. Through several more enzymatic steps, succinate becomes fumarate, which becomes malate, and finally transforms back into oxalacetate, which in turn can react with a second acetyl-CoA molecule and initiate the cycle again (**Figure 15**) (Nelson and Cox 2005). Throughout one turn of the TCA cycle, three molecules of NADH, one of $FADH_2$, and one of ATP are produced (Nelson and Cox 2005).

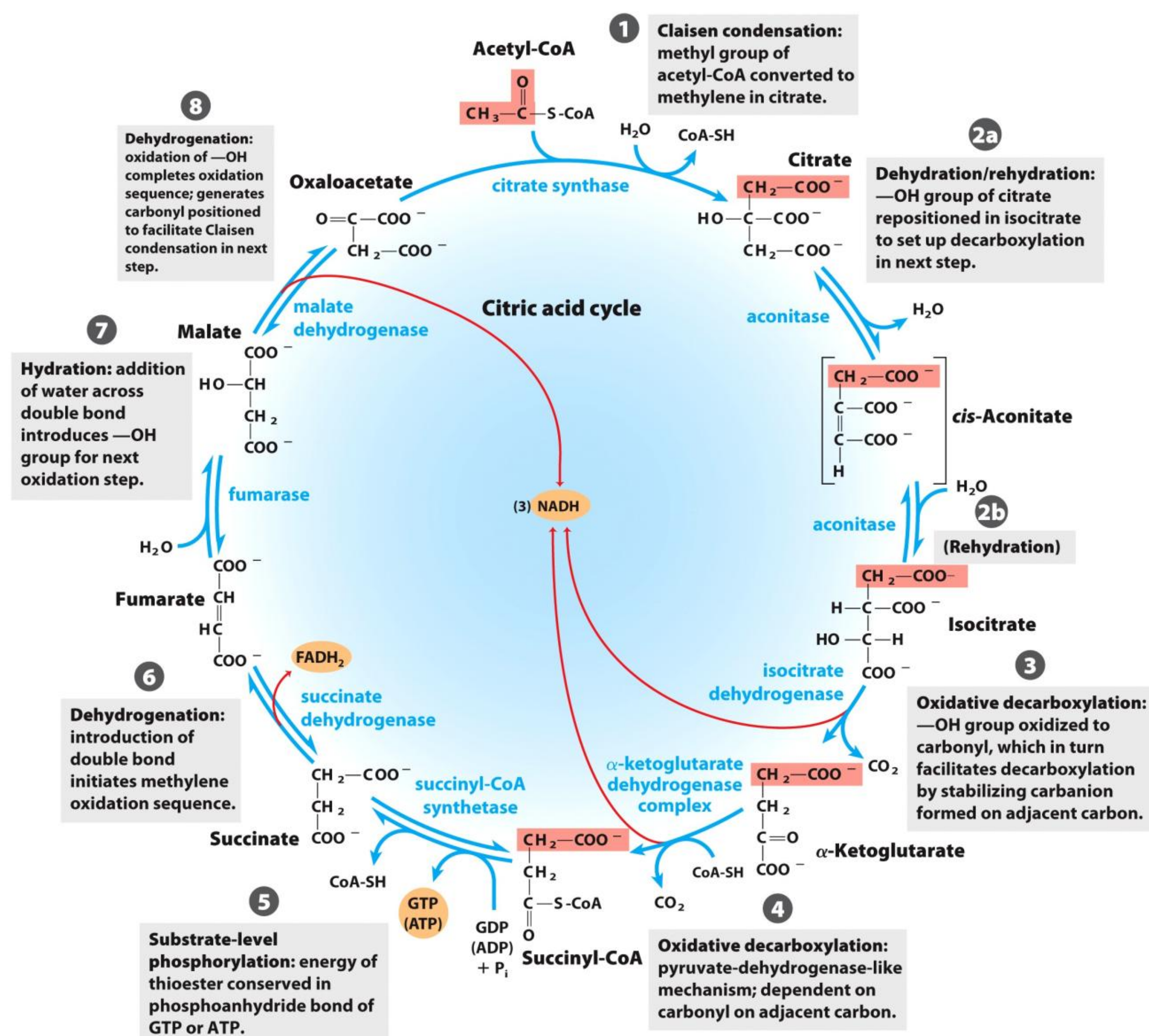


Figure 15. Overview of the Citric Acid Cycle (Nelson and Cox 2005).

2.3.1.4 Oxidative Phosphorylation and the Electron Transport Chain

The final and most energy-rich step of cellular respiration is oxidative phosphorlylation (OXPHOS), where the electrons in the NADH and $FADH_2$ are used to synthesize ATP. This process takes place in the inner membrane of the mitochondrial, which is impermeable to protons and small ions, thus maintaining mitochondria polarization (**Figure 16**). The mitochondrial membrane potential is essential to OXPHOS because it is the flow of electrons

through the electron transport chain (ETC), a chain of five membrane-bound enzyme complexes that allows for the phosphorylation of ADP into ATP.

Electrons are freed from NADH and FADH2 at Complex I while the resulting freed H+ protons are pumped into the intermembrane space, thus causing a gradient to form in the mitochondria. The electrons decoupled from NADH are bound by the different enzyme complexes to a series of electron carriers, beginning with ubiquinone, continuing through a series of cytochrome proteins (in the order of cytochrome b, c_1, c, a, and finally a_3) before finally being bound to O_2. The combined chemical and electrical gradient formed by the electron flow creates a strong proton-motive force which drives protons back into the mitochondrial matrix via ATP synthase, which in turn provides the energy needed to phosphorylate ADP into ATP (**Figure 16**) (Nelson and Cox 2005; Krauss, Israel, and Medical 2001).

The total net ATP generated through aerobic metabolism of one molecule of glucose is 30 or 32 ATP. This discrepancy in final ATP count is due to the shuttle systems used to transport the two molecules of NADH generated in the cytoplasm during glycolysis into the mitochondria. In liver, kidney, and heart cells, a malate-aspartate shuttle is used to transport NADH into the mitochondrial matrix. By using this shuttle system, these cells can recover a net 32 ATP from one molecule of glucose. However, the skeletal muscle and the brain use a slightly less efficient glycerol 3-phosphate shuttle to transport NADH into the mitochondria. This shuttle delivers the electrons from NADH to Complex III, instead of Complex I, and thus results in a net of 30 ATP per glucose molecule (Nelson and Cox 2005).

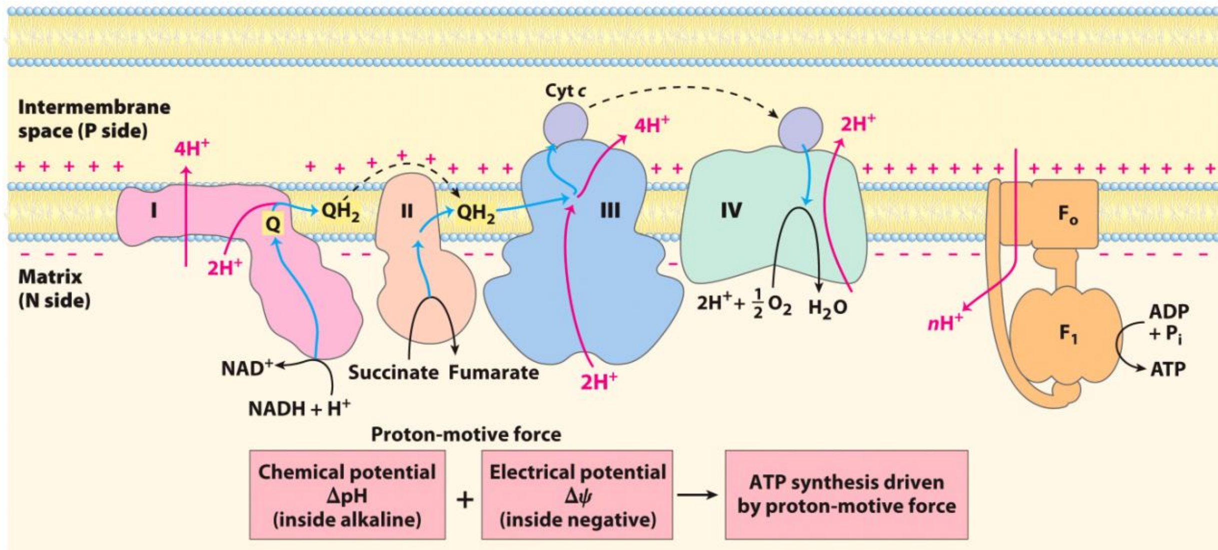


Figure 16. Overview of the Electron Transport Chain (Nelson and Cox 2005).

2.3.2 Alternative Energy Sources

During homeostasis and quiescence, cells will prioritize glucose metabolism to fulfill their energy and biosynthetic needs. However, there are times, such as times of stress, starvation, or when a cell are differentiating and thus have different metabolic needs, when alternative energy sources are metabolized (van der Windt and Pearce 2012).

2.3.2.1 Glutaminolysis

In order for amino acids to be catabolized, they must be converted into carbon intermediates which will enter the citric acid cycle. Glutamine is an abundant non-essential amino acid that enters the TCA α-ketoglutarate. Glutamine plays an important anaplerotic role in the metabolic programming of lymphocytes. Glutaminolysis begins when glutaminase converts glutamine into glutamate, a closely-related amino acid, which is in turn converted into α-ketoglutarate by glutamate dehydrogenase (Nelson and Cox 2005). Glutamine is transported into lymphocytes via the SLC (solute carrier) protein families; however, little is known about the transporter responsible for shuttling glutamine from the cytoplasm into the mitochondria (Pochini et al. 2014).

2.3.2.2 Fatty Acid Oxidation

Long-chain fatty acids have high energetic potential, where one molecule of palmitate has the potential net yield of 106 molecules of ATP (**Figure 17**). Before fatty acids can be oxidized in the mitochondria, they must be broken down into acetyl-CoA by a process called β oxidation. First, fatty acids longer than 12 carbon lengths are converted into a fatty acyl-CoA and transported into the mitochondria by carnitine palmitoyltransferase 1 (CPT1) (**Figure 17A**). Shorter fatty acids do no need to be actively transported into the mitochondria. Once inside the mitochondria, fatty acids undergo the four-step β oxidation where a two-carbon acetyl group is removed for the fatty acid chain to form acetyl-CoA. In addition to acetyl-CoA, β oxidation also produces one molecule of NADH and one of $FADH_2$. A 16-carbon palmitate molecule will yield 8 molecules of acetyl-CoA, 7 of NADH, and 7 of $FADH_2$ after β oxidation (Nelson and Cox 2005; Kennedy and Lehninger 1949).

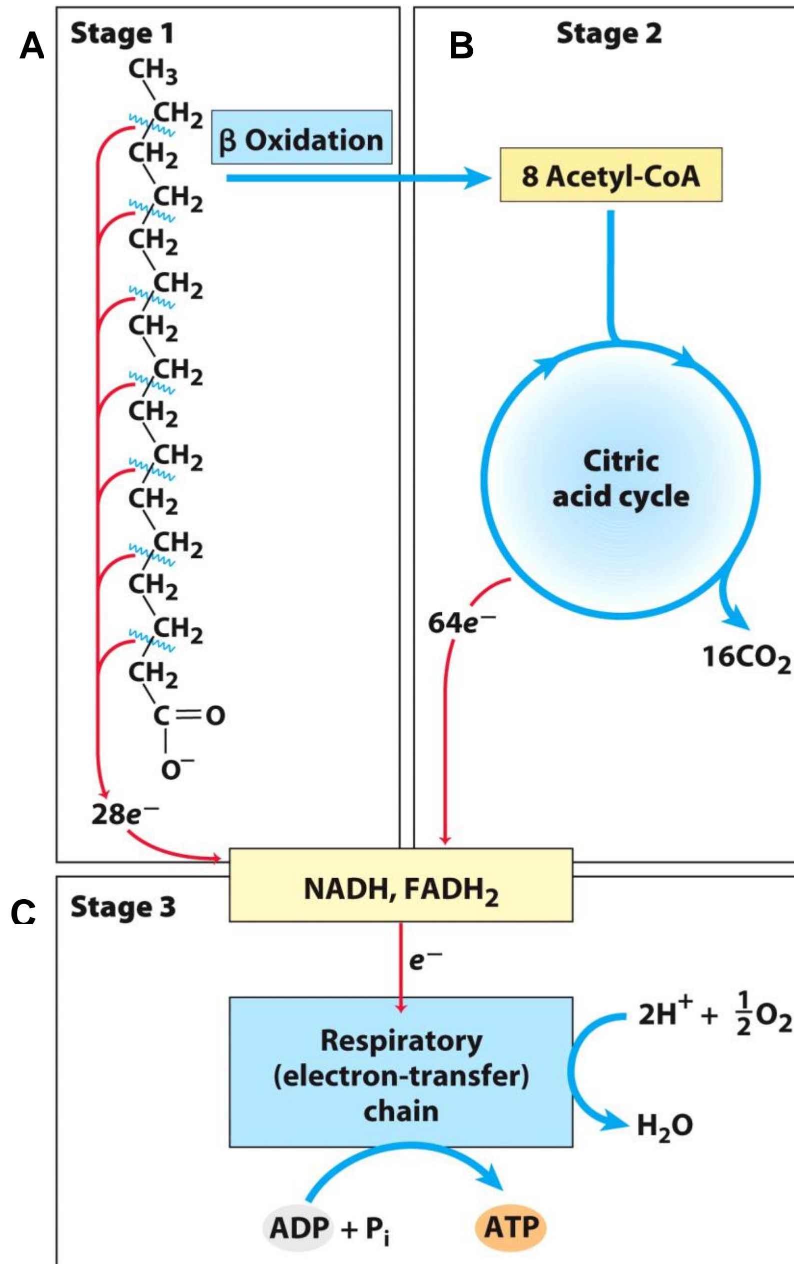


Figure 17. The Stages of Fatty Acid Oxidation (Nelson and Cox 2005). (A) β oxidation breaks down long-chain fatty acids into acetyl-CoA molecules (B) which enter into the TCA for metabolism. (C) The resulting metabolic components undergo OXPHOS to produce ATP.

2.3.3 CD8 T Cell Metabolic Programming

2.3.3.1 Metabolic Demands in Quiescent T Cells

In resting state, naive T cells rely on oxidative phosphorylation (OXPHOS) to produce ATP (Guppy, Greiner, and Brand 1993). This metabolic pathway is the most effective method to generate ATP by catabolizing metabolic intermediates derived from glucose, fatty acids, and amino acids. The low rate of energy metabolism needed by quiescent cells to maintain their housekeeping functions is instructed by cytokine-dependent signals, and preferentially IL-7 mediated signals. Indeed, IL-7 is a crucial cytokine to sustain the homeostasis of naïve cells and their survival and is also an important regulator of the glucose transporter GLUT1 (Wofford et al. 2008). The IL-7 receptor (IL-7R) is largely expressed in naïve lymphocytes and downregulated when naïve CD8 T cells differentiate into the effector phenotype. IL-7 causes JAK1 and 3 activation, which in turn activates STAT5 and the PI3K/AKT pathways (Wofford et al. 2008; Pallard et al. 1999; Crawley et al. 2014). IL-7 allows a basal glucose metabolism which has been shown to be essential for quiescent T cell survival (Wofford et al. 2008).

In addition to mitochondrial glucose oxidation, resting T cells generate energy through the β-oxidation of fatty acids, and especially quiescent memory subsets (**Figure 18A**). Fatty acid oxidation is integral to the development of memory T cells and when FAO is impeded, the memory CD8 T cell population is heavily affected (Lochner, Berod, and Sparwasser 2015; O'Sullivan et al. 2014). Pearce et al. showed that mice which lack tumor necrosis receptor-associated factor 6 (TRAF6) cannot upregulate FAO, and while these mice were able to mount a normal effector CD8 response, they were unable to form a CD8 memory population after bacterial infection (Pearce et al. 2009). Additionally, TRAF6 deficient CD8 cells could not properly activate AMP-activated kinase (AMPK); however, Metformin, an AMPK

activator, was able to rescue FAO in the TRAF6 deficient CD8 cells and promote the development of the memory subset (Pearce et al. 2009). The generation of potent CD8 memory T cells is also dependent on mammalian target of Rapamycin (mTOR) related signaling since Rapamycin, a mTOR inhibitor, promotes memory T cell differentiation (Araki et al. 2009; Sipula, Brown, and Perdomo 2006). Rapamycin treatment during the CD8 expansion phase has been shown to increase the resulting quantity of CD8 memory cells; furthermore, Rapamycin given during the contraction phase results in memory CD8 cells that are highly functional and can mount a better memory response (Araki et al. 2009; Xu et al. 2012; Araki, Youngblood, and Ahmed 2010).

2.3.3.2 Activation of CD8 T Cells Leads to Metabolic Reprogramming.

Proliferation and potent effector functions are an energetically demanding process that requires a metabolic adaptation in order to fulfill the needs of T cells. Upon activation, CD8 T cells reprogram their metabolism from OXPHOS to aerobic glycolysis and glutaminolysis (**Figure 18B**) (MacIver, Michalek, and Rathmell 2013). This glycolytic shift supports the rapid proliferation of activated lymphocytes in terms of energy requirement but also in terms of metabolic intermediates (Lunt and Vander Heiden 2011). These metabolic intermediates allow the biosynthesis of macromolecules essential for the massive cell growth and proliferation required as effector CD8 cells expand during the immune response. For example, these intermediates generated from the glycolytic pathway are used to generate nucleotides, amino acids, and lipids which in turn will be used to synthesize nucleic acids, lipids and proteins needed in order to duplicate the cellular biomass (Vander Heiden, M. G., Cantley, L.C., Thompson 2009; Lunt and Vander Heiden 2011).

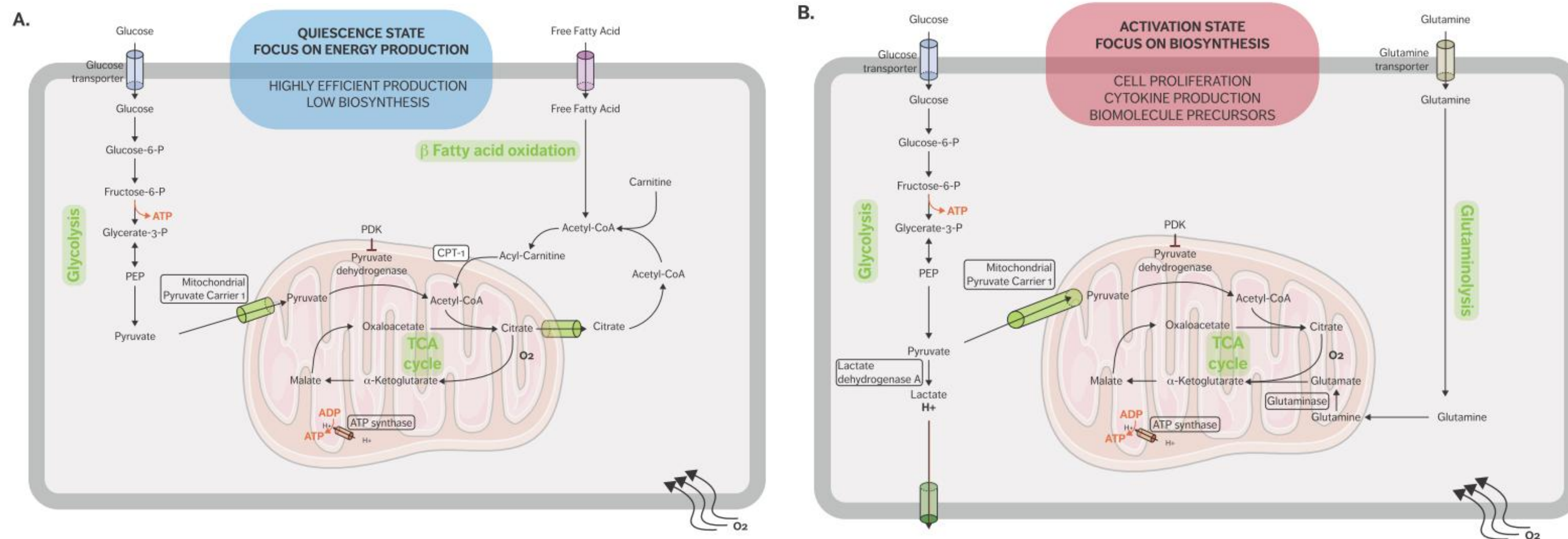


Figure 18. Metabolic Pathways Used by CD8 T Cells in Quiescent and Active States.

A. When in a quiescent state, naïve CD8 T cells fulfill their energy needs mainly through aerobic glycolysis and mitochondrial respiration. Fatty acid oxidation plays an important role during the restriction phase when effector CD8 cells differentiate into memory CD8 cells and the immune system returns to a quiescent state.

B. When stimulated, effector CD8 T cells rapidly upregulate glycolysis and glutaminolysis because the activated cells require many of the metabolic intermediates for macromolecule biosynthesis. The metabolic adaptations sustain the rapid expansion of effector CD8 T cells and support the secretion of cytokines and cytotoxic molecules.

T cell activation requires two signals: one from the T cell receptor (TCR) and the other from costimulatory receptors. TCR signaling alone is not sufficient for inducing major changes to the metabolic programming (Frauwirth et al. 2002).The increased glucose metabolism seen in activated lymphocytes is due to CD28 costimulation signaling, which in turn activates the PI3K/AKT pathway (**Figure 19**) (Frauwirth et al. 2002; Jacobs et al. 2008; Parry et al. 1997), leading to an increase of nutriment uptake, cell surface expression and function of glucose transporter. However, it is becoming apparent that there are specific metabolic adaptations dependent on the T cell subset. Gubser et al. showed that the effector memory CD8 T cells are able to switch to glycolysis more rapidly as compared to naïve CD8 T cells (Gubser et al. 2013). Furthermore, effector memory CD8 T cells are able to sustain this higher glycolytic rate. The drastic diversion of energy generation from OXPHOS to aerobic glycolysis induced by T cell activation may be over-simplified as a higher mitochondrial mass of memory CD8 T cells compared to naïve CD8 T cells has been reported and linked to a higher enhancement of both OXPHOS and glycolysis in memory CD8 T cells (van der Windt et al. 2013).

In addition to increase glycolytic activity, activated T cells also upregulate glutamine metabolism (glutaminolysis). Glutamine, a common amino acid found in human plasma, can serve as an alternative source of energy. Activated lymphocytes require glutamine for cell proliferation and cytokine secretion, and CD28 costimulation enhances glutamine uptake and increases glutamine transporter expression (Carr et al. 2010; van der Windt and Pearce 2012). Several metabolic TCA cycle intermediates such as citrate, α-ketoglutarate, oxaloacetate are crucial for proliferation as precursor for lipid and amino acids synthesis. Glutamine undergoes anaplerotic reaction to produce oxaloacetate and α-ketoglutarate metabolism through glutamate. Glutaminolysis also replenishes NADPH pool, which is also needed by proliferating lymphocytes to support lipid and nucleotide biosynthesis as well as maintaining the redox balance (van der Windt and Pearce 2012; Wasinski et al. 2014).

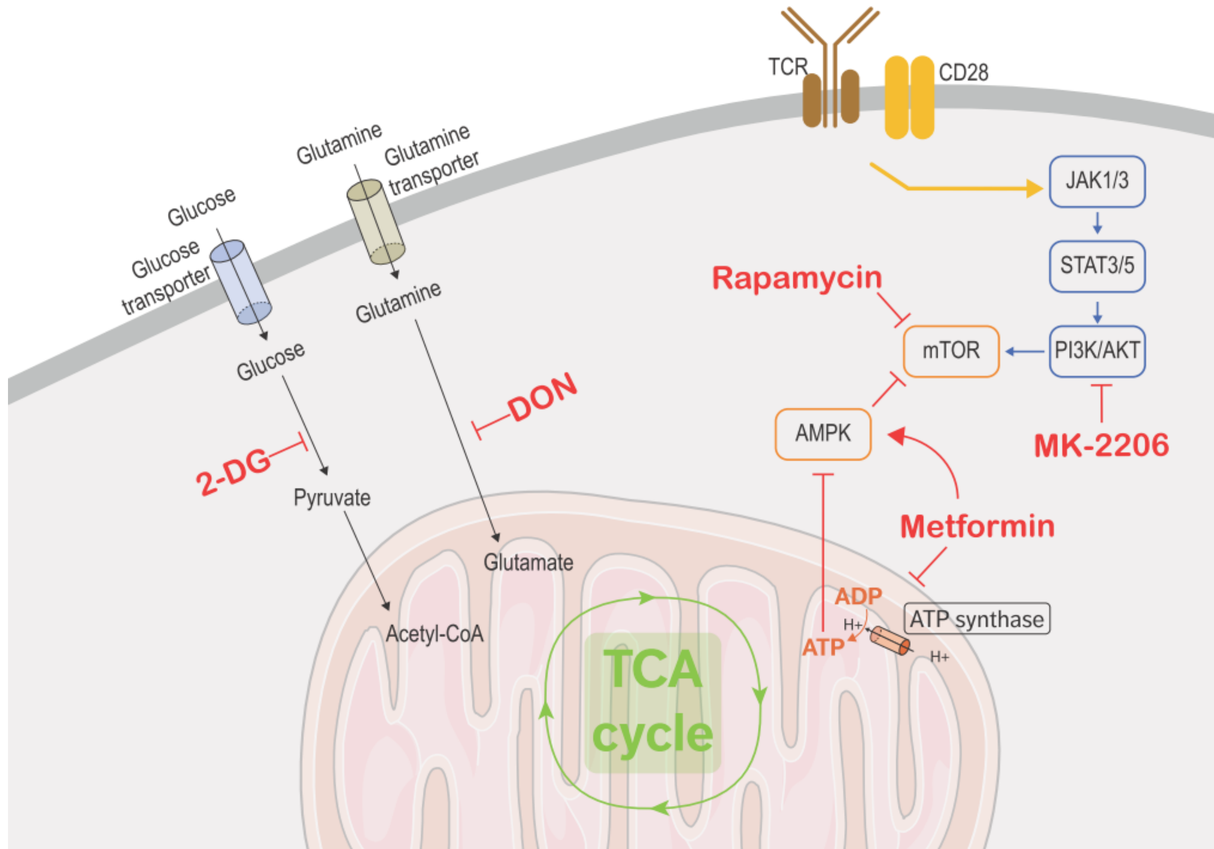


Figure 19. Immunometabolic targets. This figure depicts the different metabolic and transcriptional pathways involved in the immunometabolic functions of CD8 T cells. Black arrows are used to show the transport of glucose and glutamine into the cells where they are metabolized. The yellow arrow represents the combined signaling of the TCR and CD28 which activates CD8 effector cells and triggers the metabolic adaptations in the effector cells. The blue arrows show the transcriptional used by activated CD8 T cells to upregulate mTOR, which controls cellular metabolism and growth. Red arrows show the promotion or suppression of therapeutic molecules on the various immunometabolic pathways.

2.3.3.3 Metabolism and Immunomodulation

2.3.3.3.1 Targeting Bioenergetic Pathways

As previously discussed, glycolysis and glutaminolysis are two key metabolic pathways which are imperative for proper CD8 effector function. 2-Deoxy-D-glucose (2-DG) is a glucose analog that inhibits glycolysis by blocking hexokinase function. Many studies showed the potent effect of 2-DG in inhibiting the cytotoxic function of effector CD8 cells (O'Sullivan and Pearce 2015; Cham and Gajewski 2005; Gubser et al. 2013; Sukumar et al. 2013). In a similar fashion, blocking glutaminolysis with a glutamine antagonist such as 6-diazo-5-oxo-l-norleucine (DON) inhibits lymphocyte proliferation (R. Wang et al. 2011). Additionally, blocking glucose and glutamine transporters disrupt lymphocyte activation and affect memory differentiation (Sinclair et al. 2013; Singer et al. 2011; Anastasiou et al. 2011; Macintyre et al. 2014).

Targeting major bioenergetic pathways seems at first glance a rather dangerous mean to control an immune response and major side effects such as a massive toxicity to normal tissue is expected. Animal models and ongoing therapeutic use of metabolic interferences therapies prove that such strategy is safe and feasible. For instance, *leflunomide*, a molecule from which teriflunomide is the active metabolite, prevents *de novo* biosynthesis of pyrimidine and efficiently inhibit the development of EAE (Korn et al. 2004). In Multiple Sclerosis patients, teriflunomide, compared to a placebo, significantly reduces relapse rates, disability progression (at the higher dose), and MRI evidence of disease activity (O'Connor et al. 2011). Interestingly, whereas a link with metabolism has not been established, Leflunomide or analogues have also been shown to be effective in prolonging graft survival and even to induce tolerance in a model of heart allograft transplantation (Le Texier et al. 2011).

2.3.3.3.2 Targeting Transcriptional Regulators of Immunometabolism

Since metabolic adaptation is required to support T cells activation and function, nutrient availability or limitation will affect these processes. A recent publication has shown that adenosine monophosphate-activated protein kinase (AMPK) couples T cell function to nutrient availability (Blagih et al. 2015). AMPK is a serine-threonine kinase that is sensitive to energy levels and is activated during cellular stress. By sensing the AMP/ATP ratio, AMPK senses energy deficiency and favors pathways leading to ATP production while inhibiting ATP-consuming pathway. It increases catabolic processes and inhibits anabolic processes to increase ATP production when activated. AMPK upregulates fatty acid β-oxidation by promoting the transfer of long chain fatty acids into the mitochondria via carnitine palmitoyltransferase 1 (CPT1) (MacIver, Michalek, and Rathmell 2013). Metformin, a drug commonly used in diabetes treatment, blocks mitochondrial complex I, which has the downstream effect of promoting AMPK activity. Interestingly, Metformin fosters memory CD8 T cell differentiation in mice(Pearce et al. 2009). In agreement with these results, it has been shown that autoreactive T cells can be efficiently control by the co-administration of 2-DG and Metformin in a mouse model of Systemic lupus erythematosus (SLE) (Yin et al. 2015). However, as Meformin also inhibits OXPHOS, in vitro or in vivo administration is likely to have a broader target than solely the memory compartment.

While using metabolic interference as a medical treatment has been mainly studied in the cancer field, given the similarity of metabolic adaptation between cancer cells and activated T cells, immunometabolic regulation of CD8 T cells could be used as a mean to manipulate the CD8 T cell immune function for effective immunosuppression.

3 Results

3.1 Thesis Overview

Kidney transplantation is the best treatments for end-stage renal disease. Unfortunately, due to the risk of allograft rejection, patients need to endure life-long immunosuppression regiments. Furthermore, as the causes of chronic allograft rejection are not fully understood, chronic allograft rejection endangers the long term survival of the allograft. With the current clinical biomarkers available, rejection episodes are not detected until after they have begun to do damage to the allograft. Therefore, it is a pressing concern in the transplant community to further our understanding of the mechanisms of allograft rejection and to find new biomarkers with better reliability and predictive power.

Miqueu et al. showed that chronic kidney allograft rejection was associated with an altered peripheral TCR Vβ repertoire and an increased $CD8^+/CD4^+$ T cell ratio. Tolerant patients did not have skewed repertoires and stable patients exhibited a heterogeneous mixture of repertoire usage, ranging from unaltered to altered (Miqueu et al. 2010). Additionally, Brouard et al. showed that alterations in the TCR Vβ repertoire in transplant recipients is mainly in the CD8 compartment (Brouard et al. 2005). Furthermore, Baeten et al. demonstrated that cytotoxic CD8 T cells were involved in chronic rejections (Baeten et al. 2006). Cumulatively, this has shown that CD8 T cells play an important role in the health of kidney allografts.

The main goal of this thesis was to investigate the role of CD8 T cell subsets and the involvement of their immunometabolic programming in kidney transplant recipients and evaluate the possibility of using CD8 T cells as biomarkers of allograft rejection.

This thesis was divided into two parts. The first part was focused on characterizing the distribution of CD8 T cell subsets in the periphery of long term stable kidney transplant patients (Article 1) and evaluated the use of TEMRA CD8 T cells as predictive biomarkers of

long term allograft rejection (Article 3). Additionally, we compared three different nomenclatures which are used to identify CD8 T cell subsets in order to assess and identify the best nomenclature to be used to monitor the CD8 compartment in kidney transplant patients (Article 2).

The second part of the thesis was focused on characterizing the immunometabolic programming involved in CD8 T cell function in healthy volunteers, kidney transplant recipients and multiple sclerosis patients. We used an extracellular flux analyzer to observe the changes in the immunometabolic programming in different CD8 T cells subsets and investigated the importance of different metabolic pathways in the effector function of CD8 T cells (Article 4). Furthermore, we compiled our findings about the importance of the immunometabolism of CD8 T cells in kidney transplantation with the current research and literature in this field and wrote a mini review, which is located in the appendix.

3.2 Article 1: Expansion of Highly Differentiated Cytotoxic Terminally Differentiated Effector Memory CD8+ T Cells in a Subset of Clinically Stable Kidney Transplant Recipients: A Potential Marker for Late Graft Dysfunction.

Published in the Journal of the American Society of Nephrology in 2014 (Yap et al. 2014).

In this article, we investigated the association of the TCR Vβ repertoire and CD8 immunophenotype in long-term stable kidney graft recipients. In previous animal studies, our team has found that alterations to the TCR Vβ repertoire is associated with chronic allograft dysfunction in a rodent cardiac allograft model and that these alterations are due mainly to CD8 T lymphocytes and not CD4 lymphocytes (Ballet et al. 2009). Furthermore, Miqueu et al. showed that kidney allograft recipients suffering from chronic rejection had an altered TCR Vβ repertoire while tolerant patients had an unaltered TCR Vβ repertoire. Additionally, this study found that stable kidney allograft patients had a heterogeneous spread of TCR Vβ repertoire usage (Miqueu et al. 2010). These finding caused us to hypothesize about the association between the TCR Vβ repertoire and the immunophenotype of the CD8 cells in these stable patients and to also investigate the prognostic value of the TCR Vβ repertoire and CD8 T cells in predicting long-term allograft dysfunction.

We used polychromatic flow cytometry to characterize the CD8 T cell populations in 131 long-term stable kidney transplant patients. Patients were divided into two groups depending on the alterations to their TCR Vβ repertoire (unaltered vs. altered). We found that an altered TCR Vβ repertoire was associated with an expansion in the TEMRA $CD45RA^{+}CCR7^{-}CD27^{-}$ $CD28^{-}$ CD8 T cell subset and that patients with an increased frequency of TEMRA

$CD45RA^+CCR7^-CD27^-CD28^-$ CD8 T cells had a 1.96 fold chance increase of long-term allograft dysfunction.

CLINICAL RESEARCH www.jasn.org

Expansion of Highly Differentiated Cytotoxic Terminally Differentiated Effector Memory CD8+ T Cells in a Subset of Clinically Stable Kidney Transplant Recipients: A Potential Marker for Late Graft Dysfunction

Michelle Yap,[*†‡] Françoise Boeffard,[*†‡] Emmanuel Clave,[§] Annaick Pallier,[*†‡] Richard Danger,[*†‡] Magali Giral,[*†‡] Jacques Dantal,[*†‡] Yohann Foucher,[†ǁ] Cécile Guillot-Gueguen,[†] Antoine Toubert,[§] Jean-Paul Soulillou,[*†‡] Sophie Brouard,[*†‡] and Nicolas Degauque[*†‡]

*Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche 1064, Nantes, France; †Centre Hospitalier Universitaire de Nantes, Institut de Transplantation Urologie-Néphrologie, Nantes, France; ‡Faculté de Médecine, Université de Nantes, Nantes, France; §Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche 940, Paris, France; and ǁUniversité de Nantes, Equipe d'Accueil 4275 and Labex Transplantex, Nantes, France

ABSTRACT

Despite the effectiveness of immunosuppressive drugs, kidney transplant recipients still face late graft dysfunction. Thus, it is necessary to identify biomarkers to detect the first pathologic events and guide therapeutic target development. Previously, we identified differences in the T-cell receptor Vβ repertoire in patients with stable graft function. In this prospective study, we assessed the long-term effect of CD8$^+$ T-cell differentiation and function in 131 patients who had stable graft function. In 45 of 131 patients, a restriction of TCR Vβ diversity was detected and associated with the expansion of terminally differentiated effector memory (TEMRA; CD45RA$^+$CCR7$^-$CD27$^-$CD28$^-$) CD8$^+$ T cells expressing high levels of perforin, granzyme B, and T-bet. This phenotype positively correlated with the level of CD57 and the ability of CD8$^+$ T cells to secrete TNF-α and IFN-γ. Finally, 47 of 131 patients experienced kidney dysfunction during the median 15-year follow-up period. Using a Cox regression model, we found a 2-fold higher risk (P=0.06) of long-term graft dysfunction in patients who had increased levels of differentiated TEMRA CD8$^+$ T cells at inclusion. Collectively, these results suggest that monitoring the phenotype and function of circulating CD8$^+$ T cells may improve the early identification of at-risk patients.

J Am Soc Nephrol 25: ●●●–●●●, 2014. doi: 10.1681/ASN.2013080848

The major immunologic cause of late kidney graft failure is chronic antibody-mediated rejection (CAMR).[1] Its diagnosis relies on renal dysfunction, histologic features, and donor-specific antibodies.[2] The biologic mechanisms leading to CAMR are poorly defined. Whereas anti-donor antibodies are identified as a risk factor for graft survival,[3,4] preexisting T-cell memory is associated with high incidence and severe rejection episodes,[5] and recipients prone to acute rejection have a higher precursor frequency of alloreactive CD8 T cells than nonrejectors.[6] Using an experimental model of CAMR,[7] we previously reported that a similar T-cell receptor (TCR) Vβ selection of CD8 T cells

Received August 9, 2013. Accepted January 24, 2014.

S.B. and N.D. contributed equally to this work.

Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Jean-Paul Soulillou, Pavillon Jean Monnet, Hôtel Dieu, CHU de Nantes, 30, Bd Jean Monnet, 44093 Nantes cedex 01, France. Email: jean-paul.soulillou@univ-nantes.fr

Copyright © 2014 by the American Society of Nephrology

 ISSN : 1046-6673/2508-●●●

can be identified in the blood and graft of recipients.[8] This profile was associated with transcript coding for granzyme B (GZM-B) in the graft.[9–11] Similar observations made in the blood of CAMR patients reported a restricted TCR Vβ repertoire, an increase in IFN-γ, GZM-B, and perforin-1 (PERF-1) transcripts, and an increase in CD8 T cells.[12–14] These observations suggest that alteration in the TCR Vβ repertoire of CD8 T cells may be associated with kidney dysfunction.

We previously reported that different shapes of TCR Vβ repertoire are identified in patients with stable graft function, despite the stringent clinical criteria used to constitute a homogeneous group.[14] In this prospective study, we examined CD8 T-cell phenotype and function and the long-term clinical outcome of these patients with stable graft function (n=131) who exhibit different usage of their TCR Vβ repertoire. We found that the restriction of the TCR Vβ repertoire diversity is associated with an increase of highly differentiated terminally differentiated effector memory (TEMRA; CD45RA$^+$CCR7$^-$ CD27$^-$CD28$^-$) CD8 T cells, which are characterized by a high expression of cytotoxic molecules, PERF and GZM-B, T-bet, and CD57 and the ability to secrete TNF-α and IFN-γ. During the follow-up period, 47 patients exhibit kidney dysfunction. Using an unsupervised clustering strategy, we report that stable patients who have an increase in highly differentiated TEMRA CD8 T cells have a 2-fold higher risk of long-term graft dysfunction (P=0.06). Additional prospective clinical investigation will be necessary to provide definitive proof of the association between kidney graft dysfunction and the accumulation of highly differentiated TEMRA CD8 T cells.

RESULTS

Study Population

In total, 131 kidney transplant recipients who displayed a stable graft function (Table 1) were enrolled. At the time of inclusion, patients' TCR Vβ repertoire was analyzed, T-cell phenotype and function were characterized, and signal joint TCR excision circle (sjTREC) levels were measured (Figure 1). With more than 6 years of follow-up, the kidney graft was re-evaluated for graft dysfunction.

Reduction in TCR Vβ Repertoire Diversity Is Associated with an Increase of Highly Differentiated TEMRA (CD45RA$^+$CCR7$^-$CD27$^-$CD28$^-$) CD8 T cells

Of 131 patients (median time post-transplantation=7.78 years, range=5.01–21.66 years), 45 patients exhibited a restricted TCR Vβ repertoire (median time post-transplantation=6.55 years; range=5.11–19.58 years), and 86 patients did not (median time post-transplantation=8.10 years; range=5.01–21.66 years) (Table 1). Patients with a restricted TCR Vβ repertoire were older (P<0.001), transplanted with an older kidney (P=0.002), and more frequently treated with mycophenolate-mofetil (P=0.01) compared with patients with a diverse TCR Vβ repertoire (Table 1). All the other clinical parameters were similar between the two groups. CD8 T cells were classified as naive (CD45RA$^+$CCR7$^+$), central memory (CD45RA$^-$CCR7$^+$), effector memory (EM; CD45RA$^-$CCR7$^-$), or TEMRA (CD45RA$^+$CCR7$^-$).[15,16] CD28 and CD27 expressions were also used to identify early (CD27$^+$CD28$^+$), intermediate (CD28$^-$CD27$^+$), and late (CD28$^-$CD27$^-$)[16] differentiated cells (Supplemental Figure 1). Patients with a restricted TCR Vβ repertoire exhibit a higher frequency of CD45RA$^+$CCR7$^-$ TEMRA CD8 T cells compared with patients with a diverse TCR Vβ repertoire (52.74±2.96% versus 31.39±1.99%; P<0.001) (Figure 2A) and a decrease in CD45RA$^+$CCR7$^+$ naïve CD8 T-cell frequency (14.13±1.62% versus 29.31±1.82%; P<0.001) (Figure 2A).

A restricted TCR Vβ repertoire was associated with a marked increase in late differentiated CD27$^-$CD28 CD8 T cells (55.13±3.14% versus 23.06±2.30%; P<0.001) (Figure 2B). Finally, an increase in CD27$^-$CD28$^-$ T cells was associated with a significant decrease in CD27$^+$CD28$^+$ T cells in EM and TEMRA CD8 T cells in restricted TCR Vβ repertoire patients (P<0.001) (Figure 2C). Collectively, the restriction in TCR Vβ diversity was associated with an expansion of TEMRA cells with highly differentiated phenotype.

CD8 T Cells in Patients with Restricted TCR Vβ Repertoire Showed High Cytotoxic Molecule Expression

A significant increase of CD8 T cells expressing either GZM-B only (28.04±3.05%; P=0.007) or GZM-B and PERF (30.61±2.83%; P<0.001) was observed in patients with a restricted TCR Vβ repertoire. Three levels of expression of PERF were observed within CD8 T cells (Figure 3B). CD8 T cells with a restricted TCR Vβ repertoire exhibit a higher expression of PERF compared with patients with a diverse TCR Vβ repertoire (PERF^{hi}: 21.04±2.80% versus 7.84±0.88%; P<0.001) (Figure 3C). The enhanced expression of PERF was associated with an increase in the mean fluorescence intensity of GZM-B (diverse TCR Vβ repertoire [4375±487] versus restricted TCR Vβ repertoire [5809±283]; P=0.008) (Figure 3D). Finally, a positive correlation between the expression of PERF and GZM-B was observed (R^2=0.46, P<0.001).

High cytolytic potential can be measured using the expression of CD57.[17,18] Patients with restricted TCR Vβ repertoire display a higher frequency of CD57$^+$ CD8 T cells compared with patients with a diverse TCR Vβ repertoire (47.75±2.69% versus 26.83±1.59%; P<0.001) (Figure 3E). Collectively, these data show that restriction of TCR Vβ repertoire diversity is associated with an enrichment of CD8 T cells exhibiting markers associated with cytotoxicity.

CD8 T Cells in Patients with Restricted TCR Vβ Repertoire Expressed Higher Levels of T-Bet

Three populations could be defined based on the expression of T-bet (T-bet^{neg}, T-bet^{dull}, and T-bet^{high}).[19] Whereas the frequency of T-bet^{dull} CD8 T cells was similar between patients, patients with a restricted TCR Vβ repertoire exhibit a marked

Table 1. Summary of demographic and clinical characteristics of patients

Patient Characteristics	Global (n=131)		Diverse Stable (n=86)		Restricted Stable (n=45)		P Value
	Mean or Number	SD or Percentage	Mean or Number	SD or Percentage	Mean or Number	SD or Percentage	
Recipient age (yr)	45.21	13.53	42.41	13.44	50.56	12.15	<0.001
Donor age (yr)	36.34	14.56	33.53	14.39	41.69	13.50	0.002
At blood sampling							
Time post-transplantation (yr)	9.01	3.84	9.42	3.88	8.43	3.74	0.16
Creatinemia (μmol/L)	118	31	118	32	119	28	0.76
Proteinuria (g/24 h)	0.20	0.17	0.21	0.19	0.17	0.13	0.26
Woman recipient	48	36.6%	33	38.4%	12	26.7%	0.25
Woman donor	35	26.7%	23	26.7%	12	26.7%	1.00
Incompatibility HLA-A, -B, or -DR>4	63	48.1%	41	47.7%	22	48.9%	1.00
Induction therapy							
mAb	15	11%	9	10%	6	13%	0.77
Polyclonal antibody	54	41%	33	38%	21	47%	0.46
None	33	25%	21	24%	11	24%	1.00
Other	29	22%	22	26%	7	16%	0.27
Maintenance therapy							
Mycophenolatemofetil	72	55%	40	47%	32	71%	0.01
CSA	104	79%	66	77%	38	84%	0.37
Azathioprine	32	29%	25	29%	7	16%	0.13
FK	38	30%	26	30%	12	27%	0.84
Corticotherapy	12	9%	10	12%	2	4%	0.22

Kidney recipients under standard biotherapy immunosuppression with stable graft function and without TCR Vβ alterations (diverse stable; n=86) or with TCR Vβ alterations (restricted stable; n=45). CSA, cyclosporin A; FK, Tacrolimus.

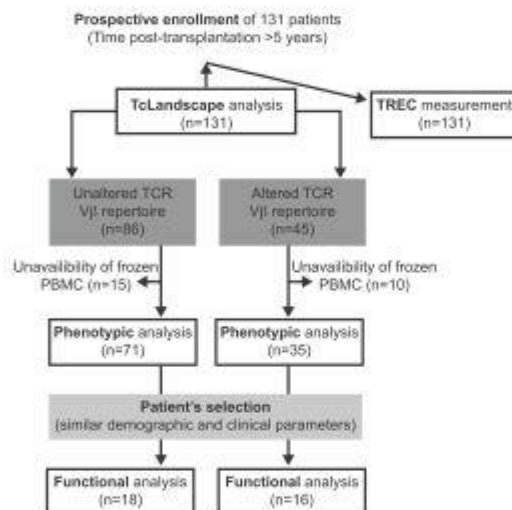


Figure 1. Description of the observational and prospective study. The number of patients is shown in parentheses.

increase in T-bet^{high} CD8 T cells (44.05±4.05% versus 25.25±1.88%; P<0.001) (Figure 4A).The expression of T-bet^{high} was positively correlated with CD57 expression (R^2=0.40; P<0.001) (Figure 4B). Most of the T-bet^{high} CD8 T cells express the effector-associated marker CD57, lose the expression of CD27 and CD28, and are preferentially found within the EM and TEMRA CD8 subset (Figure 4C). Altogether, patients with a restricted TCR Vβ repertoire exhibit T-bet^{high} CD8 T cells with an increased expression of CD57 (67.37±2.34% versus 52.86±2.13%; P<0.001) (Figure 4D) associated with a highly differentiated phenotype of CD27$^-$ CD28$^-$ T cells (75.57±2.50% versus 44.76±3.26%; P<0.001) (Figure 4D) and an increase in TEMRA CD45RA$^+$ CCR7$^-$ T cells (59.45±3.06% versus 48.00±2.67%; P=0.02) (Figure 4D).

Downregulation of CD127 by CD8 T Cells in Patients with Restricted TCR Vβ Repertoire

High expression of CD127 (IL-7Rα) allows the maintenance of a diverse repertoire of naïve CD8 T cells.[20,21] We report that the level of CD127 expression was lower in patients with a restricted TCR Vβ repertoire (Figure 5, A and B). Whereas the frequency of CD127^{dim} was similar between the two groups, patients with a restricted TCR Vβ repertoire exhibit an increase of CD127^{low} CD8 T cells (54.92±2.81% versus 31.13±2.10%; P<0.001) (Figure 5B). Of interest, CD127^{low} CD8$^+$ T cells with a restricted TCR Vβ repertoire were more differentiated compared with CD127^{low}CD8$^+$ T cells with a diverse TCR Vβ repertoire (Figure 5C). The frequency of CD27$^-$CD28$^-$ and CD45RA$^+$CCR7$^-$ was significantly higher in patients with a restricted TCR Vβ repertoire (83.75±2.45%

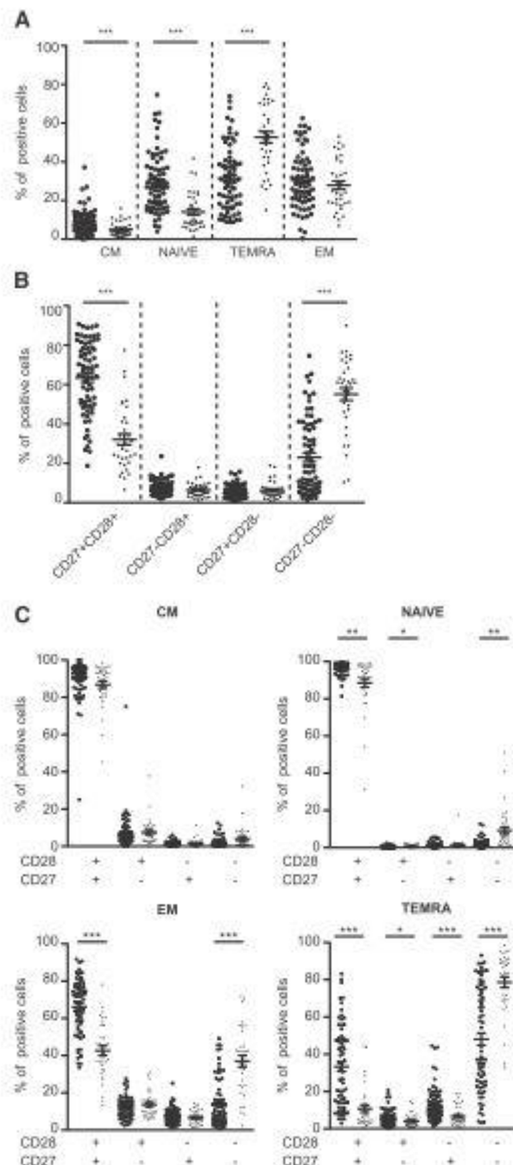


Figure 2. Reduction in TCR Vβ repertoire diversity is associated with an increase of highly differentiated TEMRA (CD45RA⁺CCR7⁻ CD27⁻CD28⁻) CD8 effector T cells. Expression of (A) CD45RA and CCR7 and (B) CD27 and CD28 was measured in CD8 T cells in PBMCs from stable patients with a diverse (n=71; open circles) or restricted (n=35; filled circles) TCR Vβ repertoire. CD3⁺CD8⁺ cells were gated by morphology and viability before being subdivided into (A) central memory (CM), naïve, TEMRA, and EM subsets based on the expression of CD45RA and CCR7 or (B) CD27 and CD28 subsets. (C) Expression of CD27 and CD28 within CM, naïve, TEMRA, and EM subsets. Each dot represents one individual, and the mean and the SEM are displayed. Comparison of the frequency of each CD8 subset was performed using a Holm multiple comparison test. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

versus 54.22±2.10%; $P<0.001$ and 75.28±2.90% versus 63.06±2.44%; $P=0.006$) (Figure 5C).

Collectively, the reduction of TCR Vβ repertoire in kidney transplant recipients is associated with a profound downregulation of CD127 in CD8 T cells with a highly differentiated phenotype.

CD8 T Cells in Patients with Restricted TCR Vβ Repertoire Exhibit Potent Effector and Cytotoxic Functions

Restriction of TCR Vβ repertoire diversity was associated with an increase in T-bet⁺ TNF-α⁺ CD8 T cells (14.52±1.64% versus 4.29±0.66%; $P<0.001$) (Figure 6A) and T-bet⁺ IFN-γ⁺ CD8 T cells (6.76±1.03% versus 1.86±0.57%; $P<0.001$) (Figure 6A) after short-term polyclonal stimulation. IFN-γ–secreting CD8 T cells were virtually all enclosed within TNF-α⁺ CD8 T cells. Polyclonal stimulation results in a fast release of cytotoxic granules as assessed by CD107a upregulation at the cell surface (7.11±2.35% versus 1.69±0.65%; $P=0.003$) (Figure 6B) in patients with a restricted TCR Vβ repertoire. Collectively, the restriction of TCR Vβ repertoire is associated with the acquisition of potent effector function markers of CD8⁺ T cells.

Reduction in Thymic Output Does Not Account for the TCR Vβ Repertoire Alteration

Given the accumulation of highly differentiated CD8 TEMRA T cells in patients with a restricted TCR Vβ repertoire, we asked whether these patients could have a defective thymic T-cell output. A generalized linear model was used to take into account potential confounding factors (recipient age and sex). The difference in sjTREC levels was not statistically significant between the two groups of patients (Supplemental Table 1).

Increase of Highly Differentiated TEMRA CD8 T Cells Is a Risk Factor for Long-Term Graft Dysfunction

Finally, we examined the association between accumulation of highly differentiated CD8 T cells and graft function outcome. A set of markers, linked phenotypically and functionally (TEMRA⁺CD27⁻CD28⁻ CD57⁺T-bet⁺GZM-B⁺PERF⁺), allows the segregation of the population into two groups of 54 and 50 patients according to the level of differentiated TEMRA CD8 T cells (Figure 7A). We assessed whether the kidney dysfunction (47 of 131 patients) (Table 2) was associated with markers expressed by CD8 T cells. Graft dysfunction was defined as significant proteinuria greater than 1 g/d (10 patients), an isolated increase of the creatinine level without proteinuria (20 patients), or both increased proteinuria and creatinine levels (17 patients). Only two patients had an isolated episode of proteinuria, six patients had an isolated episode of

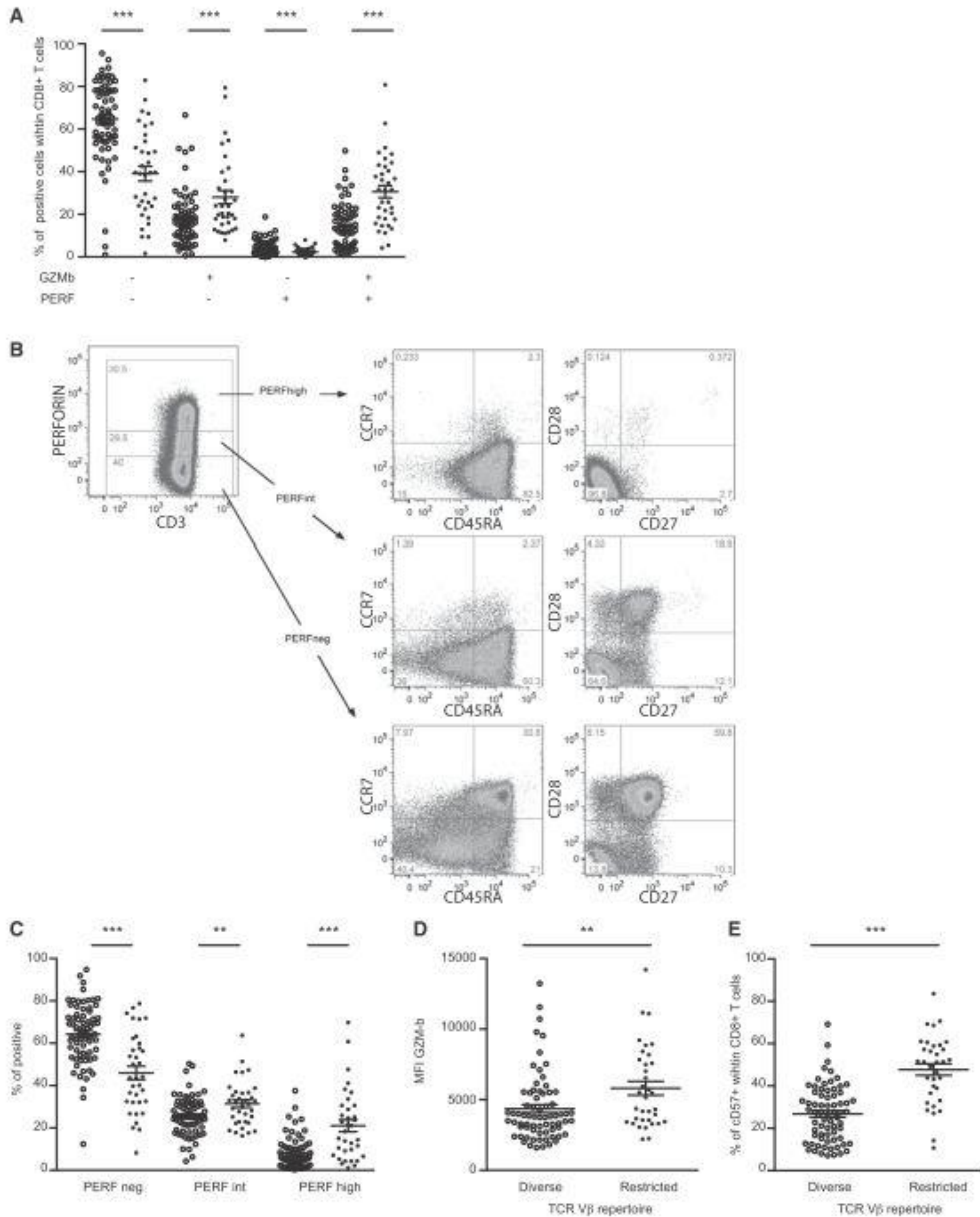


Figure 3. CD8 T cells in patients with restricted TCR Vβ repertoire showed a high expression of cytotoxic molecules (GZM-B and PERF) compared with patients with a polyclonal TCR Vβ repertoire. (A) $CD3^{+}CD8^{+}$ cells from patients with a restricted TCR Vβ repertoire (n=35; filled circles) express high amounts of GZM-B with and without the coexpression of PERF compared with cells from diverse TCR Vβ repertoire patients (n=71; open circles). (B) Three levels of PERF expression could be identified. Representative flow cytometry plots

functional degradation, and six patients displayed concomitant donor-specific antibodies (six were suspicious; *i.e.*, HLA-DQ donor typing not done). Among six patients who were subjected to a biopsy, four patients presented lesions of chronic humoral rejection, one patient had isolated transplant glomerulopathy, and one patient had acute cellular rejection after immunosuppression withdrawal. A biostatistical model was developed to adjust for the different times at which patients were enrolled post-transplantation (Table 1) and followed (median time of follow-up=15.01 years; range=5.60–29.71 years). The probability of late graft dysfunction during the 10 years after the fifth anniversary of transplantation was 41.6% (95% confidence interval, 20.7% to 57.0%) for patients with an increase of highly differentiated TEMRA CD8 T cells, whereas it was only 23.0% (95% confidence interval, 6.5% to 36.5%) for the other group of patients (Figure 7B). Independently from the time post-transplantation, recipient and donor age, recipient and donor sex, number of HLA mismatches above four, induction therapy, plasma renin activity (PRA) at inclusion, and eGFR at inclusion, patients with an increase of highly differentiated TEMRA CD8 T cells had a 1.96-fold higher risk of graft dysfunction during their follow-up and before their 15th graft anniversary (P=0.06) (Table 3). The increased risk of kidney dysfunction was observed preferentially in the first 5 years after inclusion (Figure 7B). At later time points, the increased risk was similar between the two groups.

Collectively, CD8 T-cell monitoring allows for the identification of patients with higher risk of graft dysfunction at a distance from the actual occurrence of kidney dysfunction.

DISCUSSION

Using a prospective analysis of a cohort of patients with long-term stable kidney function, we found that highly differentiated TEMRA CD8 T cells accumulate in patients with a restricted TCR Vβ repertoire. Despite exhibiting stable graft function at the time of analysis, patients with reduced TCR Vβ repertoire diversity exhibit an accumulation of highly differentiated TEMRA (CD45RA$^+$CCR7$^-$CD27$^-$CD28$^-$) CD8 T cells with all the attributes of cytotoxic and effector cells. Of interest, similar modifications were observed in the CD4 compartment (data not shown). We also report that patients with an increase of highly differentiated TEMRA CD8 T cells have higher risk of graft dysfunction (hazard ratio, 1.96; 95% confidence interval, 0.97–4.05; P=0.06). The P value may be explained by the low sample size (n=131) and incomplete data (truncated and censored observations). Rather than using only the P values, the size of the effect should be used to judge on the clinical importance of a result,[22] because the risk of incorrectly stating that accumulation of highly differentiated TEMRA is a risk factor of graft dysfunction was as low as 6%.

A subgroup of patients with stable graft function was characterized by a restriction of TCR Vβ repertoire diversity and an accumulation of GZM-B$^+$PERF^{high} CD8 T cells with high cytotoxic function. The expression of the two cytotoxic molecules was positively correlated and associated with CD57 expression, which identifies high cytotoxic potential cells.[23] Interestingly, increased PERF and GZM-B expression has also been associated with acute rejection.[24] Our data suggest that high expression of these molecules also implicates adverse chronic events. Therefore, CD8 T cells of patients with a restricted TCR Vβ repertoire have a greater potential of lysis. The identification of the potential target will be of great importance. Based on our prior work,[14] viral targets could be excluded, because we showed no link between viral infection and the shape of the TCR Vβ repertoire. Moreover, active inflammatory processes, including viral and bacterial infections, were exclusion criteria in the current study. Anti-cytomegalovirus IgG prevalence was similar in patients with highly different TCR Vβ repertoire typologies.

The accumulation of effector CD8 T cells was strengthened by the enhanced T-bet expression, which is crucial in T_H1-cell commitment and CD8 T-cell differentiation and function.[19] Moreover, T-bet^{high} CD8 T cells expressed the highest levels of PERF and GZM-B and are associated with HIV-specific cytotoxic function.[19] Interestingly, we found that T-bet^{high} expression in CD8 T cells correlated with cytotoxic potential marker CD57 expression (R^2=0.40; P<0.001). Moreover, the ability to secrete TNF-α and IFN-γ was restricted to T-bet expressing CD8 T cells, which confirmed the functionality of the TEMRA CD8 T cells within the at-risk patient group.

A profound decrease in CD127 was observed in patients with restricted TCR Vβ repertoire, suggesting that TEMRA CD8 T cells do not rely on IL-7–related homeostasis. CD127^{low} CD8 T cells were preferentially found within the CD45RA$^+$CCR7$^-$CD27$^-$CD28$^-$ subsets, a phenotype consistent with the reported prevalence of low levels of CD127 in effector CD8 T cells.[25] Downregulation of CD127 expression in CD8 T cells has been observed in latent viral or chronic viral infections[26–29] and correlated with CD8 T-cell exhaustion. We found that prolonged exposure to allogeneic antigens does not result in similar exhaustion. Patients with a diverse TCR Vβ repertoire retain high expression of CD127 on CD8 T cells; because these patients also exhibit a high frequency of naïve CD8 T cells, the retention of high expression of IL-7R is in

showing CD45RA and CCR7 or CD27 and CD28 by PERF^{high}, PERF^{int}, and PERF^{neg} CD3$^+$CD8$^+$ cells. (C and E) Frequency of (C) PERF^{high}, PERF^{int}, and PERF^{neg} and (E) CD57$^+$ and CD3$^+$CD8$^+$ cells in patients with a diverse TCR Vβ repertoire (n=71; open circles) or a restricted TCR Vβ repertoire (n=35; filled circles). (D) Level of expression of GZM-B (mean fluorescence intensity [MFI]) in patients with a diverse TCR Vβ repertoire (n=71; open circles) or a restricted TCR Vβ repertoire (n=35; filled circles). Each dot represents one individual, except for in E, and the mean and the SEM are displayed. Comparison of the frequency of the each CD8 subset was performed using a Holm multiple comparison test. **P<0.01; ***P<0.001.

agreement with the fact that the maintenance of naïve CD8 T cell pool relies on intermittent response to IL-7 and self-TCR.[21] The modulation of IL-7R expression may be caused by a switch in survival factors, leading to either the maintenance of naïve CD8 T cells with diverse TCR Vβ repertoire or the expansion of TEMRA CD8 T cells with restricted TCR Vβ repertoire. TCR Vβ repertoire restriction results in decreased CD127 expression without altering CD8 T-cell effector functions, which was exemplified by the high expression of T-bet, CD57, GZM-B, and PERF by CD127^{low} CD8 T cells. However, low expression of CD127 by TEMRA CD27$^-$CD28$^-$ CD8 T cells does not necessarily preclude their ability to respond to IL-7.[30–32]

Longitudinal monitoring of the CD8 T cells compartment will be needed to document the differentiation from naive to TEMRA cells. Before transplantation, an increase of TEMRA CD8 T cells and a decrease of naive CD8 T cells were associated with a lower risk of acute rejection.[32] The reduction of T-cell diversity may reflect a reduction of alloreactive T cells before transplantation. In contrast, we identified that the increase of TEMRA CD8 T cells and the decrease of naïve CD8 T cells were risk factors for long-term graft dysfunction. Moreover, TEMRA CD8 T cells exhibit potent effector functions, including the ability to secrete proinflammatory cytokines and cytotoxic molecules. Whereas at the early stages post-transplantation, the T-cell immunity is controlled by the immunosuppressive drugs, it is possible that there is a gradual leakage in the control of the antidonor immune response, leading to an expansion of TEMRA CD8 T cells. The continuous stimulation of the alloimmune system is evidenced by CD27 and CD28 downregulation. Controlling TEMRA CD8 T-cell function and preventing their deleterious effects will be the next challenge. Targeting the CD28-CD80/86 pathway in patients with CD80/86 antagonists (Belatacept) or using monovalent anti-CD28 mAb may not be effective on TEMRA CD8 T cells given the lack of expression of costimulatory molecules (CD28 and CD27). An interesting candidate could be anti-TNF treatment that decreases the granulysin-expressing CD8$^+$ CCR7$^-$CD45RA$^+$ effector memory T-cell population.[33,34]

Collectively, our study provides new evidence implicating blood CD8 T cells in kidney graft dysfunction. Additional prospective clinical investigation will be necessary to provide definitive proof of the association between kidney graft dysfunction and the accumulation of highly differentiated TEMRA CD8 T cells. Monitoring CD8 T-cell differentiation may be useful for identifying patients at risk of kidney dysfunction. Innovative therapies targeting the CD8 TEMRA compartment may offer new opportunities to prevent and treat kidney dysfunction.

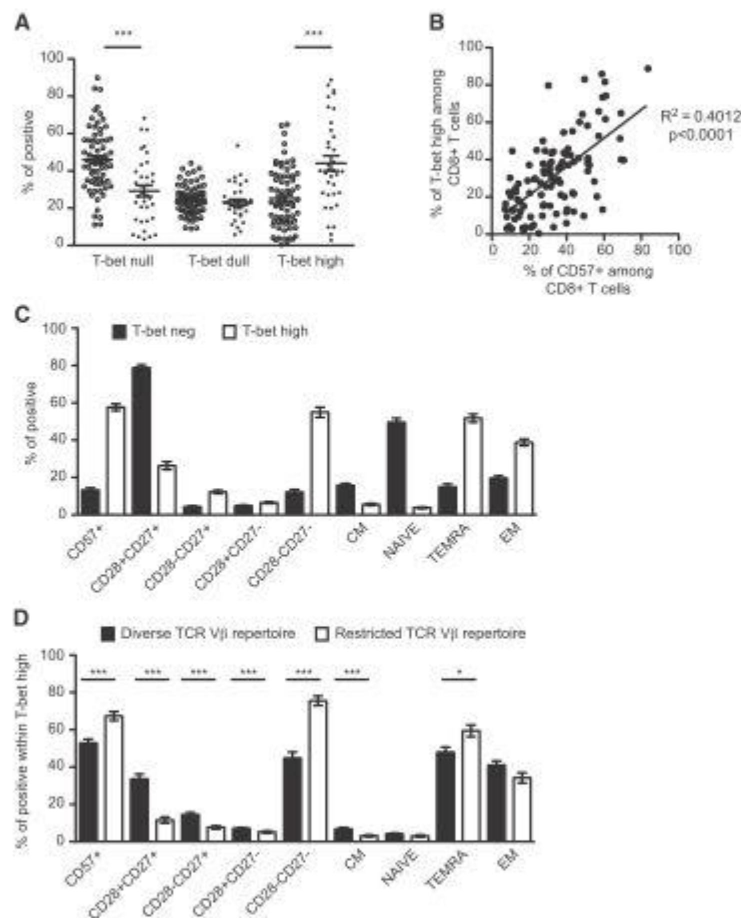


Figure 4. CD8 T cells in patients with restricted TCR Vβ repertoire expressed higher levels of T-bet than patients with diverse TCR Vβ repertoire. (A) Frequency of T-bet^{neg}, T-bet^{dull}, and T-bet^{high} CD8 T cells was measured in CD8 T cells in PBMCs from stable patients with a diverse (n=71; open circles) or restricted TR Vβ repertoire (n=35; filled circles). (B) The frequency of CD57$^+$ CD8 T cells was plotted against the frequency of T-bet^{high} CD8 T cells within all patients. Linear regression was performed to determine statistical significance, and the regression factor is indicated. (C) Phenotype of T-bet^{neg} (white bars) versus T-bet^{high} (black bars) of the whole cohort. (D) Phenotype of T-bet^{high} CD8 T cells from stable patients with a diverse (n=71; black bars) or restricted TCR Vβ repertoire (n=35; white bars). Comparison of the frequency of the each CD8 subset was performed using a Holm multiple comparison test. *P<0.05; ***P<0.001. CM, central memory.

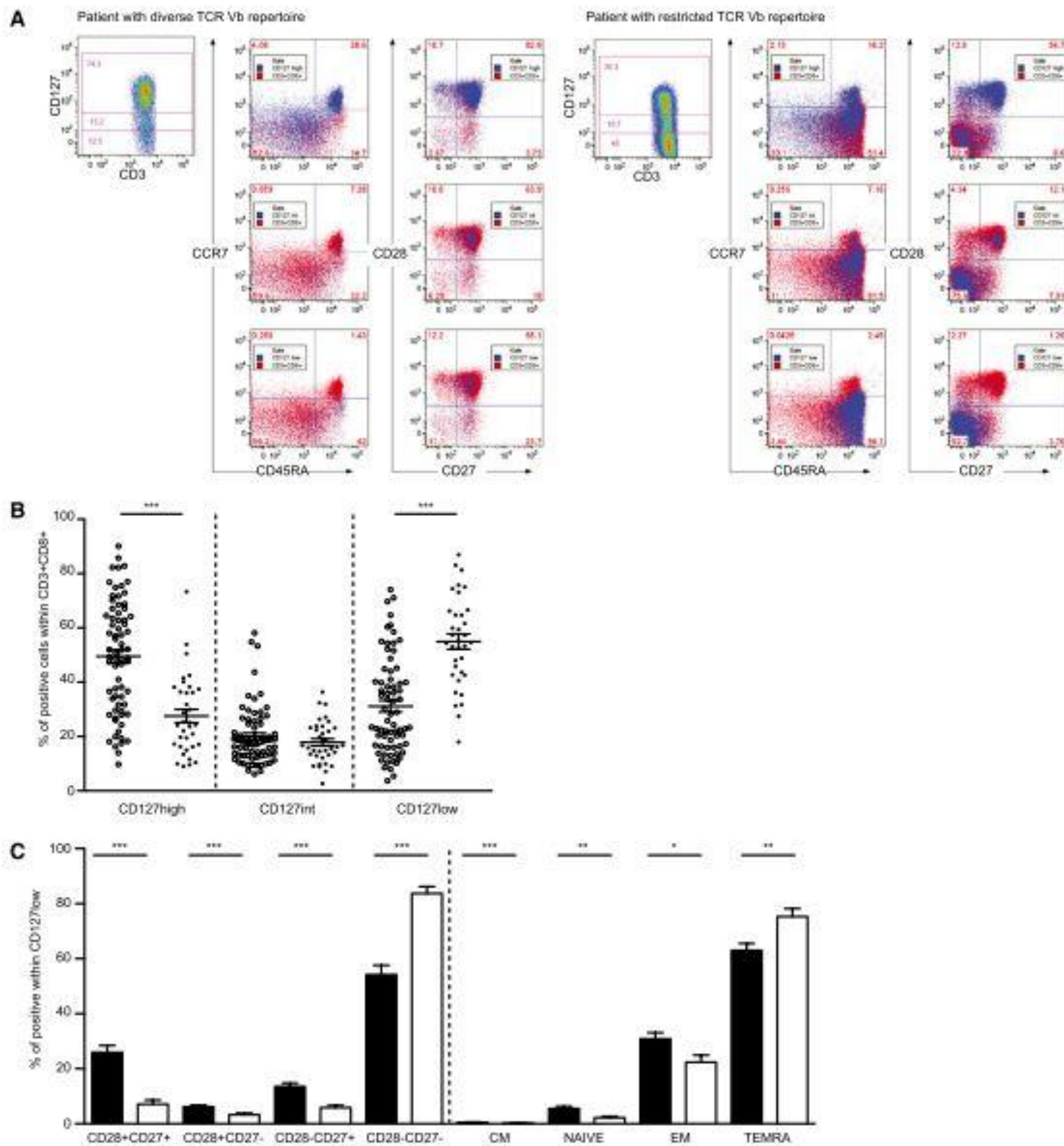


Figure 5. Downregulation of CD127 by CD8 T cells in patients with restricted TCR Vβ repertoire. (A) Three levels of CD127 expression could be identified. Representative flow cytometry plots showing CD45RA and CCR7 or CD27 and CD28 by CD127^{high}, CD127^{int}, and CD127^{low} CD3^{+}CD8^{+} cells in stable patients with a diverse (left panel) or restricted TCR Vβ repertoire (right panel). The various CD127 populations (blue) were overlaid onto dot plots (red) of total CD8^{+} T cells. (B) Frequency of CD127^{high}, CD127^{int}, and CD127^{low}CD3^{+}CD8^{+} cells in patients with a diverse TCR Vβ repertoire (*n*=71; open circles) or a restricted TCR Vβ repertoire (*n*=35; filled circles). Each dot represents one individual, and the mean and the SEM are displayed. (C) Phenotype of CD127^{low} CD8 T cells from stable patients with a diverse (*n*=71; black bars) or restricted TCR Vβ repertoire (*n*=35; white bars). The mean and the SEM are displayed. Comparison of the frequency of the each CD8 subset was performed using a Holm multiple comparison test. *$P<0.05$; **$P<0.01$; ***$P<0.001$. CM, central memory.

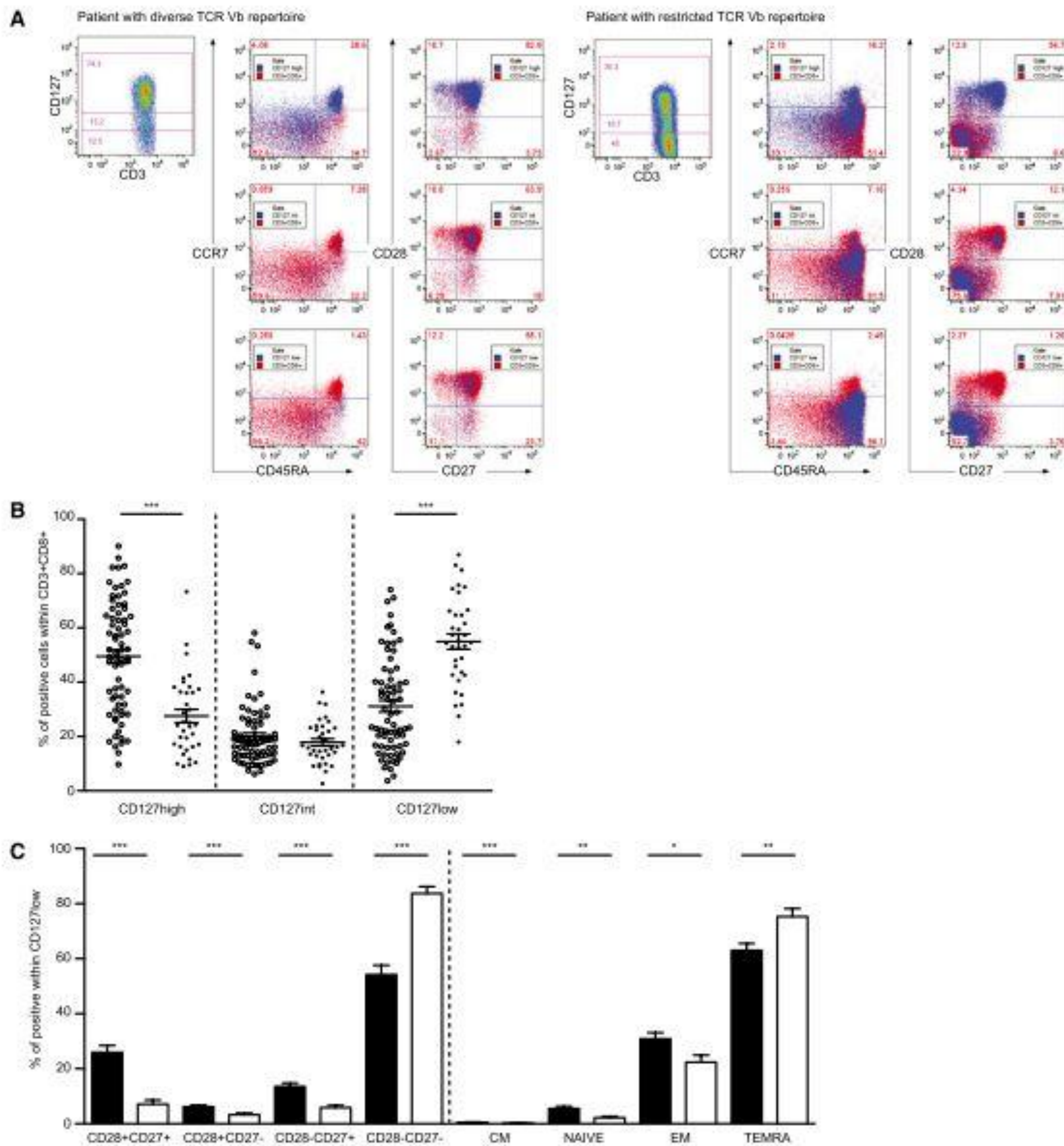


Figure 5. Downregulation of CD127 by CD8 T cells in patients with restricted TCR Vβ repertoire. (A) Three levels of CD127 expression could be identified. Representative flow cytometry plots showing CD45RA and CCR7 or CD27 and CD28 by $CD127^{high}$, $CD127^{int}$, and $CD127^{low}$ $CD3^{+}CD8^{+}$ cells in stable patients with a diverse (left panel) or restricted TCR Vβ repertoire (right panel). The various CD127 populations (blue) were overlaid onto dot plots (red) of total $CD8^{+}$ T cells. (B) Frequency of $CD127^{high}$, $CD127^{int}$, and $CD127^{low}CD3^{+}CD8^{+}$ cells in patients with a diverse TCR Vβ repertoire (n=71; open circles) or a restricted TCR Vβ repertoire (n=35; filled circles). Each dot represents one individual, and the mean and the SEM are displayed. (C) Phenotype of $CD127^{low}$ CD8 T cells from stable patients with a diverse (n=71; black bars) or restricted TCR Vβ repertoire (n=35; white bars). The mean and the SEM are displayed. Comparison of the frequency of the each CD8 subset was performed using a Holm multiple comparison test. *P<0.05; **P<0.01; ***P<0.001. CM, central memory.

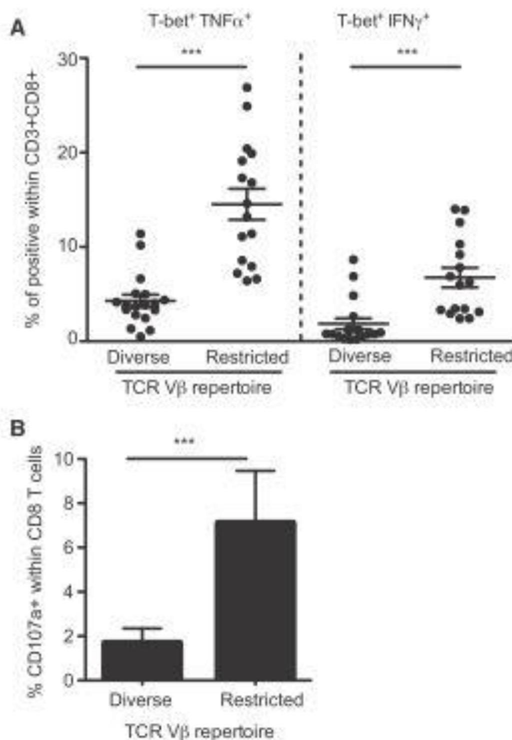


Figure 6. CD8 T cells in patients with restricted TCR Vβ repertoire exhibit potent effector functions (secretion of proinflammatory cytokines [TNF-α and IFN-γ] and cytotoxic function). (A) PBMCs from patients with a diverse TCR Vβ repertoire (n=18) or a restricted TCR Vβ repertoire (n=16) were stimulated with plate-bound anti-CD3 anti-CD28.2 mAbs for 6 hours, and expression of T-bet, IFN-γ, and TNF-α was measured on CD8 T cells. The mean and the SEM are displayed. (B) PBMCs from patients with a diverse TCR Vβ repertoire (n=15) or a restricted TCR Vβ repertoire (n=13) were stimulated with plate-bound anti-CD3 anti-CD28.2 mAbs for 6 hours, and expression of CD107a was measured on CD8 T cells. Comparison of the frequency of each CD8 subset was performed using a Holm multiple comparison test. ***P<0.001.

CONCISE METHODS

Subjects and Ethics Statement

The University Hospital Ethical Committee and the Committee for the Protection of Patients from Biologic Risks approved the study. All kidney transplant recipients gave informed consent; 131 transplant recipients who had received a first and unique kidney transplant from a deceased donor and displayed a stable graft function (Modification of Diet in Renal Disease eGFR>40 ml/min and proteinuria<1 g/24 h) for at least 5 years were enrolled. Patients were prescreened and designated as stable according to an eGFR above 40 ml/min, a stable creatinemia (±25% of the mean value of creatinemia in the year before the inclusion), and a daily proteinuria<1 g/d. Criteria of graft stability were confirmed at 3 months. Graft dysfunction was defined by at least three values of creatinemia above 25% of the basal level within the last 2 years of follow-up and/or a proteinuria>1 g/24 h. Patients received tacrolimus or cyclosporine A for maintenance therapy with or without mycophenolatemofetil, azathioprine, and/or steroids. All patients were compliant with the medical description and had not undergone an episode of rejection or ongoing infection during the monitoring period. At the inclusion, anticlass I PRA was detected for three patients, and anticlass II PRA was detected for six patients, including two patients who exhibited both anticlass I and II PRA.

Characterization of the TCR Vβ Repertoire and Identification of Restricted TCR Vβ Repertoire

The TcLandscape was performed as previously described by combining the complementary determining region 3 length distribution with each normalized amount of Vβ transcript.[35–37] A capillary sequencer (Applied Biosystems 3730)[38] was used to determine the CDR3 length distribution after amplification of the Vβ genes transcript.[39] The level of Vβ family transcripts was measured by quantitative RT-PCR and normalized by a reference gene (HPRT). TCR Vβ repertoires were stratified into diverse and restricted profiles using the rules recently developed.[14]

Study Design

One hundred thirty-one patients were prospectively recruited according to the exclusion/inclusion criteria described above (Figure 1). Patients were classified into two groups according to the alteration of the TCR Vβ repertoire (diverse stable, n=86; restricted stable, n=45).[14] Among 131 patients recruited, frozen PBMCs were available for 106 patients (diverse stable, n=71; restricted stable, n=35) and subjected to phenotypic and functional analyses at the time of inclusion. A fraction of the patients was selected to characterize the transcripts expression of a selected CD8-related genes set (diverse stable, n=17; restricted stable, n=12). The selection of the patients was done to minimize the disparity of the demographic and clinical parameters (time post-transplantation, recipient age and sex, donor age and sex, and HLA mismatches).

Blood Samples

PBMCs were separated on a Ficoll gradient layer and frozen in 10% DMSO autologous serum.

Polychromatic Flow Cytometry

Cells were analyzed with an LSRII flow cytometer (BD Immunocytometry Systems); 2×10^6 frozen PBMCs were surface-stained with antibodies specific for CD3 (BW264/56; VioBlue), CD8 (BW135/80; VioGreen), CD45RA (T6D11; APC-Vio770), CCR7 (3D12; PE-Cy7), CD27 (L128; Brilliant Violet 605), and CD28 (CD28.2; PE-CF594). In addition to this core-staining cocktail, different combinations of antibody were used: CD127 (MB15–18C9; PE), CD57 (TB03; FITC), T-bet (O4–46; PE), GZM-B (GB11; Alexa Fluor 700), and PERF (B-D48; PE). Yellow LIVE/DEAD Fixable Dead Cell Stain Kit was used to exclude dead cells from analysis. BD CompBeads stained separately with individual mAbs were used to define the compensation matrix. Data were analyzed using FlowJo Version 9.0.1 (TreeStar). All the

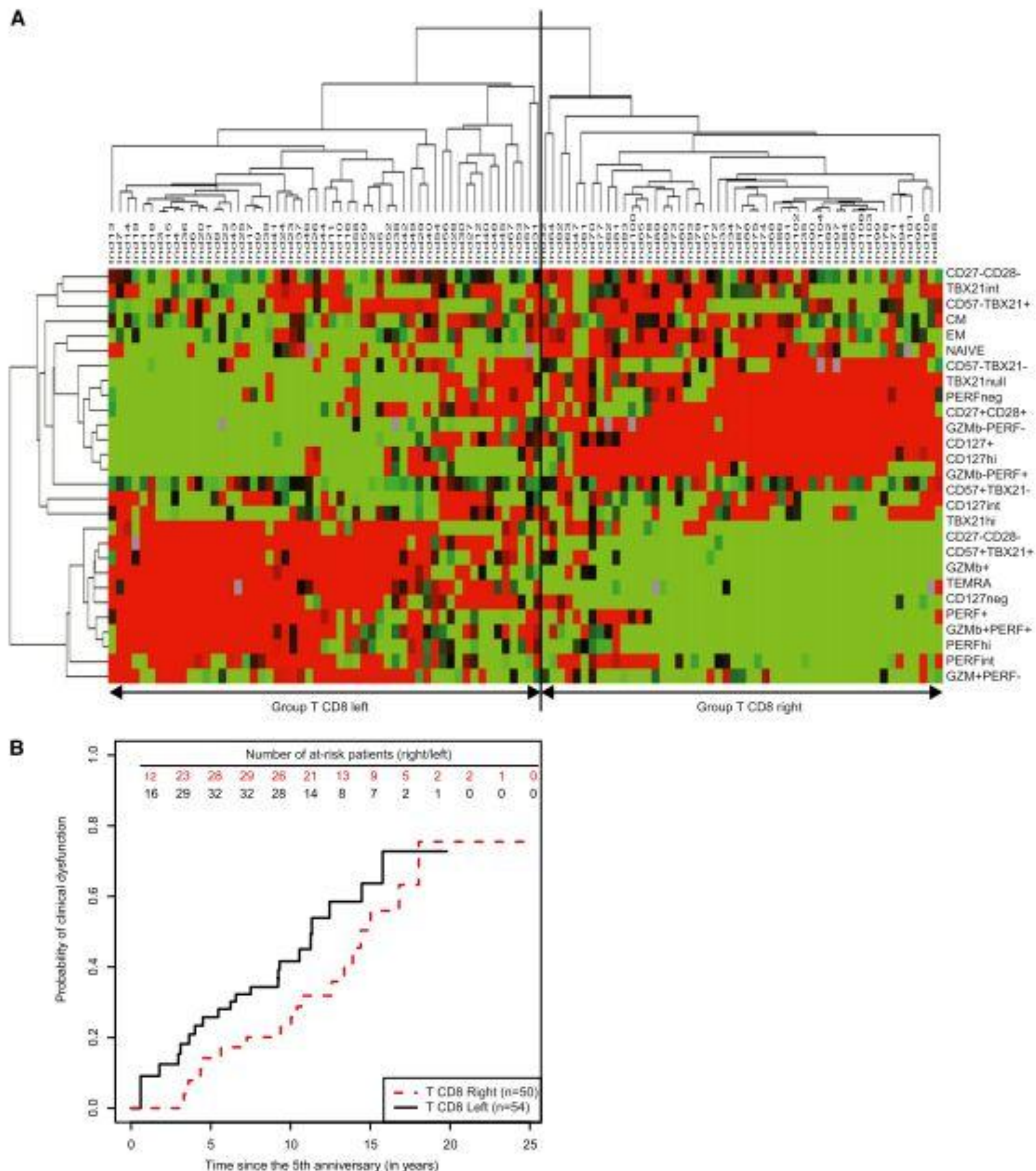


Figure 7. Identification of patients at risk of late kidney dysfunction based on the features of CD8 T cells. (A) Unsupervised clustering of kidney recipients based on CD8 T-cell markers measured by flow cytometry at the time of inclusion. Two groups of patients (referred as left and right CD8) are identified. (B) The cumulative probability of graft dysfunction was assessed in patients with stable graft function according to the shape of the TCR Vβ repertoire (restricted versus diverse TCR Vβ repertoire) and the time post-transplantation after the fifth anniversary of transplantation. At the end of the follow-up, the low remaining number of transplant recipients explained the sudden increase in kidney dysfunction. CM, central memory.

Table 2. Summary of clinical characteristics of patients with a kidney dysfunction

Patient Characteristics	Mean or Number	SD or Percentage
Time between transplantation and kidney dysfunction (yr)	13.88	4.49
Time between inclusion and kidney dysfunction (yr)	4.15	2.35
Anti-HLA antibodies	19/47	40.42%
Donor-specific antibodies	16/47	34.04%
Proteinuria>1 g/d alone	10/47	21.27%
Rise in creatinine level alone	20/47	42.55%
Proteinuria>1 g/d and rise in creatinine level	17/47	36.17%

Table 3. Multivariate analysis of the time between the fifth anniversary of transplantation and the time of graft dysfunction using a Cox model adapted to truncated data

Patient Characteristics	Coefficient	SD	Hazard Ratio (95% CI)	P Value
CD8 T cells (left arm versus right arm)	−0.68	0.37	1.96 (0.96–4.05)	0.06
Recipient age (yr)	−0.03	0.02	0.97 (0.94–1.01)	0.15
Donor age (yr)	0.04	0.02	1.04 (1.00–1.08)	0.03
Recipient sex (woman versus man)	−0.90	0.42	0.41 (0.18–0.97)	0.04
Donor sex (woman versus man)	−1.37	0.50	0.26 (0.09–0.68)	<0.01
HLA (A+B+DR) mismatches (>4 versus other)	0.46	0.45	1.58 (0.66–3.81)	0.30
eGFR level at collection (ml/min)	−0.02	0.01	0.98 (0.95–1.00)	0.06
PRA at inclusion	−0.28	0.47	0.76 (0.30–1.89)	0.56
Polyclonal induction therapy (yes versus no)	−0.10	0.40	0.91 (0.42–1.96)	0.80

The confounding variables were all reinforced in the model, and the Wald's test was used in this model to test for correlations. Patients were classified into two groups according to the clustering based on the expression of phenotypic markers. 95% CI, 95% confidence interval.

antibodies were purchased from BD Biosciences, except for CD3-VioBlue (Miltenyi), CD8-VioGreen (Miltenyi), CD45RA-APC-Vio770 (Miltenyi), CD127-PE (Miltenyi), CD57-FITC (Miltenyi), and PERF-PE (Diaclone).

Functional Assays

PBMCs were thawed and rested overnight in complete RPMI medium (10% fetal calf serum). Cell concentration was adjusted at 2×10^6 cells/ml and stimulated for 6 hours with coated anti-CD3 (3 μg/ml) and anti-CD28.2 mAb (5 μg/ml) in a final volume of 1 ml in 24-well flat-bottom plates. When indicated, PE-conjugated anti-CD107a mAb (10 μl/well) was added at the beginning of the culture. After 2 hours, monensin (1 μg/ml; in a well with anti-CD107a mAb) or brefeldin A (5 μg/ml) was added. PBMCs were stained for cell surface markers (CD3, CD8, CD45RA, CCR7, CD27, and CD28), fixed, permeabilized according to the manufacturer's procedure (eBiosciences), and then, stained for IFN-γ (AlexaFluor 700), TNF-α (FITC), and T-bet (O4–46; PE). A minimum of 1×10^4 $CD3^+CD8^+$ cells was recorded (median=8×10^5; range=1×10^5–2×10^7).

Quantification of sjTRECs by Real-Time PCR

Genomic DNA was extracted from 5×10^6 to 10×10^6 cells stored in TRIzol reagent. Quantification of thymic sjTREC was done by multiplex real-time quantitative PCR.[40] Values were normalized for the genomic copy number using albumin gene quantification. Data were expressed per 150,000 PBMCs.

Statistical Methods

Descriptive Analyses

Quantitative variables were described according to mean and SEM and compared using a *t* test; the *P* value was corrected according to Holm's procedure. Qualitative variables were compared using a Fisher exact test.

Survival Analyses.

The outcome was the time between the fifth anniversary of transplantation and the time of graft dysfunction. A nonparametric estimator of survival function for truncated and censored data was used.[41] A Cox model adapted to truncated data was used for additional multivariate analysis.[42] The Wald's test was used to evaluate the significance of hazard ratios. The model was adjusted on all possible confounding factors according to well established risk factors of renal dysfunction.

sjTREC Analyses

Multivariate analysis was used to evaluate the confounding factors using a generalized linear model with a γ-distribution and inversed link function.[43] The model was adjusted on all possible confounding factors according to well established risk factors of sjTREC modification.

CD8 T-Cell Phenotype and Function Analysis

Analysis and presentation of distributions were performed using Simplified Presentation of Incredibly Complex Evaluations (SPICE version 5.1; http://exon.niaid.nih.gov[44]). Holm multiple comparison test was used to compare the different markers.[45]

ACKNOWLEDGMENTS

We thank Corinne Douay for excellent technical assistance.

This work was supported by Grant Recherche translationnelle Pays de la Loire—Délégation Interrégionale à la Recherche Clinique Grand Ouest and the European Society of Organ Transplantation Junior

Clinical Research Grant. R.D. was supported by the Fondation Centaure, which supports a French research network in transplantation, and a grant from the Fondation pour la Recherche Médicale.

DISCLOSURES

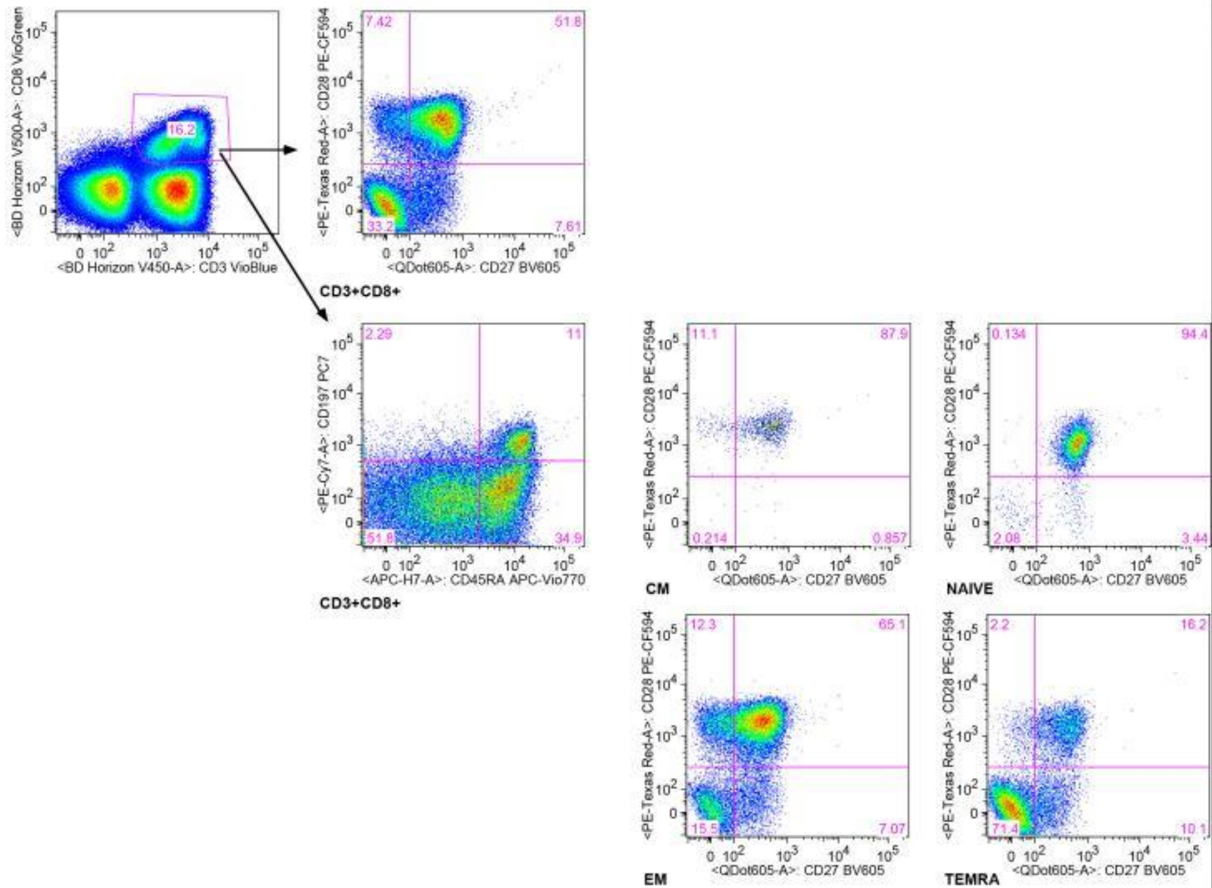
None.

REFERENCES

1. Colvin RB, Smith RN: Antibody-mediated organ-allograft rejection. *Nat Rev Immunol* 5: 807–817, 2005
2. Sis B, Mengel M, Haas M, Colvin RB, Halloran PF, Racusen LC, Solez K, Baldwin WM 3rd, Bracamonte ER, Broecker V, Cosio F, Demetris AJ, Drachenberg C, Einecke G, Gloor J, Glotz D, Kraus E, Legendre C, Liapis H, Mannon RB, Nankivell BJ, Nickeleit V, Papadimitriou JC, Randhawa P, Regele H, Renaudin K, Rodriguez ER, Seron D, Seshan S, Suthanthiran M, Wasowska BA, Zachary A, Zeevi A: Banff '09 meeting report: Antibody mediated graft deterioration and implementation of Banff working groups. *Am J Transplant* 10: 464–471, 2010
3. Hourmant M, Cesbron-Gautier A, Terasaki PI, Mizutani K, Moreau A, Meurette A, Dantal J, Giral M, Blancho G, Cantarovich D, Karam G, Follea G, Soulillou JP, Bignon JD: Frequency and clinical implications of development of donor-specific and non-donor-specific HLA antibodies after kidney transplantation. *J Am Soc Nephrol* 16: 2804–2812, 2005
4. Soulillou JP, Peyrat MA, Guenel J: Association between treatment-resistant kidney-allograft rejection and post-transplant appearance of antibodies to donor B-lymphocyte alloantigens. *Lancet* 1: 354–356, 1978
5. Poggio ED, Clemente M, Riley J, Roddy M, Greenspan NS, Dejelo C, Najafian N, Sayegh MH, Hricik DE, Heeger PS: Alloreactivity in renal transplant recipients with and without chronic allograft nephropathy. *J Am Soc Nephrol* 15: 1952–1960, 2004
6. van de Berg PJEJ, Yong SL, Koch SD, Lardy N, van Donselaar-van der Pant KAMI, Florquin S, Bemelman FJ, van Lier RAW, ten Berge IJ: Characteristics of alloreactive T cells measured before renal transplantation. *Clin Exp Immunol* 168: 241–250, 2012
7. Ballet C, Renaudin K, Degauque N, Mai HL, Boëffard F, Lair D, Berthelot L, Feng C, Smit H, Usal C, Heslan M, Josien R, Brouard S, Soulillou JP: Indirect CD4+ TH1 response, antidonor antibodies and diffuse C4d graft deposits in long-term recipients conditioned by donor antigens priming. *Am J Transplant* 9: 697–708, 2009
8. Lair D, Degauque N, Miqueu P, Jovanovic V, Guillet M, Mérieau E, Moreau A, Soulillou JP, Brouard S: Functional compartmentalization following induction of long-term graft survival with pregraft donor-specific transfusion. *Am J Transplant* 7: 538–549, 2007
9. Ashton-Chess J, Dugast E, Colvin RB, Giral M, Foucher Y, Moreau A, Renaudin K, Braud C, Devys A, Brouard S, Soulillou JP: Regulatory, effector, and cytotoxic T cell profiles in long-term kidney transplant patients. *J Am Soc Nephrol* 20: 1113–1122, 2009
10. Homs S, Mansour H, Desvaux D, Diet C, Hazan M, Buchler M, Lebranchu Y, Buob D, Badoual C, Matignon M, Audard V, Lang P, Grimbert P: Predominant Th1 and cytotoxic phenotype in biopsies from renal transplant recipients with transplant glomerulopathy. *Am J Transplant* 9: 1230–1236, 2009
11. Obata F, Yoshida K, Ohkubo M, Ikeda Y, Taoka Y, Takeuchi Y, Shinohara N, Endo T, Baba S: Contribution of CD4+ and CD8+ T cells and interferon-gamma to the progress of chronic rejection of kidney allografts: The Th1 response mediates both acute and chronic rejection. *Transpl Immunol* 14: 21–25, 2005
12. Baeten D, Louis S, Braud C, Braudeau C, Ballet C, Moizant F, Pallier A, Giral M, Brouard S, Soulillou J-P: Phenotypically and functionally distinct CD8+ lymphocyte populations in long-term drug-free tolerance and chronic rejection in human kidney graft recipients. *J Am Soc Nephrol* 17: 294–304, 2006
13. Brouard S, Le Bars A, Dufay A, Gosselin M, Foucher Y, Guillet M, Cesbron-Gautier A, Thervet E, Legendre C, Dugast E, Pallier A, Guillot-Gueguen C, Lagoutte L, Evanno G, Giral M, Soulillou J-P: Identification of a gene expression profile associated with operational tolerance among a selected group of stable kidney transplant patients. *Transpl Int* 24: 536–547, 2011
14. Miqueu P, Degauque N, Guillet M, Giral M, Ruiz C, Pallier A, Braudeau C, Roussey-Kesler G, Ashton-Chess J, Doré J-C, Thervet E, Legendre C, Hernandez-Fuentes MP, Warrens AN, Goldman M, Volk H-D, Janssen U, Wood KJ, Lechler RI, Bertrand D, Sébille V, Soulillou J-P, Brouard S: Analysis of the peripheral T-cell repertoire in kidney transplant patients. *Eur J Immunol* 40: 3280–3290, 2010
15. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A: Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708–712, 1999
16. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GMA, Papagno L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havlir DV, Richman DD, Gruener N, Pape G, Waters A, Easterbrook P, Salio M, Cerundolo V, McMichael AJ, Rowland-Jones SL: Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* 8: 379–385, 2002
17. Chattopadhyay PK, Betts MR, Price DA, Gostick E, Horton H, Roederer M, De Rosa SC: The cytolytic enzymes granyzme A, granzyme B, and perforin: Expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression. *J Leukoc Biol* 85: 88–97, 2009
18. Brenchley JM, Karandikar NJ, Betts MR, Ambrozak DR, Hill BJ, Crotty LE, Casazza JP, Kuruppu J, Migueles SA, Connors M, Roederer M, Douek DC, Koup RA: Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood* 101: 2711–2720, 2003
19. Hersperger AR, Martin JN, Shin LY, Sheth PM, Kovacs CM, Cosma GL, Makedonas G, Pereyra F, Walker BD, Kaul R, Deeks SG, Betts MR: Increased HIV-specific CD8+ T-cell cytotoxic potential in HIV elite controllers is associated with T-bet expression. *Blood* 117: 3799–3808, 2011
20. Schluns KS, Kieper WC, Jameson SC, Lefrançois L: Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo. *Nat Immunol* 1: 426–432, 2000
21. Kimura MY, Pobezinsky LA, Guinter TI, Thomas J, Adams A, Park J-H, Tai X, Singer A: IL-7 signaling must be intermittent, not continuous, during CD8+ T cell homeostasis to promote cell survival instead of cell death. *Nat Immunol* 14: 143–151, 2013
22. Young J: Understanding statistical analysis in the surgical literature: Some key concepts. *ANZ J Surg* 79: 398–403, 2009
23. Migueles SA, Osborne CM, Royce C, Compton AA, Joshi RP, Weeks KA, Rood JE, Berkley AM, Sacha JB, Cogliano-Shutta NA, Lloyd M, Roby G, Kwan R, McLaughlin M, Stallings S, Rehm C, O'Shea MA, Mican J, Packard BZ, Komoriya A, Palmer S, Wiegand AP, Maldarelli F, Coffin JM, Mellors JW, Hallahan CW, Follman DA, Connors M: Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. *Immunity* 29: 1009–1021, 2008
24. Sarwal M, Chua M-S, Kambham N, Hsieh S-C, Satterwhite T, Masek M, Salvatierra O Jr.: Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. *N Engl J Med* 349: 125–138, 2003
25. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R: Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 4: 1191–1198, 2003
26. Lang KS, Recher M, Navarini AA, Harris NL, Löhning M, Junt T, Probst HC, Hengartner H, Zinkernagel RM: Inverse correlation between IL-7 receptor expression and CD8 T cell exhaustion during persistent antigen stimulation. *Eur J Immunol* 35: 738–745, 2005
27. Boutboul F, Puthier D, Appay V, Pellé O, Ait-Mohand H, Combadière B, Carcelain G, Katlama C, Rowland-Jones SL, Debré P, Nguyen C, Autran B: Modulation of interleukin-7 receptor expression characterizes

differentiation of CD8 T cells specific for HIV, EBV and CMV. *AIDS* 19: 1981–1986, 2005
28. Golden-Mason L, Burton JR Jr., Castelblanco N, Klarquist J, Benlloch S, Wang C, Rosen HR: Loss of IL-7 receptor alpha-chain (CD127) expression in acute HCV infection associated with viral persistence. *Hepatology* 44: 1098–1109, 2006
29. Koesters SA, Alimonti JB, Wachihi C, Matu L, Anzala O, Kimani J, Embree JE, Plummer FA, Fowke KR: IL-7Ralpha expression on CD4+ T lymphocytes decreases with HIV disease progression and inversely correlates with immune activation. *Eur J Immunol* 36: 336–344, 2006
30. Wuest TY, Willette-Brown J, Durum SK, Hurwitz AA: The influence of IL-2 family cytokines on activation and function of naturally occurring regulatory T cells. *J Leukoc Biol* 84: 973–980, 2008
31. Simonetta F, Gestermann N, Martinet KZ, Boniotto M, Tissières P, Seddon B, Bourgeois C: Interleukin-7 influences FOXP3+CD4+ regulatory T cells peripheral homeostasis. *PLoS One* 7: e36596, 2012
32. Betjes MGH, Meijers RWJ, de Wit EA, Weimar W, Litjens NHR: Terminally differentiated CD8+ Temra cells are associated with the risk for acute kidney allograft rejection. *Transplantation* 94: 63–69, 2012
33. Bruns H, Meinken C, Schauenberg P, Härter G, Kern P, Modlin RL, Antoni C, Stenger S: Anti-TNF immunotherapy reduces CD8+ T cell-mediated antimicrobial activity against Mycobacterium tuberculosis in humans. *J Clin Invest* 119: 1167–1177, 2009
34. FrancoSalinas G, Mai HL, Jovanovic V, Moizant F, Vanhove B, Boeffard F, Usal C, Tak PP, Soulillou JP, Baeten D, Brouard S: TNF blockade abrogates the induction of T cell-dependent humoral responses in an allotransplantation model. *J Leukoc Biol* 90: 367–375, 2011
35. Gagne K, Brouard S, Giral M, Sebille F, Moreau A, Guillet M, Bignon JD, Imbert BM, Cuturi MC, Soulillou JP: Highly altered V beta repertoire of T cells infiltrating long-term rejected kidney allografts. *J Immunol* 164: 1553–1563, 2000
36. Sebille F, Gagne K, Guillet M, Degauque N, Pallier A, Brouard S, Vanhove B, Delsuc MA, Soulillou JP: Direct recognition of foreign MHC determinants by naive T cells mobilizes specific Vbeta families without skewing of the complementarity-determining region 3 length distribution. *J Immunol* 167: 3082–3088, 2001
37. Degauque N, Schadendorf D, Brouard S, Guillet M, Sébille F, Höhn H, Pallier A, Ruiz C, Dupont A, Chapin S, Hofmann U, Maeurer M, Soulillou JP: Blood T-cell Vbeta transcriptome in melanoma patients. *Int J Cancer* 110: 721–729, 2004
38. Pannetier C, Cochet M, Darche S, Casrouge A, Zöller M, Kourilsky P: The sizes of the CDR3 hypervariable regions of the murine T-cell receptor beta chains vary as a function of the recombined germ-line segments. *Proc Natl Acad Sci USA* 90: 4319–4323, 1993
39. Brouard S, Dupont A, Giral M, Louis S, Lair D, Braudeau C, Degauque N, Moizant F, Pallier A, Ruiz C, Guillet M, Laplaud D, Soulillou J-P: Operationally tolerant and minimally immunosuppressed kidney recipients display strongly altered blood T-cell clonal regulation. *Am J Transplant* 5: 330–340, 2005
40. Clave E, Busson M, Douay C, Peffault de Latour R, Berrou J, Rabian C, Carmagnat M, Rocha V, Charron D, Socié G, Toubert A: Acute graft-versus-host disease transiently impairs thymic output in young patients after allogeneic hematopoietic stem cell transplantation. *Blood* 113: 6477–6484, 2009
41. Pan W, Chappell R: A nonparametric estimator of survival functions for arbitrarily truncated and censored data. *Lifetime Data Anal* 4: 187–202, 1998
42. Therneau TM,Grambsch PM: *Modeling Survival Data: Extending the Cox Model (Statistics for Biology and Health)*, 1st Ed., Springer
43. McCullagh P, Nelder JA: *Default: Generalized Linear Models; Chapman & Hall/CRC Monographs on Statistics & Applied Probability*, 2nd Ed., Chapman and Hall/CRC
44. Roederer M, Nozzi JL, Nason MC: SPICE: Exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry A* 79: 167–174, 2011
45. Holm S: A simple sequentially rejective multiple test procedure. *Scand J Stat* 6: 65–70, 1979

This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2013080848/-/DCSupplemental.



Supplementary Figure 1 | Gating strategy for phenotypic characterization of CD8 T cells.

	Coef	SE	Wald	p-value
Intercept	0.0079	0.0034	2.35	0.0205
Restricted-STABLE vs. Diverse-STABLE	0.0001	0.0005	0.30	0.7625
Recipient age (years)	-0.0003	0.0001	-2.31	0.0227
Recipient gender (Female vs. Male)	-0.0009	0.0004	-1206	0.0416

Supplementary Table 1 | Multivariate analysis of sjTREC levels using a generalized linear model with a gamma distribution and inversed link function.

3.3 *Article 2: The Benefits of Using CD45RA and CD28 to Investigate CD8 Subsets in Kidney Transplant Recipients*

Article *in Press* with the American Journal of Transplantation

The most commonly used nomenclature for identifying naïve, EM, CM, and TEMRA CD8 cell subsets is that which was proposed by F. Sallusto et al (Sallusto et al. 1999). However, in the recent years, we have noticed an increase in the use of alternative nomenclatures to identify the CD8 subsets. Furthermore, we noticed a shift in usage the nomenclature CD45RA & CD27 for identifying subsets, which was originally proposed by van Lier et al. as a means to identify naïve, effector, and memory CD8 subsets (D Hamann et al. 1997). However, in more recent articles, this nomenclature has begun to mimic the nomenclature of Sallusto et al. and been used to identify naïve, EM, CM, and TEMRA CD8 subsets without examining if indeed these subsets are equivalent.

Due to this evolution of CD8 nomenclatures, as well as the emergence of anti-CD28 pharmaceutical therapies coming into the transplant field, we decided to perform a comparison study in kidney transplant recipients to examine the similarities and differences of using three CD8 nomenclatures: CD45RA & CCR7, CD45RA & CD27, and CD45RA & CD28. We found that all three nomenclatures can identify naïve and TEMRA subsets with similar characteristics, but there are differences in the resulting EM and CM subsets. We found that the $CD45RA^{-}CD27^{+}$, which was originally described by van Lier et al. as memory CD8 cells but now is labeled CM CD8 cells (Henson et al. 2014; Shen et al. 2010), do not possess many features which one attributes to CM, and instead appear to be EM cells who are at an early stage of differentiation. Furthermore, we are also able to see this distinction of early versus late stages of differentiation of EM in the CD45RA & CD28 nomenclature. This

suggests that this nomenclature may be useful to investigate the role of CD8 cell subsets in transplant patients who are being treated with the next generation of anti-CD28 therapies.

THE BENEFITS OF USING CD45RA AND CD28 TO INVESTIGATE CD8 SUBSETS IN KIDNEY TRANSPLANT RECIPIENTS

Michelle Yap[1,2], Gaelle Tilly[1,2], Magali Giral[1,2,3,4,5], Sophie Brouard[1,3,4,5] and Nicolas Degauque[1, 3]

[1]INSERM, UMR 1064, Nantes, F-44093, France

[2]Université de Nantes, Faculté de Médecine, Nantes, F-44035, France

[3]CHU de Nantes, ITUN, Nantes, F-44093, France

[4]CIC biothérapie, Nantes, F-44035, France

[5]CHU Nantes, CRB, Nantes, F-44093, France

The authors have declared that no conflict of interest exists.

Corresponding author

Nicolas Degauque

Pavillon Jean Monnet

Hôtel Dieu - CHU de Nantes

30, Bd Jean Monnet

44093 Nantes Cedex 01

France

Phone +33 (0)240087410

Fax +33 (0)240087411

Email nicolas.degauque@univ-nantes.fr

Abstract

The deleterious role of CD8 T cells in kidney graft outcome has regained interest over the years and memory T cells are considered as one of the main hurdle to achieve transplantation success. Monitoring the CD8 immune response in transplant recipients involved a heterogeneous combination of markers but the justification of their choice is rarely stated. Whereas the number of parameters is not an issue in phenotypic analysis, functional assays have to accommodate the cell number with the narrowing of the subset. The aim of the study was to investigate the similarities and differences of the subsets identified using three nomenclatures (CD45RA and CCR7/CD27/CD28) in kidney transplant recipients with stable graft function. We found that all three nomenclatures can identify Naïve and TEMRA CD8 with similar features. Whereas CM CD8 could only be documented using CCR7 and CD45RA, the characteristics of EM CD8 will differ according to the nomenclature. We found that the use of the CD45RA & CD28 gives the benefit of examining two EM populations at early and late differentiation states. This systematic comparison provides a cohesive layout of the advantages of using these nomenclature strategies in kidney transplant recipients in order to guide the choice of their use.

Introduction

The deleterious role of CD8 T cells in kidney graft outcome has regained interest over the years. Memory T cells are now under scrutiny and are considered as one of the main hurdle to achieve transplantation success and even transplantation tolerance (1). Higher incidence of rejection has been associated with the pre-existing memory T cells (2) or a higher precursor frequency of alloreactive CD8 T cells (3). Heterogeneous combination of markers could be found in the literature of immune-monitoring of CD8 subsets in kidney transplant recipients such as the use of CD45RO and CCR7 (4), CD45RO and CD27 (5,6), CD45RA and CD27 (7), CD45RA, CCR7 and CD28 (8,9), CD45RO, CCR7 and CD28 (10), CD45RA, CCR7, CD27 and CD28 (11,12). The inclusion of CD28 in the set of markers has gained a growing interest with the advent of costimulation blockade therapies and especially those targeting CD28 – CD80/CD86 pathway(13). On which ground one nomenclature is chosen over another is rarely mentioned despite the fact that the functionality of the CD8 subsets will be greatly influenced by the markers chosen. While the description of the CD8 compartment will benefit from a larger number of markers, functional assay required fewer markers to accommodate the balance between the precision of CD8 subsets and the number of cells needed for this assay. Moreover, current FACS-sorters allows only for the purification of 4 populations. Therefore, it is important to choose the right CD8-markers to address the appropriate research question. As aforementioned, the justification of the use of one combination over another is rarely stated which ultimately could lead to the misuse of the markers. For instance, there appears to have been an evolution in the subsets identified using CD45RA and CD27. While this nomenclature was pioneered as being able to identify naïve, effector and memory subsets(14), over time it has begun to be misused as a means to identify naïve, CM, EM, and TEMRA (7,15,16). The aim of the study was to investigate the similarities and differences of the subsets identified using three nomenclatures (CD45RA

&CCR7, CD45RA & CD27, and CD45RA & CD28) in a large cohort of kidney transplant recipients with stable graft function and standard immunosuppressive regimen. This systematic comparison aims to provide a cohesive layout of the advantages of using these nomenclature strategies in kidney transplant recipients in order to guide the choice of the use of one over another nomenclature. The monitoring of CD8-compartment of kidney transplant recipients will also benefit from an enlighten choice of CD8-related markers.

Materials and methods

Subjects and Ethics statement

Peripheral blood mononuclear cells (PBMC) were prospectively collected from 73 kidney transplant recipients in the DIVAT biocollection (www.divat.fr) and stored in the Biological Resource Center of the Nantes University Hospital, F-44093, France (BRIF : BB-0033-00040). All donors were informed of the final use of their blood and signed an informed consent. The University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks approved the study for patients. All kidney transplant patients have normal graft function at 12 months post-transplantation (creatinemia less than 150 μmol/L and proteinuria less than 0.4g/24h at the time of sampling). Clinical characteristics are shown in **Table 1**.

Blood samples

PBMC were separated on a Ficoll gradient layer and frozen in DMSO-10% autologous serum.

Polychromatic flow cytometry

Multi-colored flow cytometry was performed on whole PBMC using a LSRII (BD Bioscience). 3×10^{6}frozen PBMCs were surface stained with antibodies specific for CD3 (BW264/56; VioBlue), CD8 (BW135/80; VioGreen), CD45RA (T6D11; APC-Vio770), CCR7 (3D12; PE-Cy7), CD27 (L128; Brilliant Violet 605) and CD28 (CD28.2; PE-CF594). In addition to this core-staining cocktail, different combinations of antibody were used CD127 (MB15-18C9; PE), CD57 (TB03; FITC), T-bet (O4-46; PE), Granzyme B (GB11; AlexaFluor 700), and Perforin (B-D48; PE). Intracellular staining was performed using the Foxp3/Transcription Factor Staining Buffer Set according to the manufacture instructions (eBioscience). Yellow LIVE/DEAD Fixable Dead Cell Stain Kit was used to exclude dead

cells from analysis. BD CompBeads stained separately with individual mAbs were used to define the compensation matrix. Data were analyzed using FlowJo Version 9.7.6. All the antibodies were purchased from BD Biosciences except for CD3-VioBlue, CD8-VioGreen, CD45RA-APC-Vio770, CD127-PE, and CD57-FITC (Miltenyi) and Perforin-PE (Diaclone).Lymphocytes were first gated for morphology then for viability. Next, $CD3^+CD8^+$ cells were located and then cells were gated using CD45RA &CCR7, CD45RA& CD27, or CD45RA &CD28. On the subsets of each nomenclature, the percentage of cells positive for each phenotypic marker was analyzed. Of note, there was not additional stimulation used to detect the expression of any markers.

Statistical analyses

Statistics were analyzed using Graphpad Prism. Unpaired T-test with Welch's correction was used and a p-value of less than 0.05 was considered significant.

Results

Characteristics of the kidney transplant cohort

The clinical and demographic characteristics of the population are shown in **Table 1**. Patients were transplanted between November 2006 and December 2011. All the patients signed informed consent. Among the 73 kidney transplant recipients alive with a functional kidney graft at 1 year post-transplantation, 95.9% were recipients of a first graft. At the time of sampling (i-e 12 months post-transplantation), mean±sd of creatinemia and proteinuria was 109±21 µmol/L and 0.18±0.13 g/24h respectively.

Characterization of CD8 Subsets by combining CD45RA and one additional maker (CCR7, CD27 orCD28)

We investigated the differences in gating strategies used to identify different CD8 subsets using a combination of CD45RA with CD27, CD28, or CCR7. The three different strategies, CD45RA&CCR7, CD45RA&CD27, and CD45RA&CD28 were compared using flow cytometry data collected from whole PBMC from 73 stable kidney transplant patients (TX). After gating for morphology, viability, and $CD3^{+}CD8^{+}$ cells, the three different strategies were used to identify the different CD8 subsets (**Supplementary Figure 1**). The expression of remaining two antibodies that were missing from each nomenclature was also analyzed in both cohorts. In all 3 nomenclatures, Naïve CD8 cells and TEMRA cells are both positive for CD45RA whereas Effector Memory (EM) and Central Memory (CM) cells do not express CD45RA. A second marker (CCR7, CD27 or CD28) is necessary to separate naïve cells from TEMRA cells within the $CD45RA^{+}$ compartment and EM from CM within the $CD45RA^{-}$ compartment.

The use of CD8 T cells was heterogeneous within the 73 kidney transplant recipients (**Figure 1A**). Whereas the frequency of CM has low variation across patients (mean±sd 5.6±4.3), the frequency of naïve, TEMRA and EM exhibit divergent repartition (mean±sd27.7±17.1, 25.8±16.4 and 22.0±7.1 respectively; **Figure 1A**). Two groups of patients could be identified: $TEMRA^{high}$ consisted of patients with a high frequency of TEMRA CD8 T cells and lower frequency of naïve and EM T cells; $TEMRA^{low}$ consisted of patients with a lower frequency of TEMRA CD8 T cells and a higher frequency of naïve and EM T cells. Of note, the clinical characteristics was similar between patients with high or low frequency of TEMRA CD8 T cells.

We took advantage of the heterogeneity of CD8 T cell subsets to first assess the influence of CCR7, CD27 and CD28 on the ability to decipher the 4 CD8 subsets, using the CCR7 & CD45RA nomenclature as the nomenclature of reference. The frequencies of naïve CD8 T cells (defined as $CD45RA^{+}CCR7^{+}$) and TEMRA CD8 T cells (defined as $CD45RA^{+}CCR7^{-}$) were on average similar when the expression of CD27 or CD28 was used (**Figure 1B**). The $CD45RA^{+}CD27^{+}$ and $CD45RA^{+}CD28^{+}$ cell subsets highly correlated with the naïve CD8 T cells (Spearman coefficient r = 0.91 and 0.98, respectively; for both p<0.0001; **Figure 1B**). Like the naïve subsets, $CD45RA^{+}CD27^{-}$ and $CD45RA^{+}CD28^{-}$ cells correlated very well with TEMRA cells (Spearman coefficient r = 0.84 and 0.95, respectively; for both p<0.0001; **Figure 1B**). In contrast, the EM and CM showed little correlation with the analogous subsets identified with CD27 and CD28. The linear correlation of EM ($CD45RA^{-}CCR7^{-}$) was low when compared to $CD45RA^{-}CD27^{-}$ and $CD45RA^{-}CD28^{-}$(Spearman coefficient r 0.49 (p<0.0001) and 0.22 (p = 0.0675) respectively; **Figure 1B**). Similarly, $CD45RA^{-}CD27^{+}$ and $CD45RA^{-}CD28^{+}$ have a Spearman coefficient r equal to 0.71 and 0.64, respectively (for both, p<0.0001), when correlated to CM cells (**Figure 1B**). The high correlation in the naïve and TEMRA subsets and the low correlation in the EM and CM subsets shows that all three

nomenclatures can easily and dependably identify naïve and TEMRA CD8 populations with similar characteristics; however, there are discrepancies in the expression of co-stimulatory markers CD27 and CD28, as well as CCR7 in the EM and CM subsets. Finally, we tested whether the differences across the 3 nomenclatures could be explained by the time of sampling or by the immune-challenge induced by kidney transplantation. The time of sampling does impact the distribution of CD8 subsets (identified according to the expression CD45RA and CCR7; **Supplementary Figure 2**). The ability of the 3 nomenclatures to define CD8 subsets exhibits a similar efficiency when blood samples from Healthy Volunteers (HV; n=16) were used (**Supplementary Figure 3**). Collectively, our results show that co-stimulatory markers CD27 and CD28 as well as CCR7 could be used to define naïve and TEMRA CD8 T cells whereas the EM and CM population exhibit heterogeneous expression of these markers.

Naïve and TEMRA CD8 cell characteristics remain consistent across the three different nomenclatures.

We then aimed to better characterize within each subset of CD8 T cells the expression of additional markers (CD127, PERF, GZMB, CD57 and T-bet) and thus confirmed the identity of one subset identified using the 3 different nomenclatures.CD45RA$^+$ cells that also co-express the second marker (CCR7, CD27 or CD28) exhibit all the features attributed to naïve cells (**Figure 2A**). Expression of CD127 was detected on all cells whereas very low expression of cytotoxic molecules (PERF and GZMB), effector associated transcription factor (T-bet) and markers of activation/senescent (CD57) was found. A small increase of GZMB$^+$, CD57$^+$ and T-bet$^+$ frequency was observed in CD45RA$^+$CD27$^+$ T cells as compared to those of CD45RA$^+$CCR7$^+$ or CD45RA$^+$CD28$^+$. The magnitude of this increase expression of effector-associated markers is low and explains the trend of a higher frequency of naïve cells when CD27 or CD28 markers were used (**Supplemental Table 1**).TEMRA cells moderately

expressed CD127 and expressed high levels of PERF, GZMB, CD57, and TBET (**Figure 2B**). Altogether, we found that the expression of these markers was not significantly different within the three groups. Of interest, whereas the range of frequency of naïve and TEMRA CD8 T cells varies from low to high (**Figure 1A**), the population identified by the 3 nomenclatures is homogeneous regarding the expression of effector-associated molecules (**Figure 2A** and **B**).

Intra-subset differences in characterization profiles EM subsets.

EM CD8 T cells exhibit as expected a high expression of T-bet, regardless the nomenclature used (**Figure 2C**). The range of expression of cytotoxic molecules and CD127 was large when EM CD8 T cells were identified as $CD45RA^-CCR7^-$(**Figure 2C**) despite the more narrow dispersion of the frequency of EM across the kidney transplant recipients (**Figure 1A**). The use of costimulatory molecules to identify EM highlights a more differentiated population with especially a higher frequency of $PERF^+$ and $GZMb^+$ (**Figure 2D**). A gradient of differentiation could be observed between $CD27^-$ and $CD28^-$ $CD45RA^-$ CD8 T cells (**Figure 2D**). The expression of cytotoxic molecules (PERF and GZMb) and effector-associated marker (CD57 and T-bet) were significantly higher in $CD28^-$ compartment and the expression of CD127 was lower (**Figure 2D**). Moreover, the use of CD28 to identify EM allows restraining the heterogeneity in the level of expression of effector-associated molecules (**Figure 2C**). For instance the mean±sd of GZMb was 44.6%±20.3 for $CD45RA^-CCR7^-$ and 84.5%±11.6 for $CD45RA^-CD28^-$ (**Figure 2C**). Collectively, the use of CD28 over CCR7 allows to increase the homogeneity of the EM CD8 subsets.

Costimulatory-based nomenclature identified EM CD8 T cells with intermediate and advanced differentiation.

We observed that the frequency of CD8 T cells within the CD45RA^{-} fraction differs when CD27 or CD28 expression is used as compared to the use of CCR7 (**Figure 1B**). Not only the frequency of subsets differs as compared to CCR7 counterpart, but we also report that the expression of CD27 and CD28 varies within the CD45RA^{-} fraction. A higher frequency of CD45RA^{-}CD28^{+} and a lower expression of CD45RA^{-}CD28^{-} were observed as compared to the CD45RA^{-}CD27^{+} and CD45RA^{-}CD27^{-} respectively (**Figure 3A**). In contrast, the frequency of CD8 subsets within the CD45RA^{+} fraction did not differ according to the use CD27 or CD28 (**Figure 3A**). As previously reported by Appay et al., the expression of CD27 and CD28 has been assigned to different levels of differentiation(17), with a gradual loss of CD27 and CD28 respectively in the context of viral infection. Our results confirmed this observation as EM CD45RA^{-}CD28^{-} expressed a more differentiated phenotype as compared to EM CD45RA^{-}CD27^{-} with a higher magnitude of expression of cytotoxic molecules and effector-associated molecules and a lower expression of CD127 (**Figure 3B**).

Collectively, the nomenclature of CD45RA & CD28 allows for the identification of two EM subsets with different characteristics that cannot be observed using the other two nomenclatures.

Discussion

Viral stimulation or allogeneic transplantation leads to the generation of CD8 memory T cells that constitutes a major hurdle to achieve long-term acceptance of allogeneic graft even in the context of standard immunosuppressive regimen. Accelerated kinetic of rejection in animal that were previously grafted had been reported decades ago (18) and numerous examples of cross-reactivity of CD8 T cells between viral peptide and allogeneic HLA have been reported(19). We have also recently reported that an accumulation of TEMRA CD8 T cells in kidney transplant recipients with stable graft function is associated with an increase risk of kidney dysfunction (11). Given their key role in anti-donor immune response, the phenotypic and functional monitoring of CD8 T cells in kidney transplant recipients require an objective choice of cell surface markers. Given the heterogeneity of markers used in the transplantation literature (3,4,7-12,20-22), it is difficult to understand the choice over one set of markers over another. In the current study, we took advantage of the inclusion of a larger number of kidney transplant recipients with stable graft function to describe the pros & cons of the association of CD45RA with CCR7, CD27 or CD28 to purify CD8 subsets. As expected (11,12), a heterogeneous repartition of naïve, TEMRA, EM and CM was observed in the 73 enrolled transplant recipients. We provide evidences that within the $CD45RA^+$ fraction, naïve and TEMRA CD8 T cell can be identified with CCR7, CD27 or CD28 markers and the expression of additional phenotypic markers (GZM-b, PERF, CD127, T-bet and CD57) is on average similar. In contrast, we further confirmed that CM CD8 T cells could only be identified with the expression of CCR7. Within the $CD45RA^-$ fraction, the use of CD28 allows to identify effector memory with strong biases in their effector program, ranging from early differentiation in the $CD45RA^-CD28^+$ T cells to highly differentiated $CD45RA^-CD28^-$ T cells. The magnitude of differentiation of $CD8^+CD45RA^-$ T cells is lower when EM cells are identified as $CD45RA^-CCR7^-$ T cells.

A drift has been observed in the literature to accommodate the use of CD45RA and CD27 with the original description of CD8 subsets based on CD45RA and CCR7. For instance, $CD8^{+}CD27^{-}$ T cells had been described as CM (7,15,16,23) in healthy volunteers as well as in clinical settings including transplantation despite the fact that these cells are lacking CCR7 expression. Indeed, the characteristics of the subsets that were identified using either $CD45RA^{-}CCR7^{+}$ or $CD45RA^{-}CD27^{+}$ were in sharp contrast. The frequencies of these two subsets were drastically different, with a mean±sd of 5.7% ± 4.3 of $CD45RA^{-}CCR7^{+}$ and 21.8% ± 12.8 of $CD45RA^{-}CD27^{+}$. Moreover, $CD45RA^{-}CCR7^{+}$ cells were mostly $CD27^{+}$, whereas $CD45RA^{-}CD27^{+}$ did not express CCR7. A careful appraisal of the marker used in each study is thus needed to avoid the report of erroneous data and their associated conclusion.

In the context of costimulation blockade strategies in kidney transplantation, the monitoring of CD28 has gained an increase interest. The FDA-approved Belatacept (CTLA4-Ig) has been proposed as an alternative to calcineurin inhibitor (13). The introduction of this new molecule has lead to the identification of costimulation blockade resistant patients in which a sizable frequency of $CD8^{+}CD28^{-}$ T cells could be evidenced (24). The loss of CD28 expression in T cells could be driven by repeated antigen stimulation leading to the generation of highly antigen-experienced $CD8^{+}CD28^{-}$ T cells (25). $CD8^{+}CD28^{-}$ T cells have been shown to exhibit polyfunctional cells including multiple cytokines secretion and high level of cytotoxic molecules (PERF and GZMb) in the context of alloreactive assays (8). The frequency of $CD8^{+}CD28^{-}$ T cells is increased in patients with biopsy-proven chronic antibody mediated rejection (12). We also have recently reported that an accumulation of TEMRA CD8 T cells in kidney transplant recipients with stable graft function is associated with an increase risk of kidney dysfunction (11). Collectively, these results highlight that $CD8^{+}CD28^{-}$ T cells are pathogeneic cells involved in kidney graft rejection that are necessary to monitor. We

reported here that the use of CD45RA and CD28 markers offers thus several benefits to characterize the CD8 compartment in kidney transplant recipients, including the ability to identify early and late-differentiated EM CD8 T cells.

Acknowledgments

The International Fellowship Grant from ProGreffe Fondation supports M.Yap. This work was funded by a grant from the ITMO Santé Publique and was realized in the context of the IHU-Cesti project which received French government financial support managed by the National Research Agency via the "Investment Into The Future" program ANR-10-IBHU-005. The IHU-Cesti project is also supported by Nantes Metropole and the Pays de la Loire Region. This work was also supported by the FP7 VISICORT project that has received funding from the European Union’s Seventh Framework Programme for research, technological development and demonstration under grant agreement no 602470.

References

1. Valujskikh A, Li XC. Frontiers in Nephrology: T Cell Memory as a Barrier to Transplant Tolerance. J Am Soc Nephrol. 2007 Jun 28;18(8):2252–61.

2. Poggio ED, Clemente M, Riley J, Roddy M, Greenspan NS, Dejelo C, et al. Alloreactivity in renal transplant recipients with and without chronic allograft nephropathy. J Am Soc Nephrol. American Society of Nephrology; 2004 Jul;15(7):1952–60.

3. van de Berg PJEJ, Yong SL, Koch SD, Lardy N, van Donselaar-van der Pant KAMI, Florquin S, et al. Characteristics of alloreactive T cells measured before renal transplantation. Clin Exp Immunol. Blackwell Publishing Ltd; 2012 May;168(2):241–50.

4. Meijers RWJ, Litjens NHR, de Wit EA, Langerak AW, van der Spek A, Baan CC, et al. Cytomegalovirus contributes partly to uraemia-associated premature immunological ageing of the T cell compartment. Clin Exp Immunol. 2013 Dec;174(3):424–32.

5. Kamburova EG, Koenen HJPM, van den Hoogen MWF, Baas MC, Joosten I, Hilbrands LB. Longitudinal Analysis of T and B Cell Phenotype and Function in Renal Transplant Recipients with or without Rituximab Induction Therapy. Borrow R, editor. PLoS ONE. 2014 Nov 13;9(11):e112658.

6. Ortiz YM, Arias LF, Alvarez CM, García LF. Memory phenotype and polyfunctional T cells in kidney transplant patients. Transplant Immunology. 2013 Mar;28(2-3):127–37.

7. Couzi L, Pitard V, Netzer S, Garrigue I, Lafon ME, Moreau J-F, et al. Common Features of γδ T Cells and CD8 + αβ T Cells Responding to Human Cytomegalovirus Infection in Kidney Transplant Recipients. J Infect Dis. 2009 Nov;200(9):1415–24.

8. Lo DJ, Weaver TA, Stempora L, Mehta AK, Ford ML, Larsen CP, et al. Selective targeting of human alloresponsive CD8+ effector memory T cells based on CD2 expression. Am J Transplant. Blackwell Publishing Inc; 2011 Jan;11(1):22–33.

9. Trzonkowski P, Zilvetti M, Chapman S, Więckiewicz J, Sutherland A, Friend P, et al. Homeostatic Repopulation by CD28−CD8+ T Cells in Alemtuzumab-Depleted Kidney Transplant Recipients Treated With Reduced Immunosuppression. Am J Transplant. 2008 Jan 15;8(2):338–47.

10. Betjes MGH, Meijers RWJ, de Wit EA, Weimar W, Litjens NHR. Terminally Differentiated CD8+ Temra Cells Are Associated With the Risk for Acute Kidney Allograft Rejection. Transplantation. 2012 Jul;94(1):63–9.

11. Yap M, Boeffard F, Clave E, Pallier A, Danger R, Giral M, et al. Expansion of highlydifferentiatedcytotoxicterminallydifferentiatedeffectormemory CD8+ T cells in a subset of clinically stable kidney transplant recipients: a potential marker for late graft dysfunction. J Am Soc Nephrol. 2014 Aug;25(8):1856–68.

12. Baeten D, Louis S, Braud C, Braudeau C, Ballet C, Moizant F, et al. Phenotypically and functionally distinct CD8+ lymphocyte populations in long-term drug-free tolerance and chronic rejection in human kidney graft recipients. J Am Soc Nephrol. 2006 Jan;17(1):294–304.

13. Vincenti F, Larsen C, Durrbach A, Wekerle T, Nashan B, Blancho G, et al. Costimulation blockade with belatacept in renal transplantation. N Engl J Med. 2005 Aug 25;353(8):770–81.

14. Hamann D, Baars PA, Rep MH, Hooibrink B, Kerkhof-Garde SR, Klein MR, et al. Phenotypic and functional separation of memory and effector human CD8+ T cells. J Exp Med. 1997 Nov 3;186(9):1407–18.

15. Henson SM, Lanna A, Riddell NE, Franzese O, Macaulay R, Griffiths SJ, et al. p38signalinginhibits mTORC1-independent autophagy in senescent human CD8+ T cells. 2014 Aug 1;124(9):4004–16.

16. Shen T, Zheng J, Xu C, Liu J, Zhang W, Lu F, et al. PD-1 expression on peripheral CD8+ TEM/TEMRA subsets closely correlated with HCV viral load in chronic hepatitis C patients. Virol J. 2010;7(1):310.

17. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GMA, Papagno L, et al. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med. 2002 Apr;8(4):379–85.

18. Gordon RD, Mathieson BJ, Samelson LE, Boyse EA, Simpson E. The effect of allogeneic presensitization on H-Y graft survival and in vitro cell-mediated responses to H-yantigen. J Exp Med. 1976 Sep 1;144(3):810–20.

19. Smith C, Miles JJ, Khanna R. Advances in direct T-cell alloreactivity: function, avidity, biophysics and structure. Am J Transplant. 2012 Jan;12(1):15–26.

20. Kirk AD, Guasch A, Xu H, Cheeseman J, Mead SI, Ghali A, et al. Renal Transplantation Using Belatacept Without Maintenance Steroids or Calcineurin Inhibitors. Am J Transplant. 2014 Mar 31;14(5):1142–51.

21. Ducloux D, Courivaud C, Bamoulid J, Bisaccia V, Roubiou C, Crepin T, et al. Alloimmune Responses and Atherosclerotic Disease After Kidney Transplantation. Transplantation. 2015 Jan;99(1):220–5.

22. Traitanon O, Gorbachev A, Bechtel JJ, Keslar KS, Baldwin WM III, Poggio ED, et al. IL-15 Induces Alloreactive CD28 −Memory CD8 T Cell Proliferation and CTLA4-Ig Resistant Memory CD8 T Cell Activation. Am J Transplant. 2014 May 19;14(6):1277–89.

23. Di Mitri D, Azevedo RI, Henson SM, Libri V, Riddell NE, Macaulay R, et al. Reversible senescence in human CD4+CD45RA+CD27- memory T cells. J Immunol. 2011 Sep 1;187(5):2093–100.

24. Xu H, Perez SD, Cheeseman J, Mehta AK, Kirk AD. The Allo- and Viral-Specific Immunosuppressive Effect of Belatacept, but Not Tacrolimus, Attenuates With Progressive T Cell Maturation. Am J Transplant. 2014 Jan 28;14(2):319–32.

25. Strioga M, Pasukoniene V, Characiejus D. CD8+ CD28− and CD8+ CD57+ T cells and their role in health and disease. Immunology. Blackwell Publishing Ltd; 2011 Jun 29;134(1):17–32.

Figure Legends

Figure 1. The identification of all CD8 subsets except CM and EM can be realized using the 3 nomenclatures in kidney transplant recipients. A. The usage of CD8 subsets identified by the expression of CD45RA and CCR7 is heterogeneous across the 73 kidney transplant recipients. The frequency of naïve, TEMRA, EM and CM CD8 T cells is shown and the relative proportion of each subset for each patient is display by a connecting line. The frequency of the 4 CD8 subsets is shown in the two groups of patients that can be identified according to their high or low frequency of TEMRA. B. Using the CD45RA and CCR7 nomenclature as the nomenclature of reference, the frequency of the 4 CD8 subsets was compared with those using CD45RA and CD28 (open triangle) or CD45RA and CD27 (open circle). The correlation of the linear regression was assessed using Spearman Test and the linear regression is display (dash line, CD45RA and CD28; solid line, CD45RA and CD27).

Figure 2. Naïve, TEMRA but not EM CD8 T cells identified by the 3 nomenclatures exhibit similar phenotypic markers. The expression of cytotoxic molecules, CD127, T-bet and CD57 was analyzed in naïve (A), TEMRA (B) and EM (C) CD8 T cells. Whereas naïve and TEMRA exhibit the expected phenotype, the expression of the analyzed markers was heterogeneous. Of note, the use CD28 marker allows to minimize this heterogeneity. For each patient, the percentage of variation of the 5 markers was compared between the 3 nomenclatures using CCR7-based nomenclature as reference.

Figure 3. CD28 expression allows the identification of intermediate and advanced stage of differentiated EM cells. (A) The percentage of variation between $CD27^-$ and $CD28^-$based nomenclature was compared for the 4 CD8 subsets. (B) The expression of PERF, GZMb, CD127, CD57 and T-bet was compared within the $CD45RA^-$ fraction according to the

expression or not of CD27 and CD28. CD27-based nomenclature was used as the reference nomenclature.

Supplementary Figure 1. FACS gating strategy to identify CD8 subsets. Viable cells were selected by gating on Yellow negative cells and lymphocytes were then identified by morphology (FSC vs. SSC), and $CD3^{+}CD8^{+}$ were selected. CD8 subsets were identified by using quadrant gates on CD45RA & CCR7, CD45RA & CD27, or CD45RA &CD28.

Supplementary Figure 2. The time of sampling does not alter the frequency of CD8 T cell subsets. The frequencies of CD8 subsets identified according to CD45RA and CCR7 expression were analyzed according to the year of sampling.

Supplementary Figure 3. Naïve and TEMRA CD8 can be identified using the 3 nomenclatures in healthy volunteers. The frequencies of CD8 subsets identified using the 3 nomenclatures were compared in PBMC retrieved from healthy volunteers (n=16). Using the CD45RA and CCR7 nomenclature as the nomenclature of reference, the frequency of the 4 CD8 subsets was compared with those using CD45RA and CD28 (open triangle) or CD45RA and CD27 (open circle). The correlation of the linear regression was assessed using Spearman Test and the linear regression is display (dash line, CD45RA and CD28; line, CD45RA and CD27).

		Mean or Number	SD or Percentage
Recipient Age (years)		48.38	11.42
Recipient Gender (Male)		42	58%
Deceased Donors		73	100%
Creatinine at 12 months post-TX		108.96	20.93
Proteinuria at 12 months post-TX		0.18	0.13
Maintenance Therapy	Tacrolimus	64	88%
	Cyclosporine A	6	8%
	Mycophenolic Mofetil	68	93%
	Azathioprine	2	3%
	Rapamycin	1	1%
	Corticosteroids	16	22%
Induction Therapy	Lymphocyte Depleting Therapy	17	23%
	Lymphocyte Non-Depleting Therapy	56	77%

Table 1. Summary of demographic and clinical characteristics of patients.

	Second	mean±sd	Generic	CCR7+	CD27+	CD28+

	marker	(%)	subset name	mean±sd (%)	mean±sd (%)	mean±sd (%)
CD45RA+	CCR7+	24.8±16.4	Naïve	++	90.6±13.1	97.0±4.1
	CD27+	29.3±16.5	Naïve	76.1±18.4	++	83.3±14.6
	CD28+	29.2±16.7	Naïve	79.3±16.9	78.9±16.6	++
	CCR7-	27.2±16.9	TEMRA	--	14.8±14.0	19.8±16.6
	CD27-	26.5±16.6	TEMRA	12.7±17.4	--	15.5±18.9
	CD28-	24.1±16.2	TEMRA	9.7±9.9	10.5±11.2	--
CD45RA-	CCR7+	6.8±6.1	CM	++	75.5±17.8	95.9±4.4
	CD27+	21.8±12.8	EM	42.0±16.6	++	86.9±9.7
	CD28+	29.3±15.3	EM early	37.5±15.5	54.6±17.4	++
	CCR7-	23.5±8.9	EM	--	30.0±17.3	57.1±22.7
	CD27-	22.4±11.9	EM	11.0±9.6	--	45.9±24.2
	CD28-	17.4±12.3	EM late	5.4±5.6	14.7±13.0	--

Supplementary Table 1. Comparison of subsets as identified by their expression of CD45RA and a secondary marker and their generic subset name.

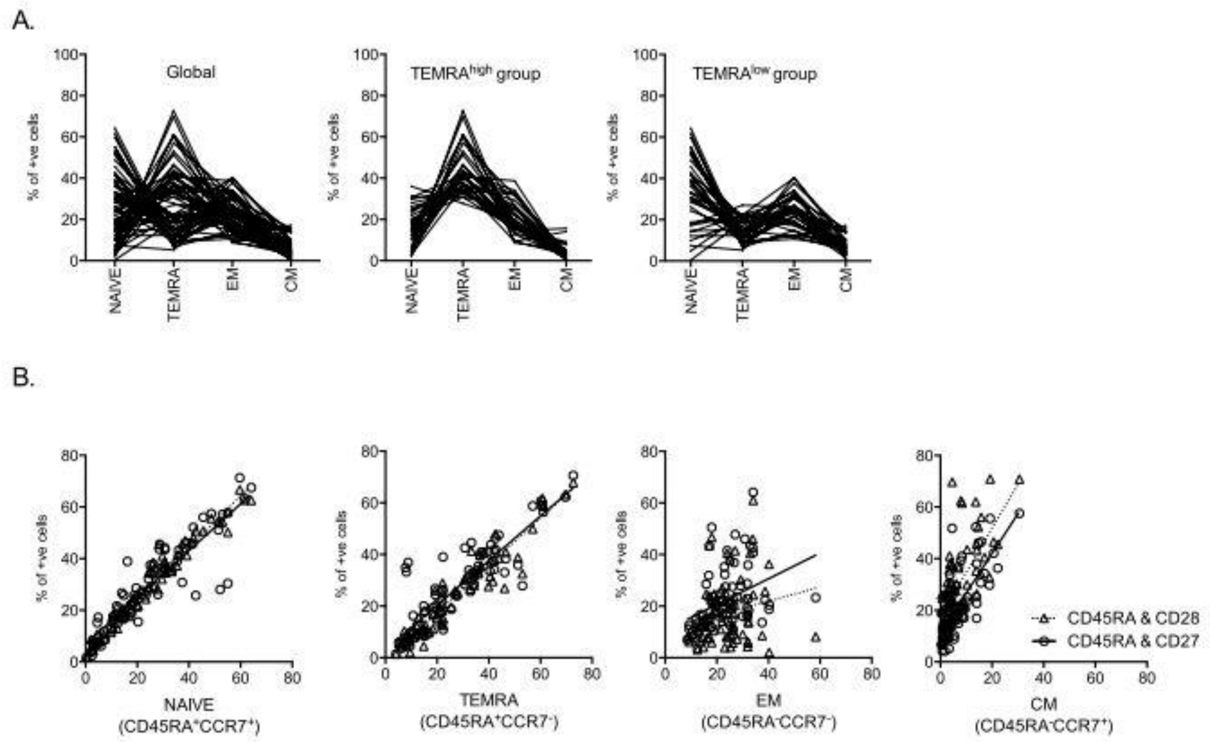


Figure 1

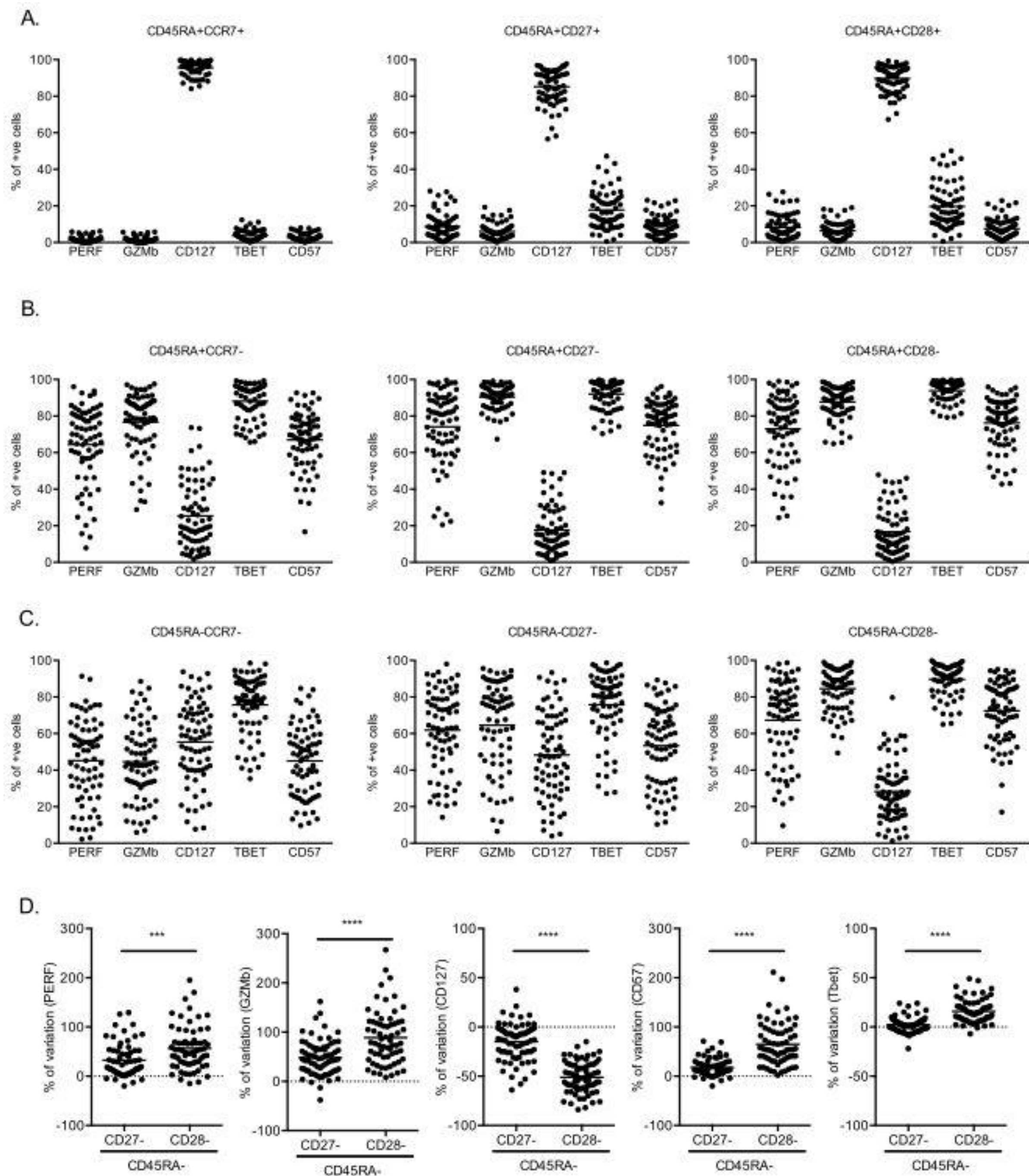


Figure 2

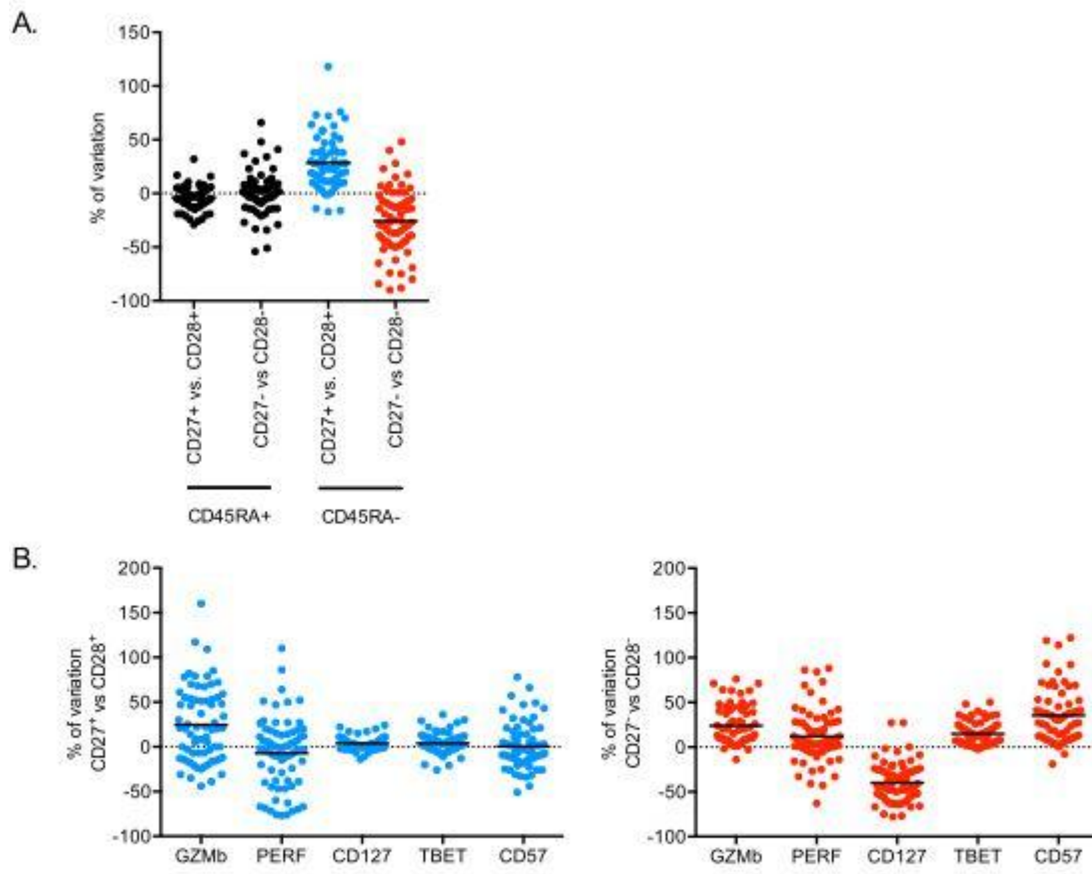
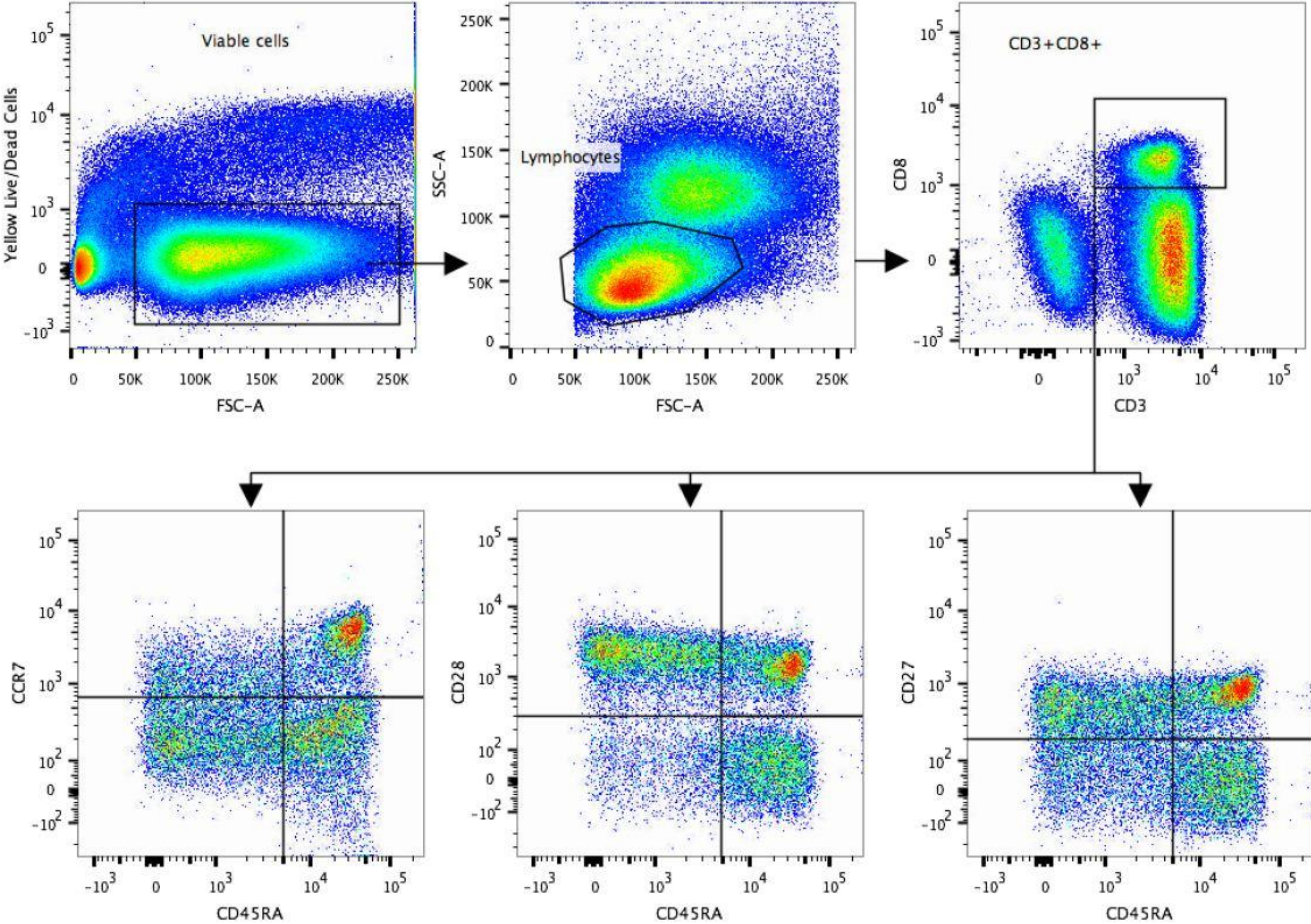
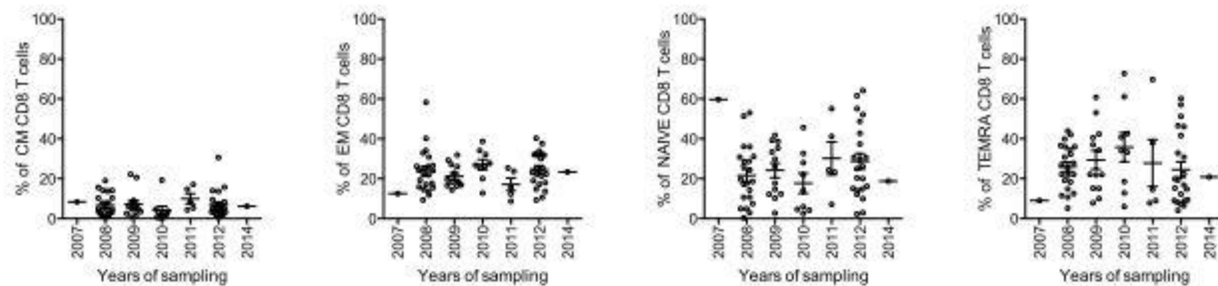


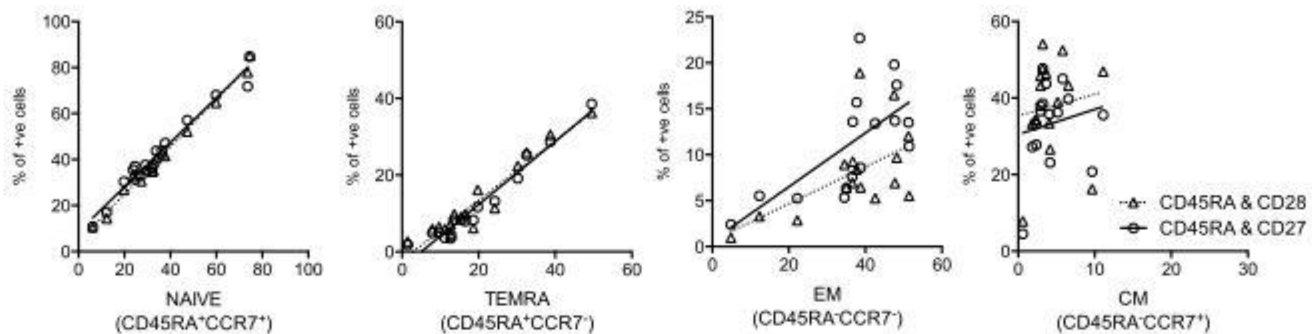
Figure 3



Supplemental Figure 1.



Supplemental Figure 2.



Supplemental Figure 3.

3.4 Article 3: Inclusion of CD8 Monitoring Improves the Prognostic Capacities of the Kidney Failure Transplant Score

Article under review with the Journal of Leukocyte Biology

This article is the follow up study to two previous studies performed by our group. The Kidney Transplant Failure Score (KTFS), which was developed by Y. Foucher and colleagues, uses clinical parameters collected at one year post-transplantation to calculate a predictive value to indicate the likelihood of a patient being at risk of long term graft failure (Foucher et al. 2010). Also, we found that patients with an increased frequency of TEMRA $CD45RA^{+}CCR7^{-}CD27^{-}CD28^{-}$ CD8 T cells had a 1.96 fold chance increase of long-term allograft dysfunction (Yap et al. 2014). In this follow up study, we examined the immunophenotype of 161 kidney transplant patients who were not included in the original KTFS study to investigate if including immunological biomarkers into the KTFS would be able to improve the prognostic power of the clinical parameter based score. Using the same readout and endpoints as the initial KTFS study, we assessed if a composite KTFS would be a better predictor of graft failure and return to dialysis compared to the original KTFS. We found that including the frequency of $CD45RA^{+}CCR7^{-}$ TEMRA CD8 T cells into the KTFS increase the AUC from 0.64 (95% CI 0.48 – 0.80) to 0.69 (95% CI 0.54 – 0.83). By including the frequencies of $CD27^{+}CD28^{-}$, $GZMb^{+}PERF^{+}$ and $GZMb^{-}PERF^{+}$ to the KTFS calculations, the AUC for prognostic up to 6-years post transplantation increased to 0.76 (95% CI 0.62 – 0.90). This new biomarker-based KTFS allowed for the better classification of 26.1% of the kidney transplant patients. These results show that the updated KTFS is an innovative tool for clinicians which will help predict patients at risk of chronic kidney graft loss and that TEMRA, $CD27^{+}CD28^{-}$, $GZMb^{+}PERF^{+}$ and $GZMb^{-}PERF^{+}$ CD8 cells are biomarkers for kidney allograft health.

INCLUSION OF CD8 MONITORING IMPROVES THE PROGNOSTIC CAPACITIES OF THE KIDNEY TRANSPLANT FAILURE SCORE

Nicolas Degauque[*,1,2] Michelle Yap[*,1,3], Gaëlle Tilly[1,3], Pierrick Guerif[2], Magali Giral[1,2,3,4,5], Sophie Brouard [*,1,2,4,5] and Yohann Foucher[*, 2,6]

[1]INSERM, UMR 1064, Nantes, F-44093, France

[2]CHU de Nantes, ITUN, Nantes, F-44093, France

[3]Université de Nantes, Nantes, F-44035, France

[4]CIC biothérapie, Nantes, F-44035, France

[5]CHU Nantes, CRB, Nantes, F-44093, France

[6]Université de Nantes, SPHERE laboratory EA4275, Nantes, F-44035, France

[*] ND, MY, SB and YF contribute equally to the work and should be considered as co-first authors and co-last authors respectively. Names are by alphabetical order for first and last.

Running Title. Kidney Graft Failure Score

Corresponding author

Magali Giral

INSERM UMR 1064 - ITUN

Hôtel Dieu - CHU de Nantes

30, Bd Jean Monnet

44093 Nantes cedex 01

France

Phone +33 (0)240087410

Fax +33 (0)240087411

Email magali.giral@chu-nantes.fr

Abstract

Beside the classical monitoring of kidney graft function using creatinemia or proteinuria levels, the assessment of other non-invasive biomarkers have been proposed to identify patients at-risk of kidney rejection. To reach a true clinical utility, the prognostic capacities of a biomarker has to be higher than other available metrics such as clinical-based scoring system. We have previously shown that an increase in TEMRA CD8 T cells is associated with a 2-fold higher risk of kidney dysfunction. In this study, we evaluate if the monitoring of CD8-related biomarkers could improve the prognostic capacities of a clinical-based scoring system (Kidney Transplant Failure Score; KTFS). 161 kidney-transplant recipients have been prospectively enrolled and followed for more than 6 years. At the end of the follow-up time, 14 patients returned to dialysis. Targeted analysis of CD8 T cell phenotypic markers have been performed on blood samples retrieved 12 months post-transplantation. We show that the prognostic capacities of graft failure by the KTFS could be improved by the inclusion of CD8-related biomarker with an increase of the area under the ROC of 0.12 (95%CI 0.00–0.26; p=0.0321). As a consequence, the improvement of prognostic power results in a better reclassification of more than 25% of patients (26.1% of patients (NRI, 95%CI 3.2–49.8; p=0.0321)). This result not only validates our strategy for improving the efficiency of recipient’s follow-up, but also point-out the need to develop new therapy to restrain TEMRA CD8 T cells.

Introduction

The improvement of kidney allograft transplantation has grounded the need to adapt the recipient follow-up regarding his susceptibility of long term graft failure[1]. Numerous assessments of biological markers in the blood, urine and kidney biopsies have been proposed in the literature. Whole-transcriptomic analysis of kidney biopsies have been extensively used, notably by the Edmonton group lead by Halloran[2-4]. The improvement of the classification of kidney graft rejection has been one of the major assets of biopsy-based scoring. However, a wider use of kidney graft biopsy is limited by the invasiveness of the technique, reproducibility issues related to small size of the biopsy and the lack of proven long-term benefit[5,6]. Numerous non-invasive biomarkers of rejection have been proposed including several blood[7-9] and urine[10,11] biomarkers, suggesting some promise in predicting long-term transplant outcome.

Obviously, personalized medicine is not a new concept for physicians as their daily practice specifically consists in driving patients according to their susceptibility of graft failure. Numbers of clinical parameters are available to guide such medical decision-making. Kidney transplant function, estimated using either serum creatinine or other estimated glomerular filtration rate (eGFR) equations, remains the most simple and pertinent biomarkers widely used to evaluate long-term graft failure risk yet. Other parameters such as proteinuria, marginal donor characteristics, or history of previous transplantation offer crucial complementary information. To help physicians in the synthesis of these multiple parameters, composite scores have been developed[1213]. Several years ago, we developed the Kidney Transplant Failure Score (KTFS) based on 8 parameters collected within the first year of transplantation[13] (Patent N°0959043, 2010). The KTFS is associated with an area under the time-dependent ROC curve (AUC) of 0.78 (CI95% = [0.71, 0.86]) for a prognostic up to eight

years post-transplantation. Its usefulness to drive the patient follow-up is under study in a randomized health-economics clinical trial[1].

In accordance with Braun et al.[14] and Moore et al.[15], we believe that complex predictors associating clinical, biological and immunological biomarkers may have a true clinical utility if their prognostic capacities are higher than other simple and available metrics, clinical-based scoring system for instance. We have recently showed that an increase of highly differentiated $CD45RA^{+}CCR7^{-}$ TEMRA CD8 T cells is associated with a 2-fold higher risk of kidney graft dysfunction in a study of kidney transplant recipients with a functioning kidney graft for more than 5-years[16]. In this study, we hypothesize that the prognostic capacities of the KTFS can be improved by monitoring a small set of CD8-related blood biomarkers. We show that combining well accepted pre- and post-transplant risk factor of graft loss and the frequency of CD8-related biomarkers may constitute a way for improving the prognostic capacities of clinical-based scoring systems.

Material and Methods

Subjects and Ethics statement

Peripheral blood mononuclear cells (PBMC) were prospectively collected from 161 kidney transplant recipients in the DIVAT biocollection (www.divat.fr) and stored in the Biological Resource Center of the Nantes University Hospital, F-44093, France (BRIF : BB-0033-00040). All donors were informed of the final use of their blood and signed an informed consent. The University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks approved the study for patients. The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the 'Declaration of Istanbul on Organ Trafficking and Transplant Tourism'. Clinical characteristics are shown in **Table 1**. Kidney transplant recipients were enrolled using the following inclusion criteria: adult recipients of kidney graft from heart-beating deceased donors with a functional transplant on the first anniversary of transplantation. Only patients without missing values for the original KTFS were retained (i.e. patients for whom creatinemia at 3 and 12 months, recipient gender and age, number of previous transplantation, last donor creatinemia value and the number of rejection episodes during the first year post-transplantation). Finally, the availability of a frozen PBMC at 12±6 months was used to select the patients. Of note, the sampling time was of 12±2 months for more than 75% of patients (**Supplementary Figure 1**).Finally, none of the patients included in Foucher et al. study [13] and in Yap et al. study [16] was included in the present cohort.

Blood samples

PBMC were separated on a Ficoll gradient layer and frozen in DMSO-10% autologous serum.

Polychromatic flow cytometry

Cells were analyzed with a LSRII flow cytometer (BD Immunocytometry Systems). 2×10^6 frozen PBMCs were surface stained with antibodies specific for CD3 (BW264/56; VioBlue), CD8 (BW135/80; VioGreen), CD45RA (T6D11; APC-Vio770), CCR7 (3D12; PE-Cy7), CD27 (L128; Brilliant Violet 605) and CD28 (CD28.2; PE-CF594). In addition to this core-staining cocktail, different combinations of antibody were used CD127 (MB15-18C9; PE), CD57 (TB03; FITC), T-bet (O4-46; PE), Granzyme B (GB11; Alexa Fluor 700), and Perforin (B-D48; PE). Yellow LIVE/DEAD Fixable Dead Cell Stain Kit was used to exclude dead cells from analysis. BD CompBeads stained separately with individual mAbs were used to define the compensation matrix. Data were analyzed using FlowJo Version 9.7.6 (TreeStar). All the antibodies were purchased from BD Biosciences except for CD3-VioBlue, CD8-VioGreen, CD45RA-APC-Vio770, CD127-PE, and CD57-FITC (Miltenyi) and Perforin-PE (Diaclone).Representative gating schemes are shown in **Supplementary Figure 2**.

Statistical analyses

Outcome definition. The graft survival represents the primary outcome of the study and was defined as the time between the transplantation and the return in dialysis (death censored).

Associations with graft survival. The survival curves were obtained using the Kaplan-Meier estimator [17]. The raw and KTFS-adjusted associations between the biomarkers and the time-to-event were described by Hazard Ratios (HR) obtained by using proportional hazard Cox models [18]. The KTFS was an offset variable, i.e. regression coefficient equals to 1 rather than estimate the regression coefficient related to the KTFS. Each biomarker was analyzed separately.

Prognostic of graft failure. The objective was to update the KTFS using biomarkers to improve its prognostic capacities. The variables were selected using Cox regression with Lasso penalization [19]. The tuning parameter, i.e. the number of selected variables, was

estimated by 5-fold cross validation. A benefit of using this methodology is to avoid the selection of the explicative variables by using the p-values, which may not constitute the most relevant indicator of prognostic capacities [20-22].

The novel biological and clinical scoring system was the sum of the KTFS value and the biomarkers values multiplied by their relative regression coefficients. The corresponding improvement of the prognostic capacities related to biomarkers was evaluated by two different methods [23]: the Net Reclassification Index (NRI) and the increase of the area under the time-dependent ROC curve (AUC) for a prognostic up to 6 years post-transplantation [24]. For the NRI estimation, as mentioned by Muhlenbruch et al. [25], the choice of the number of categories and corresponding cut-offs has a major impact on the results. Our choice was driven according to the clinical application, i.e. improving the existing binary classification. Up to date, the low-risk group is defined by a KTFS value lower than 4.17 [13] that is a risk to return to dialysis below 2%, 5%, 7% and 9% at 3, 4, 5 and 6 years post-transplantation. The 95% confidence intervals (CI) of prognostic indicators were obtained non-parametrically from 1000 bootstrap replications.

Software. All the statistical analysis were performed using R version 3.1 . The package *ROCt* version 0.9 was used for computing the time-dependent ROC curves. The package *nriccens* version 1.2 was used for computing the NRI.

Results

Characteristics of the kidney transplant cohort

The clinical and demographic characteristics of the population are shown in **Table 1**. Patients were transplanted between February 2007 and December 2011. Among the 161 kidney transplant recipients alive with a functional kidney graft at 1 year post-transplantation (baseline of the study), 14 returned to dialysis at the end of the follow-up and 7 died. The mean follow-up time was 4.4 years (range 0.1 - 6.2). The 6-year graft survival was 84.7% (95% CI 77.3 – 92.9). 91.9% of patients received a first kidney transplant. The mean donor age was 51.9 years (range 13 – 82) and 62.1% were male.

Description of the KTFS prognostic capacities

We first evaluate the predictive values of the KTFS on the 161 patients. Patients stratified at 1 year post-transplantation as patients with low-risk (KTFS $\leq$ 4.17) and high-risk (KTFS > 4.17) of graft failure exhibited significant different graft survival ($p<0.05$; **Figure 1**). At 3-year post-transplantation (i.e. 2 years after the calculation of the KTFS), the graft survival was of 100% for patients classified as low-risk whereas the graft survival for patients at high-risk was of 87.5% (95% CI 77.8 – 98.4). At 6-year post-transplantation, the graft survival was 88.2% (95% CI 80.1 – 97.0) and 76.0% (95% CI 60.2 – 95.9) for patients with low risk and high risk of graft failure respectively. Regardless a specific KTFS cut-off, the AUC for such a prognostic at 6 years was 0.64 (95% CI 0.48 – 0.80).

Early increase in TEMRA CD8 T cells is associated with an increased risk of graft failure

We have previously reported that an increase of highly differentiated TEMRA $CD45RA^{+}CCR7^{-}$ CD8 T cells in patients with a stable graft function for more than 5 years is a risk-factor of graft failure[16]. We assessed the association between the risk of kidney graft

failure (a more stringent clinical criteria than those used in our previous study) and the early monitoring of CD8-related markers. Taking advantage of the DIVAT bio-collection and the prospective storage of PBMC from kidney transplant recipients, we monitored at the first anniversary of the transplantation the frequency of CD8 subsets using phenotypic markers CD45RA and CCR7 (naïve, $CD45RA^+CCR7^+$; TEMRA, $CD45RA^+CCR7^-$; EM, $CD45RA^-CCR7^-$; CM, $CD45RA^-CCR7^+$), markers of differentiation (CD27 and CD28), expression of cytotoxic molecules (GZMb and PERF), IL-7 receptor and markers associated with the secretion of pro-inflammatory cytokines (T-bet and CD57). Stringent quality controls were implemented to ensure the accuracy and the reproducibility of the measure of each parameter. Independently of the KTFS, the 1-year frequencies of TEMRA, $CD27^-CD28^-$ and $GZMb^+PERF^-$ were associated with an increase in the risk of graft failure (KTFS-adjusted Hazard Ratio (HR) equaled 1.32, 1.29 and 1.53 respectively; **Table 2**). In contrast, an increase frequency of $CD27^+CD28^+$, $GZMb^-PERF^-$ and $CD57^-Tbet^-$ was observed with a lower risk of **graft failure** (KTFS-adjusted HR 0.71, 0.74 and 0.73 respectively; **Table 2**). The associations between these 6 biomarkers were then assessed (**Figure 2**). As expected, a strong positive correlation was observed between the percentage of TEMRA CD8 T cells and those of $CD27^-CD28^-$. In contrast, the frequency of TEMRA CD8 T cells was negatively correlated with the frequencies of $CD27^+CD28^+$, $GZMb^-PERF^-$ and $CD57^-Tbet^-$. Overall, the frequency of TEMRA was highly correlated with 4 out 5 biomarkers ($CD27^+CD28^+$, $CD27^-CD28^-$, $GZMb^-PERF^-$ and $CD57^-TBET^-$).

Inclusion of CD8-related biomarkers improved the prognostic power of the KTFS

The association between the percentage of TEMRA CD8 T cells and the kidney graft survival independently of the KTFS prompted us to hypothesize that the ability to prognosis of graft failure could be improve by combining the KTFS with the frequency of TEMRA CD8 T cells assessed at the same time than the KTFS calculation at one year of follow-up. The capacities

to predict the graft failure 6-years post-transplantation are indeed improved when the percentage of TEMRA CD8 T cells are included (**Figure 3**). The AUC increases from 0.64 (95% CI 0.48 – 0.80) when the KTFS is used as a single predictor alone to 0.69 (95% CI 0.54 – 0.83) with TEMRA CD8 T cells. We and others have shown that the selection of the variables in a prognostic context by using usual indicators such as HR and corresponding 95% CI may not be the optimal option[20-22]. To test whether the prognosis capacities of a composite KTFS could be further improved, we therefore select the potential predictor of kidney graft failure using the penalized Lasso regression strategy[19]. This strategy allows a more efficient selection of potential predictors, especially in the context of highly correlated predictors as observed for CD8-related biomarkers (**Figure 2**). By 5-fold maximizing the cross-validation criteria, 3 biomarkers may be retained in addition to the KTFS: the frequencies of $CD27^{+}CD28^{-}$, $GZMb^{+}PERF^{+}$ and $GZMb^{-}PERF^{+}$ to predict graft failure. Of interest, all five variables that could be included in the scoring model were independent (**Supplementary Figure 3**) and thus each variable brings complementary information regarding the risk of graft failure. The biomarker-updated KTFS has an AUC for prognostic up to 6-years post transplantation at 0.76 (95% CI 0.62 – 0.90; **Figure 3**) as compared to 0.64 with KTFS asa single predictor (95% CI 0.48 – 0.80; **Figure 3**). The AUC increased by 0.12 (95% CI 0.00 – 0.26; p=0.0321; **Figure 4A**). The improvement of the prediction of kidney graft failure was also illustrated by a better classification of 26.1% of patients (NRI, 95% CI 3.2 – 49.8; p=0.0321; **Figure 4B**).

Discussion

The ability to stratify the patients during the first year post-transplantation according to their long-term graft survival is an important challenge. Identifying as early as possible patients with a high risk of kidney graft failure offers the opportunity to physicians to change their standard of care of kidney transplant recipients, as for instance to adjust the immunosuppression, to test alternative or to prescribe innovative therapies. One way to address this challenge is to use clinical-based scoring systems. A clinical trial (Clinical Trial Registry NCT01615900) is ongoing to test the efficiency of the Kidney Transplant Failure Score[13] for improving the recipient follow-up after one year post-transplantation[1]. Patients with a higher risk of graft failure (KTFS >4.17) will be follow-up with 6 visits at hospital and 6 video conferencing every year, whereas patients with a lower risk (KTFS ≤4.17) of graft failure will be interviewed only once at hospital and 3 times by video conferencing. The aim from the physician perspective is to allocate more time to patients that potentially need it and to improve the quality-of-life for patients at low-risk of graft failure. The implementation of biomarkers into the KTFS could improve the predictive capacity of the KTFS for still increase its efficiency. But biomarkers may also ultimately guide the introduction of alternative immunosuppressive regimens targeting more efficiently alloreactive immune cells.

The aim of our study was to validate such approach by implementing in the KTFS the measurement of CD8-related markers, that have been associated with long term graft outcome and/or graft failure [16]. In contrast to the initial AUC value obtained in Foucher et al. report [13], the estimated AUC obtained in the current study was lower. These apparent lower prognostic capacities is only explained by the small sample size, especially the low number of patients returning to dialysis, resulting in a high sample to sample fluctuation. In the literature, various criteria have been used to define a poor graft outcome, including decrease of kidney function estimated by the eGFR. It is important to stress that the endpoint of the current study has to be

the same than for the KTFS (i-e return to dialysis), and the use the eGFR to increase the number of events cannot be considered as an alternative option. The inclusion of TEMRA CD8 frequency and other CD8 subsets ($CD27^{+}CD28^{-}$, $GZMb^{+}PERF^{+}$ and $GZMb^{-}PERF^{-}$) improve the predictive capacities of the KTFS. Indeed, the AUC for prognostic up to 6-years post transplantation increases by 0.12 (95% CI 0.00 – 0.26; p=0.0321) when CD8-related biomarkers were implemented. As a consequence, the improvement of prognostic power results in a better reclassification of more than 25% of patients. This result not only validates our strategy for improving the efficiency of recipient's follow-up, but also point-out the need to develop new therapy to restrain TEMRA CD8 T cells. The main objective of the current study was to evaluate the ability of the monitoring of CD8 T cell to improve the evaluation of the patient susceptibility to return to dialysis. Despite the promising result obtained, the clinical utility of such composite score is beyond the scope of the study. A randomized clinical trial (NCT01615900) is ongoing and is aiming to improve the efficiency of the follow-up of patients based on the stratification of patients at 12 months post-transplantation [1].

161 kidney transplant recipients have been included in this study, which remains an important sample size for a study related to novel biomarkers in comparison with the literature in this field in kidney transplantation [26-28]. We therefore described an important improvement of the prognostic capacities to predict graft failure related to the implementation of CD8-related phenotypes. As only 14 patients returned to dialysis, we were not able to divide our initial cohort into learning and validation groups. Nevertheless, major arguments justify that our results are true positive: 1) we demonstrated in a previous work based on independent recipients that these CD8-related biomarkers were accumulated in patients with chronic antibody mediated rejection[29], 2) we also demonstrated that such accumulation is associated with a higher risk of graft failure in a independent population of kidney transplant recipients

recruited more than 5 years post-transplantation[16], and 3) we measured only few CD8-related biomarkers to limit as much as possible the over-fitting and the increase of the false positive rate.

The deleterious role of CD8 T cells in kidney graft outcome and not only in early events post-transplantation has regained interest over the years. Memory T cells are now under scrutiny and are considered as one of the main hurdle to achieve transplantation success and even transplantation tolerance. Pre-existing memory T cells is associated with high incidence and severe rejection episodes[30], and recipients prone to acute rejection have a higher precursor frequency of alloreactive CD8 T cells than non-rejectors[31]. Using an experimental model of ABMR[32], we previously reported that CD8 T cells in the blood exhibit an altered TCR Vβ repertoire and that a similar TCR Vβ selection of CD8 T cells can be identified in the blood and graft of recipients[33]. This profile was associated with transcript coding for cytotoxic molecule, granzyme B (GZM-B), in the graft[8,34,35]. Similar observations made in the blood of ABMR patients reported a restricted TCR Vβ repertoire, an increase in IFN-γ, GZM-B and perforin (PERF-1) transcripts and in the frequency of $CD8^+CD28^-$ T cells[29,36,37]. More recently, in a prospective study, we found that a 1.96 fold higher risk of kidney graft failure was observed in patients with an increase of differentiated TEMRA CD8 T cells at the inclusion[16]. Several groups including our own, have thus highlighted the involvement of alloreactive CD8 T cells in kidney graft dysfunction.

The efficacy of calcineurin inhibitors (CNI) on controlling adaptive immunity is obvious but such strategy seems to be less effective in the control of pathogenic $CD8^+CD28^-$ T cells as the rise of these pathogenic CD8 T cells is observed in kidney transplant recipients treated with CNI for maintenance therapy[16,29]. Costimulation blockade has emerged over the last decades as an alternative to treatment with CNI. For instance, similar 5-year graft survival is obtained with the use of Belatacept but with an improvement of the renal function and a reduction in

side effect as compared to CNI[38,39]. Of note, an increased rate of acute rejection is observed[40] and alloreactive $CD8^+CD28^-$ T effector memory cells are hypothesized to be critical mediators of Belatacept-resistant rejection[41]. Nevertheless, whereas association of Belatacept and ICOS blockade had shown promising results in mice[42], it failed to control Belatacept resistant rejection in non-human primate[43]. These results illustrate the need to intensive the search of effective blockade of alloreactive $CD8^+CD28^-$ T cells and probably not only based on costimulatory blockade but also to identify such biomarkers directly in cohorts of transplanted recipients and that extrapolation from mice model is not always possible.

Collectively, we have shown that the combination of CD8-related biomarkers with clinical-parameters based KTFS allows to better predict patients at-risk of kidney graft failure and to target those with a more specific immunologic risk. Such score, after further validation on large external cohorts, could be useful as decision tool in the clinical management of kidney transplant recipients.

Disclosure

None of the authors have financial interests that might influence the present study.

Acknowledgments

The International Fellowship Grant from ProGreffe Foundation supports M. Yap. This work was funded by a grant from the ITMO Santé Publique (A13053NS), supported by a grant from the French National Research Agency (ANR-11-JSV1-0008-01) and in part by the RTRS Fondation de Coopération Scientifique CENTAURE. This work was realized in the context of the IHU-Cesti project which received French government financial support managed by the National Research Agency via the "Investment Into The Future" program ANR-10-IBHU-005. The IHU-Cesti project is also supported by Nantes Metropole and the Pays de la Loire Region. The Biological Resource Center of the Nantes University Hospital, F-44093, France (BRIF : BB-0033-00040) secures the quality of the biological samples.

References

1. Foucher Y, Meurette A, Daguin P, Bonnaud-Antignac A, Hardouin J-B, Chailan S, Neau K, Papuchon E, Gaboriau S, Legendre C, Morelon E, Tessier P, Giral M: A personalized follow-up of kidney transplant recipients using video conferencing based on a 1-year scoring system predictive of long term graft failure (TELEGRAFT study): protocol for a randomized controlled trial. *BMC Nephrol* 16: 6, 2015

2. Einecke G, Reeve J, Sis B, Mengel M, Hidalgo L, Famulski KS, Matas A, Kasiske B, Kaplan B, Halloran PF: A molecular classifier for predicting future graft loss in late kidney transplant biopsies. 120: 1862–1872, 2010

3. Reeve J, Einecke G, Mengel M, Sis B, Kayser N, Kaplan B, Halloran PF: Diagnosing Rejection in Renal Transplants: A Comparison of Molecular- and Histopathology-Based Approaches. *Am J Transplant* 9: 1802–1810, 2009

4. Halloran PF, Pereira AB, Chang J, Matas A, Picton M, de Freitas D, Bromberg J, Seron D, Sellarés J, Einecke G, Reeve J: Potential Impact of Microarray Diagnosis of T Cell-Mediated Rejection in Kidney Transplants: The INTERCOM Study. *Am J Transplant* 13: 2352–2363, 2013

5. Thaunat O, Legendre C, Morelon E, Kreis H, Mamzer-Bruneel M-F: To biopsy or not to biopsy? Should we screen the histology of stable renal grafts? *Transplantation* 84: 671–676, 2007

6. Wilkinson A: Protocol transplant biopsies: are they really needed? *Clin J Am Soc Nephrol* 1: 130–137, 2006

7. Ashton-Chess J, Le Mai H, Jovanovic V, Renaudin K, Foucher Y, Giral M, Moreau A, Dugast E, Mengel M, eacute MR, Danger R, Usal C, Smit H, Guillet M, Gwinner W, Le

Berre L, Dantal J, Soulillou J-P, Brouard S: Immunoproteasome beta subunit 10 is increased in chronic antibody-mediated rejection. *Kidney Int.* 77: 880–890, 2010

8. Ashton-Chess J, Dugast E, Colvin RB, Giral M, Foucher Y, Moreau A, Renaudin K, Braud C, Devys A, Brouard S, Soulillou J-P: Regulatory, effector, and cytotoxic T cell profiles in long-term kidney transplant patients. *J. Am. Soc. Nephrol* 20: 1113–1122, 2009

9. Ashton-Chess J, Giral M, Mengel M, Renaudin K, Foucher Y, Gwinner W, Braud C, Dugast E, Quillard T, Thebault P, Chiffoleau E, Braudeau C, Charreau B, Soulillou JP, Brouard S: Tribbles-1 as a Novel Biomarker of Chronic Antibody-Mediated Rejection. *J. Am. Soc. Nephrol* 19: 1116–1127, 2008

10. Anglicheau D, Sharma VK, Ding R, Hummel A, Snopkowski C, Dadhania D, Seshan SV, Suthanthiran M: MicroRNA expression profiles predictive of human renal allograft status. *Proc Natl Acad Sci U S A* 106: 5330–5335, 2009

11. Rabant M, Amrouche L, Lebreton X, Aulagnon F, Benon A, Sauvaget V, Bonifay R, Morin L, Scemla A, Delville M, Martinez F, Timsit MO, Duong van Huyen JP, Legendre C, Terzi F, Anglicheau D: Urinary C-X-C Motif Chemokine 10 Independently Improves the Noninvasive Diagnosis of Antibody-Mediated Kidney Allograft Rejection. *J. Am. Soc. Nephrol.* 1–12, 2015

12. Shabir S, Halimi J-M, Cherukuri A, Ball S, Ferro C, Lipkin G, Benavente D, Gatault P, Baker R, Kiberd B, Borrows R: Predicting 5-year risk of kidney transplant failure: a prediction instrument using data available at 1 year posttransplantation. *Am. J. Kidney Dis.* 63: 643–651, 2014

13. Foucher Y, Daguin P, Akl A, Kessler M, Ladrière M, Legendre C, Kreis H, Rostaing L, Kamar N, Mourad G, Garrigue V, Bayle F, de Ligny BH, Büchler M, Meier C, Daurès JP,

Soulillou J-P, Giral M: A clinical scoring system highly predictive of long-term kidney graft survival. *Kidney Int.* 78: 1288–1294, 2010

14. Braun WE, Schold JD: Transplantation: strength in numbers-predicting long-term transplant outcomes. Nat Rev Nephrol. 7: 135–136, 2011

15. Moore J, He X, Liu X, Shabir S, Ball S, Cockwell P, Inston N, Little MA, Johnston A, Borrows R: Mortality prediction after kidney transplantation: comparative clinical use of 7 comorbidity indices. *Exp Clin Transplant* 9: 32–41, 2011

16. Yap M, Boeffard F, Clave E, Pallier A, Danger R, Giral M, Dantal J, Foucher Y, Guillot-Gueguen C, Toubert A, Soulillou J-P, Brouard S, Degauque N: Expansion of highly differentiated cytotoxic terminally differentiated effector memory CD8+ T cells in a subset of clinically stable kidney transplant recipients: a potential marker for late graft dysfunction. *J. Am. Soc. Nephrol* 25: 1856–1868, 2014

17. Kaplan EL, Meier P: Non-parametric estimation from incomplete observations. *Journal of the American Statistical Association* 53: 457–481, 1958

18. Cox D: Regression models and life-tables. Journal of the Royal Statistical Society. Series B Methodological 34: 187–220, 1972

19. Tibshirani R: Regression shrinkage and selection via the lasso: a retrospective. *Journal of the Royal Statistical Society. Series B Methodological* 73: 273–282, 2011

20. Ware JH: The limitations of risk factors as prognostic tools. *N. Engl. J. Med.* 355: 2615–2617, 2006

21. Foucher Y, Combescure C, Ashton-Chess J, Giral M: Prognostic markers: data misinterpretation often leads to overoptimistic conclusions. *Am J Transplant* 12: 1060–1061, 2012

22. Dantan E, Combescure C, Lorent M, Ashton-Chess J, Daguin P, Classe J-M, Giral M, Foucher Y: An original approach was used to better evaluate the capacity of a prognostic marker using published survival curves. *J Clin Epidemiol* 67: 441–448, 2014

23. Pencina MJ, D'Agostino RB, D'Agostino RB, Vasan RS: Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. *Stat Med* 27: 157–72– discussion 207–12, 2008

24. Heagerty PJ, Lumley T, Pepe MS: Time-dependent ROC curves for censored survival data and a diagnostic marker. *Biometrics* 56: 337–344, 2000

25. Mühlenbruch K, Heraclides A, Steyerberg EW, Joost H-G, Boeing H, Schulze MB: Assessing improvement in disease prediction using net reclassification improvement: impact of risk cut-offs and number of risk categories. *Eur. J. Epidemiol.* 28: 25–33, 2013

26. Valke LLFG, van Cranenbroek B, Hilbrands LB, Joosten I: Soluble CD30 does not predict late acute rejection or safe tapering of immunosuppression in renal transplantation. *Transplant Immunology* 32: 18–22, 2015

27. Millán O, Rafael-Valdivia L, Segundo DS, Boix F, Castro-Panete MJ, López-Hoyos M, Muro M, Valero-Hervás D, Rimola A, Navasa M, Muñoz P, Miras M, Andrés A, Guirado L, Pascual J, Brunet M: Should IFN-γ, IL-17 and IL-2 be considered predictive biomarkers of acute rejection in liver and kidney transplant? Results of a multicentric study. *Clinical Immunology* 154: 141–154, 2014

28. Shabir S, Girdlestone J, Briggs D, Kaul B, Smith H, Daga S, Chand S, Jham S, Navarrete C, Harper L, Ball S, Borrows R: Transitional B lymphocytes are associated with protection from kidney allograft rejection: a prospective study. *Am J Transplant* 15: 1384–1391, 2015

29. Baeten D, Louis S, Braud C, Braudeau C, Ballet C, Moizant F, Pallier A, Giral M, Brouard S, Soulillou J-P: Phenotypically and functionally distinct CD8+ lymphocyte populations in long-term drug-free tolerance and chronic rejection in human kidney graft recipients. *J. Am. Soc. Nephrol.* 17: 294–304, 2006

30. Poggio ED, Clemente M, Riley J, Roddy M, Greenspan NS, Dejelo C, Najafian N, Sayegh MH, Hricik DE, Heeger PS: Alloreactivity in renal transplant recipients with and without chronic allograft nephropathy. *J. Am. Soc. Nephrol.* 15: 1952–1960, 2004

31. van de Berg PJEJ, Yong SL, Koch SD, Lardy N, van Donselaar-van der Pant KAMI, Florquin S, Bemelman FJ, van Lier RAW, Berge ten IJM: Characteristics of alloreactive T cells measured before renal transplantation. *Clin Exp Immunol.* 168: 241–250, 2012

32. Ballet C, Renaudin K, Degauque N, Mai HL, Boëffard F, Lair D, Berthelot L, Feng C, Smit H, Usal C, Heslan M, Josien R, Brouard S, Soulillou JP: Indirect CD4 +TH1 Response, Antidonor Antibodies and Diffuse C4d Graft Deposits in Long-Term Recipients Conditioned by Donor Antigens Priming. *Am J Transplant* 9: 697–708, 2009

33. Lair D, Degauque N, Miqueu P, Jovanovic V, Guillet M, Mérieau E, Moreau A, Soulillou JP, Brouard S: Functional Compartmentalization Following Induction of Long-Term Graft Survival with Pregraft Donor-Specific Transfusion. *Am J Transplant* 7: 538–549, 2007

34. Homs S, Mansour H, Desvaux D, Diet C, Hazan M, Buchler M, Lebranchu Y, Buob D, Badoual C, Matignon M, Audard V, Lang P, Grimbert P: Predominant Th1 and Cytotoxic Phenotype in Biopsies from Renal Transplant Recipients with Transplant Glomerulopathy. *Am J Transplant* 9: 1230–1236, 2009

35. Obata F, Yoshida K, Ohkubo M, Ikeda Y, Taoka Y, Takeuchi Y, Shinohara N, Endo T, Baba S: Contribution of CD4+ and CD8+ T cells and interferon-gamma to the progress of chronic rejection of kidney allografts: the Th1 response mediates both acute and chronic rejection. *Transplant Immunology* 14: 21–25, 2005

36. Brouard S, Le Bars A, Dufay A, Gosselin M, Foucher Y, Guillet M, Cesbron-Gautier A, Thervet E, Legendre C, Dugast E, Pallier A, Guillot-Gueguen C, Lagoutte L, Evanno G, Giral M, Soulillou J-P: Identification of a gene expression profile associated with operational tolerance among a selected group of stable kidney transplant patients. *Transplant Int* 24: 536–547, 2011

37. Miqueu P, Degauque N, Guillet M, Giral M, Ruiz C, Pallier A, Braudeau C, Roussey-Kesler G, Ashton-Chess J, Dore J-C, Thervet E, Legendre C, Hernandez-Fuentes MP, Warrens AN, Goldman M, Volk H-D, Janssen U, Wood KJ, Lechler RI, Bertrand D, Sébille V, Soulillou J-P, Brouard S: Analysis of the peripheral T-cell repertoire in kidney transplant patients. *Eur. J. Immunol.* 40: 3280–3290, 2010

38. Rostaing L, Vincenti F, Grinyó J, Rice KM, Bresnahan B, Steinberg S, Gang S, Gaite LE, Moal MC, Mondragón-Ramirez GA, Kothari J, Pupim L, Larsen CP: Long-term belatacept exposure maintains efficacy and safety at 5 years: results from the long-term extension of the BENEFIT study. *Am J Transplant* 13: 2875–2883, 2013

39. Vanrenterghem Y, Bresnahan B, Campistol J, Durrbach A, Grinyo J, Neumayer H-H, Lang P, Larsen CP, Mancilla-Urrea E, Pestana JM, Block A, Duan T, Glicklich A, Gujrathi S, Vincenti F: Belatacept-based regimens are associated with improved cardiovascular and metabolic risk factors compared with cyclosporine in kidney transplant recipients (BENEFIT and BENEFIT-EXT studies). *Transplantation* 91: 976–983, 2011

40. Vincenti F, Larsen C, Durrbach A, Wekerle T, Nashan B, Blancho G, Lang P, Grinyo J, Halloran PF, Solez K, Hagerty D, Levy E, Zhou W, Natarajan K, Charpentier B, Belatacept Study Group: Costimulation blockade with belatacept in renal transplantation. *N. Engl. J. Med.* 353: 770–781, 2005

41. Lo DJ, Weaver TA, Stempora L, Mehta AK, Ford ML, Larsen CP, Kirk AD: Selective targeting of human alloresponsive CD8+ effector memory T cells based on CD2 expression. *Am J Transplant* 11: 22–33, 2011

42. Schenk AD, Gorbacheva V, Rabant M, Fairchild RL, Valujskikh A: Effector Functions of Donor-Reactive CD8 Memory T Cells Are Dependent on ICOS Induced During Division in Cardiac Grafts. *Am J Transplant* 9: 64–73, 2008

43. Lo DJ, Anderson DJ, Song M, Leopardi F, Farris AB, Strobert E, Chapin S, Devens B, Karrer E, Kirk AD: A Pilot Trial Targeting the ICOS-ICOS-L Pathway in Nonhuman Primate Kidney Transplantation. *Am J Transplant* 15: 984–992, 2015

Table 1. Demographic and clinical characteristics of the kidney transplant recipients at baseline (N=161)

Demographic and clinical characteristics	Mean or Number (range or %)
Recipient age (years)	50.6 (18 to 77)
Male recipient	104 (64.6%)
Donor age (years)	51.9 (13 to 82)
Male donor gender	100 (62.1%)
3-month Creatinemia (µmol/L)	146.4 (61 to 355)
1-year Creatinemia (µmol/L)	143.3 (62 to 353)
1-year Proteinuria (g/day)	0.4 (0 to 10)
Last donor Creatinemia (µmol/L)	90.7 (33 to 813)
Retransplantation	13 (8.1%)
Acute rejection episode during the first year post-transplantation	18 (11%)

Table 2. Relationship between the biomarkers and the time to return to dialysis

Markers	Mean	Range	Raw HR	95% CI	Adj. HR	95%CI
$CD45RA^{+}CCR7^{+}$ Naïve	1.64	0.04 - 6.03	0.73	0.44 -1.23	0.81	0.47 - 1.39
$CD45RA^{-}CCR7^{+}$ CM	0.80	0.06 - 3.98	0.87	0.34 - 2.23	0.68	0.28 - 1.66
$CD45RA^{-}CCR7^{-}$ EM	2.51	0.38 - 5.89	0.77	0.48 - 1.24	0.65	0.41 - 1.03
$CD45RA^{+}CCR7^{-}$ TEMRA	4.37	0.48 - 8.70	1.23	0.94 - 1.60	1.32	1.02 - 1.70
$CD127^{+}$	5.07	0.41 - 9.57	0.89	0.70 - 1.13	0.80	0.63 - 1.01
$CD27^{+}CD28^{+}$	3.82	0.40 - 8.64	0.78	0.59 - 1.04	0.71	0.53 - 0.97
$CD27^{-}CD28^{+}$	1.23	0.08 - 7.08	1.16	0.77 - 1.75	0.92	0.55 - 1.54
$CD27^{+}CD28^{-}$	0.69	0.03 - 3.19	0.16	0.03 - 0.87	0.20	0.03 - 1.38
$CD27^{-}CD28^{-}$	4.25	0.18 - 9.14	1.19	0.95 - 1.50	1.29	1.03 - 1.63

$GZMb^{+}PERF^{+}$	3.70	0.12 - 8.82	1.13	0.90 - 1.43	1.21	0.96 - 1.52
$GZMb^{-}PERF^{+}$	1.40	0.00 - 7.60	1.00	0.62 - 1.63	0.89	0.58 - 1.37
$GZMb^{+}PERF^{-}$	0.85	0.01 - 6.38	1.29	0.92 - 1.81	1.53	1.07 - 2.21
$GZMb^{-}PERF^{-}$	4.05	0.29 - 9.48	0.78	0.59 - 1.02	0.74	0.56 - 0.97
$CD57^{+}Tbet^{+}$	3.54	0.00 - 8.46	1.14	0.88 - 1.46	1.23	0.96 - 1.59
$CD57^{-}Tbet^{+}$	2.78	0.02 - 7.47	0.97	0.62 - 1.52	1.12	0.78 - 1.61
$CD57^{+}Tbet^{-}$	0.34	0.00 - 4.77	1.28	0.79 - 2.07	1.17	0.65 - 2.12
$CD57^{-}Tbet^{-}$	3.35	0.17 - 8.01	0.82	0.61 - 1.11	0.73	0.54 - 0.98

Figure Legends

Figure 1. Kidney graft survival in patients stratified according to the KTFS. Patients were stratified 12 months post-transplantation according to their KTFS value in low risk (KTFS ≤ 4.17) and high risk (KTFS > 4.17) and the survival of their kidney graft was assessed using the Kaplan-Meier estimator. The number of patients at-risk was computed every year.

Figure 2. Description of the associations between the 6 biomarkers significantly associated with the graft survival independently of the KTFS. The coefficient of linear correlation is shown is shown in the upper right panel. Individual values are shown for each pair of biomarkers in the lower left panel.

Figure 3. Inclusion of CD8-related biomarkers improves the prognosis capacities of the KTFS. Time-dependent ROC curves to predict kidney graft outcome 6-years post-transplantation using KTFS alone (black line), KTFS and the percentage of TEMRA CD8 T cells (long dashed line) or KTFS and the percentage of $CD27^{+}CD28^{-}$, $GZMb^{+}PERF^{+}$, $GZMb^{-}PERF^{-}$ (4-variables updated KTFS; short dashed line).

Figure 4. Improvement of the prognosis power of kidney transplant outcome using the composite KTFS. The prognostic capacities of the KTFS used alone or in combination with 3 CD8-related biomarkers at 3, 4, 5 and 6 years post-transplantation were evaluated and the difference in the area under the ROC curve between the two scores was computed (**A**). The percentage of improvement of the reclassification of patients according to their kidney graft outcome was assessed according to the Net Reclassification Index (NRI) (**B**).

Supplementary Figure 1. Distribution of the post transplantation times of biomarker collections.

Supplementary Figure 2. FACS gating strategy to identify CD8 subsets. Yellow negative cells (i-e viable cells) were first gated, lymphocytes were then identified by morphology (FSC vs. SSC) and CD3+CD8+ were selected. Expression of various markers were analyzed.

Supplementary Figure 3. Complementarity of the information bring by the selected predictors. The predictors selected by the Lasso penalized Cox model are analyzed according to their regression coefficients to evaluate their independency. The coefficient of linear correlation is shown is shown in the upper right panel. Individual values are shown for each pair of biomarkers in the lower left panel.

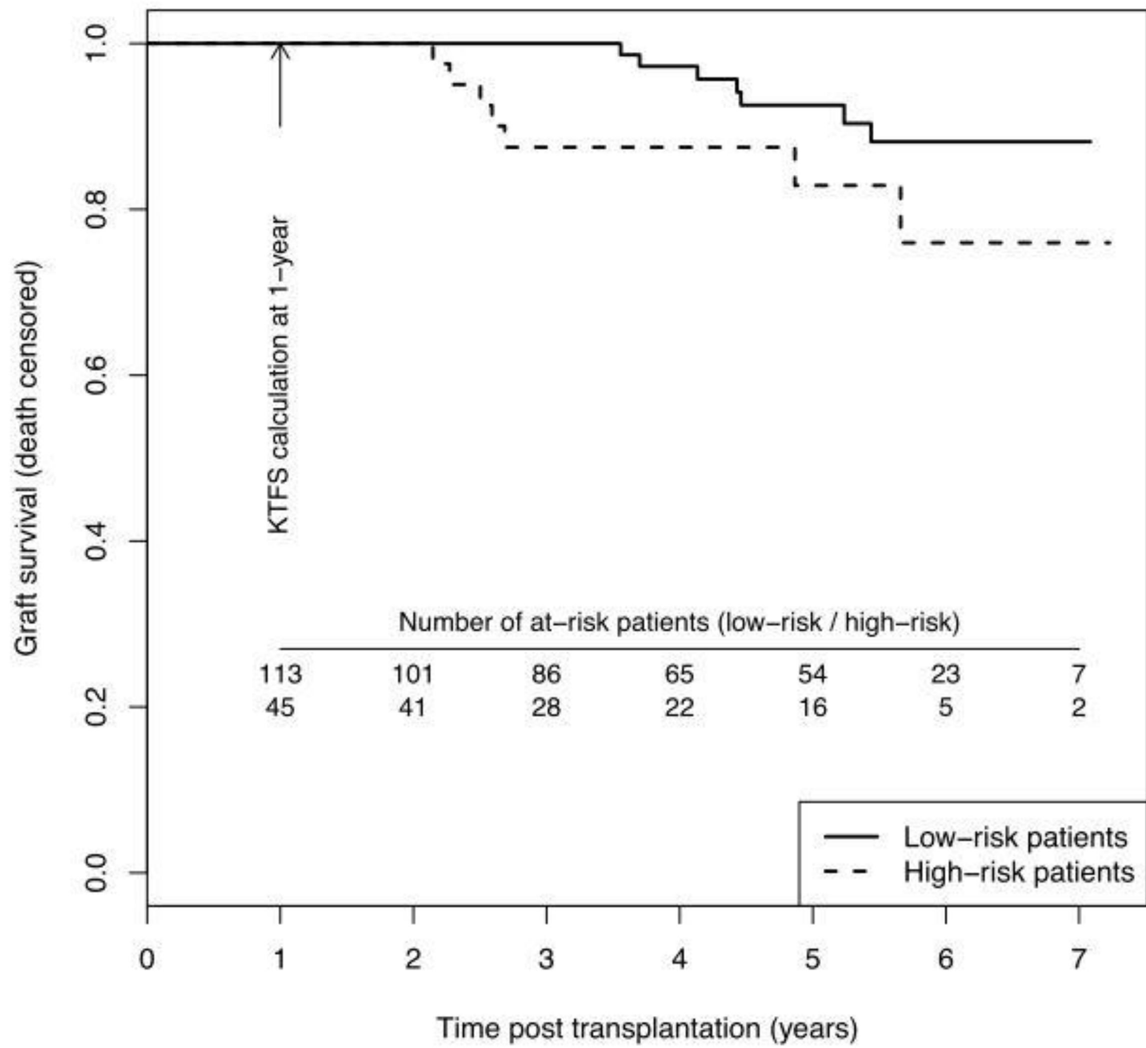


FIGURE 1

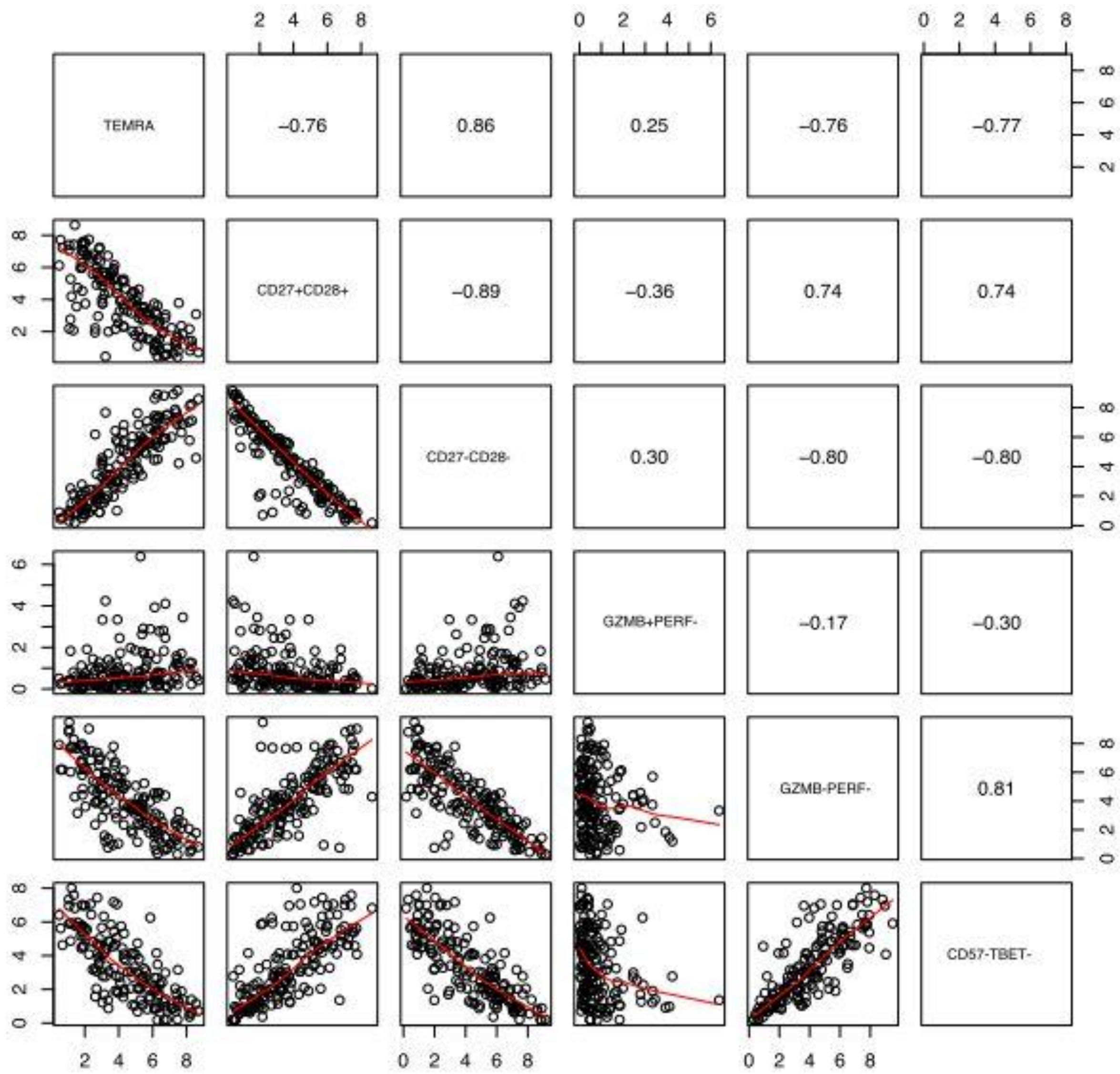


FIGURE 2

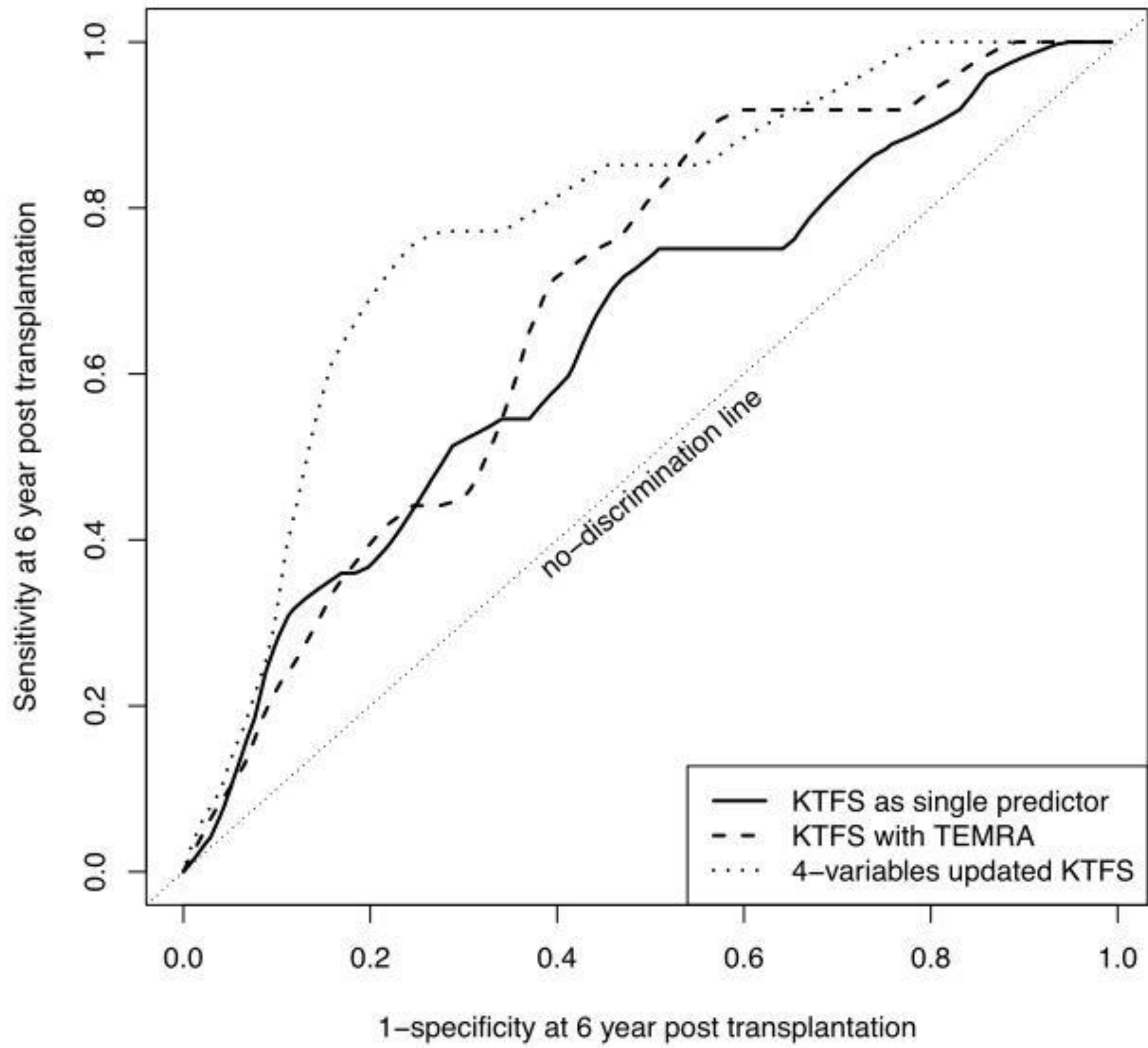


FIGURE 3

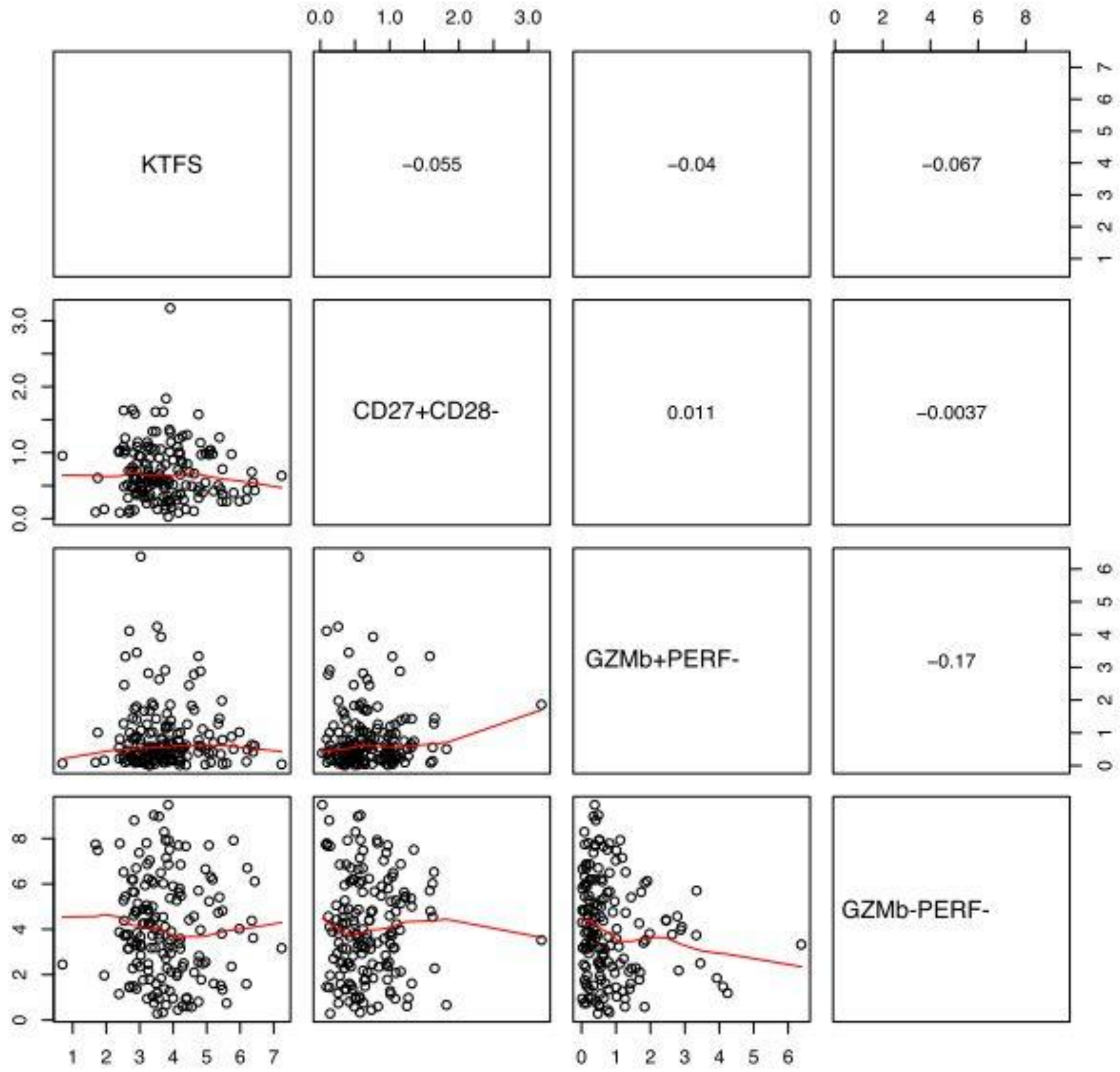


FIGURE 4

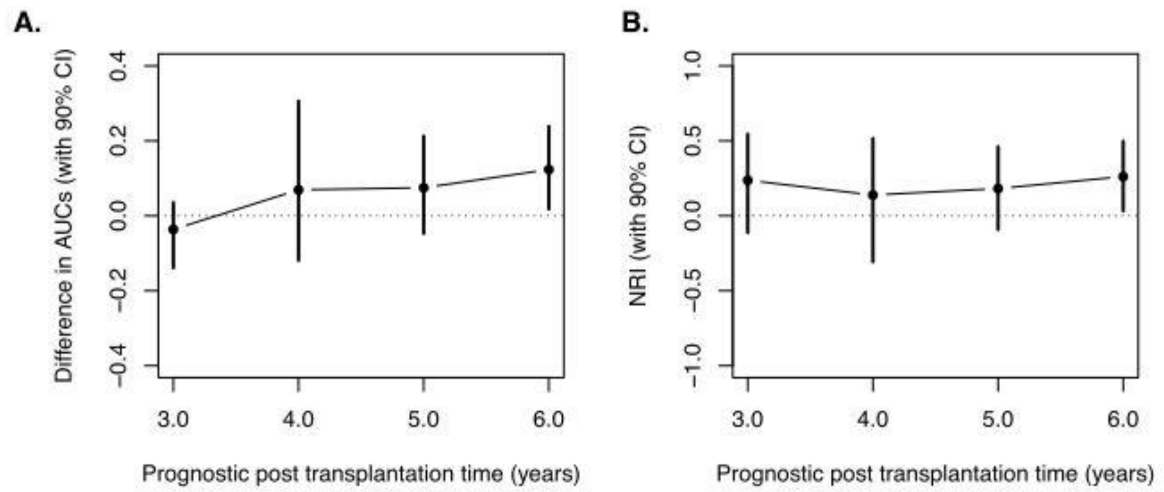


FIGURE 5

3.5 Article 4: TEMRA CD8 T Cells Rapidly Engages a Sustained Glycolytic Switch to Sustain Their Potent Effector Functions

In preparation for submission

TEMRA CD8 cells have been long considered senescent. Convention asserted that these cells possessed an exhausted phenotype and little proliferative abilities (Arosa 2002; Brenchley et al. 2003; Henson et al. 2014). However, some researchers have found contrary results and show that under different culture conditions, TEMRA CD8 T cells act in similar fashion to EM CD8 cells, thus suggesting that their label as terminally-differentiated cells does not equate to cellular exhaustion (Chiu, Fann, and Weng 2006; Chong et al. 2008; Strioga, Pasukoniene, and Characiejus 2011).

Our previous finding which showed that an increased in TEMRA CD8 T cells in kidney transplant patients indicated that TEMRA CD8 cells are important to the rejection (Yap et al. 2014). Furthermore, others have shown that this subset also plays important roles in other pathologies, such as multiple sclerosis and bone regeneration (Salou et al. 2015; Reinke et al. 2013; Strioga, Pasukoniene, and Characiejus 2011). While there has been renewed interest in investigating the ties between the metabolic programming and the immune function in lymphocytes (van der Windt and Pearce 2012; MacIver, Michalek, and Rathmell 2013), there is still much to discover about the metabolic programming involved in the different CD8 cell subsets, especially in TEMRA CD8 cells. Furthermore, investigation into the metabolics of these cells in pathological settings is lacking. Therefore we investigated the immunometabolic machinery involved in naïve, EM and TEMRA CD8 cell immune functions in healthy volunteers, kidney transplant recipients, and multiple sclerosis patients.

We first confirmed that the CD45RA^{+}CD28^{-} TEMRA cells did not conform to the conventional definition of senescent cells and could, in fact, rapidly upregulate STAT5 upon stimulation, as well as readily proliferate in response to TCR and to cytokine stimulation. Also, we found that these cells possess high basal levels of ATP, which they can rapidly utilize when stimulated, before replenishing them 24 hours later. Furthermore, they possessed well-polarized and functional mitochondria. TEMRA CD8 cells adjusted their metabolic programming in a similar manner to EM cells, in that they rapidly upregulated glycolytic activity upon stimulation and maintained high levels of glycolysis long after the initial stimulation. Additionally, glycolysis was shown to be important for pro-inflammatory cytokine release in CD8 cells, in not only healthy volunteers, but also in kidney transplant recipients and multiple sclerosis patients.

TEMRA CD8 T CELLS RAPIDLY ENGAGES A SUSTAINED GLYCOLYTIC SWITCH TO SUSTAIN THEIR POTENT EFFECTOR FUNCTIONS

Gaëlle Tilly[a,b*], Michelle Yap[a,b*], Magali Giral[a,c], Lola Jacquemont[a,c], Pierrick Guerrif[c], Bryan Nicol[a,b], Alexandra Garcia[a,b],David-Axel Laplaud[a,c], Sophie Brouard[a,c], Claire Pecqueur[d] and Nicolas Degauque[a,c]

[a]INSERM, UMR 1064, Nantes, F-44093, France

[b]Université de Nantes, Faculté de Médecine, Nantes, F-44035, France.

[c]CHU de Nantes, ITUN, Nantes, F-44093, France

[d]INSERM, UMR 892, Nantes, F-44093, France

[*] GT and MY contribute equally to the work and should be considered as co-first authors

Corresponding author

Nicolas Degauque

Pavillon Jean Monnet

Hôtel Dieu - CHU de Nantes

30, Bd Jean Monnet

44093 Nantes cedex 01

France

Phone +33 (0)240087410

Fax +33 (0)240087411

Email nicolas.degauque@univ-nantes.fr

Running Title.

Abstract Word Count words

Text Word Count words

Abstract

Effector memory CD8 T cells are able to respond faster to a second antigen stimulation thanks to their ability to switch rapidly to glycolytic mode. Among effector memory, those that re-expressed CD45RA (TEMRA) have not been well characterized as for their bio-energetic potential and function. Recent report suggested that TEMRA CD8 T cells exhibit defective mitochondria and immune-senescence. We found that TEMRA CD8 T cells ($CD45RA^{+}CD28^{-}$) exhibit many characteristics of EM CD8 T cells ($CD45RA^{-}CD28^{+}$), including the ability to proliferate upon TCR and to engage and sustain a high glycolytic rate upon polyclonal activation. In steady-state, TEMRA CD8 exhibit functional mitochondria and high ATP content that is rapidly used upon antigen stimulation to sustain their effector functions. TEMRA CD8 T cells are able to respond to common-γ chain cytokine stimulation without pre-activation and IL-2, IL-7 or IL-15 enhance the proliferation induced by anti-TCR stimulation. The secretion of pro-inflammatory cytokines (IL-2, IFN-γ and TNF-α) by TEMRA can be abrogated by the provision of 2-Deoxy-D-glucose whereas alteration of mitochondria respiration does not. Finally, we provide evidences that chronic in vivo stimulation with low-grade (multiple sclerosis) and high-grade (allogeneic kidney transplantation) stimulation leads to an increase of well-polarized mitochondria in TEMRA CD8 T cells while maintaining a need for glycolysis for pro-inflammatory cytokines secretion. These data suggest that, thanks to their high content in inflammatory cytokines and cytotoxic molecules, TEMRA CD8 T cells are in a ready-to-respond state that could engage their bioenergetic machinery to rapidly and efficiently sustain proliferation and effector functions.

Introduction

Protection against intracellular pathogens and cancer relies on an effective CD8 response. Upon activation, antigen-specific naïve CD8 T cells clonally grow and differentiate into cytotoxic effector CD8 T cells. Once the pathogen is being successfully cleared, only a fraction of effector CD8 T cells will survived and generate effective antigen-specific memory CD8 T cells. It has been recently highlighted that the ability of memory CD8 T cells to quickly respond to second stimulation relies on their bioenergetics advantages(1, 2).As quiescent cells, naïve and memory CD8 T cells rely primarily on oxidative phosphorylation to support their energy need (3). However, memory CD8 T cells exhibit several unique bioenergetics features that allow their rapid response upon re-stimulation. Memory CD8 T cells have a greater mitochondrial mass which allow a rapid metabolic response involving both oxidative phosphorylation and aerobic glycolysis(1). These long-lived T cells require mitochondrial fatty acid oxidation (FAO) for survival (4), using mostly newly synthesized lipids from extracellular glucose rather than using external long-chain fatty acid (5).Ligation of TCR on effector memory CD8 T cells induces a rapid and sustained glycolytic switch that precedes clonal expansion(2). These metabolic features and in particular glycolysis inhibition, can influence both the generation of memory CD8 T cells and their anti-tumor function (6). The bioenergetic profiles have been primarily performed by comparing the properties of naïve CD8 T cells and effector memory CD8 T cells in human settings and in rodents. However, several subsets of CD8 T cells with distinct functions can be distinguished. TEMRA CD8 T cells are terminally differentiated T cells that re-express CD45RA with contradictory observations published regarding their functionality. On one hand, TEMRA CD8 T cells have been shown to accumulate with age or chronic antigen stimulation and as such leads to the senescence of the immune system (7-9). On the other hand, we and others have shown that

TEMRA CD8 T cells are involved in a wide variety of pathogenic processes, including kidney transplant rejection (10) and bone regeneration (11).

In the present study, we found that TEMRA CD8 T cells ($CD45RA^{+}CD28^{-}$) exhibit characteristics of EM CD8 T cells ($CD45RA^{-}CD28^{+}$), including the ability to proliferate upon TCR only stimulation, to engage and sustain a high glycolytic rate upon polyclonal activation. TEMRA CD8 T cell exhibit a well-polarized mitochondria and a high ATP reservoir pool that could be rapidly engage and later reconstituted to sustain their activation. Finally, we identified that the source of energy differentially regulates the secretion of pro-inflammatory cytokines by TEMRA in healthy volunteers and immune-challenged patients. The secretion of IL-2, IFN-γ and TNF-α by TEMRA was abrogated by the provision of 2-Deoxy-D-glucose (2-DG) in the 3 groups. Whereas, inhibition of mitochondria respiration inhibits IFN-γ and TNF-α secretion in healthy volunteers and immune-challenged patients, inhibition of mitochondria respiration enhances IL-2 secretion by TEMRA from patients with multiple sclerosis. These data show that TEMRA CD8 T subsets are not immune-senescent cells and that their cytopathogenic function are sustained by bioenergetics features shared with EM CD8 T cells and that may be differentially involved according to the immunological situation.

Material & Methods

Subjects and Ethics statement

Peripheral blood mononuclear cells (PBMC) were collected from 10 kidney transplant recipients (**Table 1A**), 24 patients with multiple sclerosis (**Table 1B**) and 19healthy volunteers. All donors were informed of the final use of their blood and signed an informed consent. The University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks approved the study for patients. Healthy volunteers were enrolled by the Etablissement Français du Sang (EFS, Nantes, France) within the context of a research contract. Clinical characteristics are shown in **Table 1**.

Blood samples

PBMC were separated on a Ficoll gradient layer and frozen in DMSO-10% autologous serum.

Polychromatic flow cytometry

Cells were analyzed with a LSRII flow cytometer (BD Immunocytometry Systems). 2×10^6frozen PBMCs were surface stained with antibodies specific for CD3 (BW264/56; VioBlue), CD8 (BW135/80; VioGreen), CD45RA (T6D11; APC-Vio770), CCR7 (3D12; PE-Cy7), CD27 (L128; Brilliant Violet 605) and CD28 (CD28.2; PE-CF594). In addition to this core-staining cocktail, different combinations of antibody were used CD127 (MB15-18C9; PE), CD57 (TB03; FITC), T-bet (O4-46; PE), Granzyme B (GB11; Alexa Fluor 700), and Perforin (B-D48; PE). Yellow LIVE/DEAD Fixable Dead Cell Stain Kit was used to exclude dead cells from analysis. BD CompBeads stained separately with individual mAbs were used to define the compensation matrix. Data were analyzed using FlowJo Version 9.7.6 (TreeStar). All the antibodies were purchased from BD Biosciences except for CD3-VioBlue,

CD8-VioGreen, CD45RA-APC-Vio770, CD127-PE, and CD57-FITC (Miltenyi) and Perforin-PE (Diaclone).

Cell culture

TEMRA, NAÏVE and EM CD8 T cells were FACS-sorted according to the expression of CD45RA and CD28 using an ARIA cell-sorter flow cytometer. Before FACS sorting, CD8 T cells were purified in some instance by negative selection using CD8 T cell Isolation Kit and an AutoMACS pro (Miltenyi Biotech). Highly purified CD8 T cell subsets were stained with Cell Proliferation Dye eFluor450 (eBioscience), plated in 96-flat bottom plates and cultured for 5 days in TexMACS medium (Miltenyi). Anti-CD3 (OKT3; 1ug/mL) was previously coated for at least 2h. Various cytokines were added to the culture and were all purchased from Miltenyi. Cell proliferation was assessed according to the frequency of CPDlow cells or using the dedicated Proliferation module of FlowJo software. Annexin V was used to assess the apoptosis. Antibodies against IL-2 (5344.111; PE; BD Biosciences), IFN-γ (B27; Alexa 700; BD Biosciences), and TNF-α (cA2; FITC; Miltenyi) were used to assess cytokine expression by purified CD8 subsets 48h after stimulation with anti-CD3 (OKT3; 1ug/mL) and anti-CD28 (CD28.2; 2ug/mL) in the presence or not of 2-DG (50mM; Sigma) or metformin (50µM; Sigma).

Quantification of mitochondria and mitochondrial membrane potential assessment.

To quantify mitochondrial mass, PBMCs were first incubated with 100nM MitoTracker Red (Invitrogen) for 30’ at 37°C 5% CO_2 and then stained using CD3 (HIT3a;PE), CD8 (BW135/80;VioGreen), CD45RA (T6D11;APC-Vio770) and CD28 (CD28.2; FITC).Polarization of mitochondrial membrane was assessed using JC-1 (Invitrogen). PBMCs were first incubated with 2µM JC-1 for 30’ at 37°C 5% and then stained with CD3 (BW264/56; VioBlue), CD8 (BW135/80; VioGreen), CD45RA (T6D11; APC-Vio770) and

CD28 (CD28.2; APC). Unfixed samples were immediately analyzed with a LSRII flow cytometer.

Phosphorylation of STAT5 and STAT3

Phosphorylation of STAT3 and STAT5 was measured using the protocol developed by Goldeck et al. (12). PBMC were incubated in TexMACS at 37°C/5% $C0_2$ for 2h, washed once at RT and the final concentration was adjusted to $1x10^7$ cells/mL. 10^6 PBMC were incubated in a final volume of 100uL in a 96-U bottom plate for 30’ at 37°C/5% $C0_2$. Pre-warmed cytokines (IL-2, IL-7, IL-15, IL-6, IL-10 and IL-21) were added at a final concentration of 10ng/mL except for IL-2 (300UI/mL) for 15’ at 37°C/5% $C0_2$. Reaction was rapidly stopped by transferring the plate on ice and the addition of 100uL of cold FACS buffer. After permeabilization and fixation with Cytofix/Cytoperm buffer (BD Biosciences), PBMC were stained for cell surface markers (CD3, clone HIT3a, PE-Cy5.5; CD8, VioGreen; CD45RA, clone T6D11, APC-Vio770; CD28, clone CD28.2, PE-CF594) in Perm/Wash buffer for 30’ at 4°C. Cells were further permeabilized by adding cold BD Perm Buffer III, washed with cold FACS Buffer and stain with anti-pSTAT5 (pY694; clone 47; Alexa 647) and anti-pSTAT3 (pY705; clone 4; Alexa 488) for 30’ at RT. Stability of the staining of cell-surface markers upon the use of BD Perm Buffer III was ensured in preliminary experiments. All cytokines were purchased from Miltenyi.

Quantification of ATP

Purified CD8 T subsets cells were incubated in TexMacs at $4x10^6$ cells/ml and stimulated for various time with PMA (50 ng/ml; Sigma) and Ionomycin (500ng/ml; Sigma). 15 µl of each cells suspension (i-e $6x10e^4$ cells) were used for luminometric ATP measurement using Apo Biovision kit according to manufacturer’s instructions (Clinisciences). Luminometry was measured with a VICTOR multilabel plate reader (Perkin Elmer).

Metabolic assays

OCR and ECAR were measured using Seahorse XF24 or XF96 analyzers in purified CD8 T cell subsets ($4x10^5$ or $2.5x10^5$ purified cells respectively) that were allowed to rest for 2h after FACS-sorting in TexMACS buffer at 37°C/5% $C0_2$. The assay was performed in Seahorse XF-base medium supplemented with 10mM glucose (Sigma), 2mM glutamine (Life Technologies) and 1mM pyruvate (Life Technologies). Mitochondrial stress assay was performed by adding successively oligomycin (1.5µM; Sigma), CCCP (1µM; Sigma) and Antimycin A + Rotenone (1µM each; Sigma). To assess OCR and ECAR upon polyclonal stimulation, PMA (50ng/mL; Sigma) and Ionomycin (500ng/mL; Sigma) were added 75' after the start of the experiment. 2-DG (250mM; Sigma) and oligomycin (1.5µM; Sigma) were added before the stimulation with PMA-Iono.

Real-time quantitative PCR

RNA were extracted using RNeasy Micro Kit (Qiagen) and total RNA was reverse-transcribed using a classical MMLV cDNA synthesis (Invitrogen). Quantitative real-time PCR was performed using a ViiA 7 Real-Time PCR System (Applied Biosystems) and all TaqMan primer-probe sets were purchased as ''Assay-on-Demand'' from Applied Biosystems. Transcript levels were calculated according to the $2^{-\Delta Ct}$ method as described by Applied Biosystems and normalized to the expression of 18S.

Statistical analyses

Statistics were analyzed using Graphpad Prism. Mann-Whitney U test, Kruskall-wallis test followed by Dunn's post-hoc test and paired Wilcoxon test were used when suitable and is mentioned within the legend figures. Exact p-values are mentioned or using *. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Results

Characterization of TEMRA cells

A wide array of markers was screened at the protein and the mRNA levels to finely identify TEMRA cells withinCD8 T cell subsets. As terminally differentiated CD8 T cells, TEMRA cells characterized by a $CD45RA^{+}CD28^{-}$phenotype (13) did not express secondary lymphoid organ chemokine receptor CCR7, CD27 costimulatory molecule and IL-7 receptor (CD127) (**Figure 1A**). Their differentiation status was further confirmed by a high co-expression of effector-associated transcriptional factor T-bet and effector/senescent marker CD57. Furthermore, under resting condition, they expressed cytotoxic molecules Perforin (PERF) and Granzyme B (GZMb) (**Figure 1A**). In contrast, NAÏVE ($CD45RA^{+}CD28^{+}$) and EM ($CD45RA^{-}CD28^{+}$) CD8 T cells expressed high level of CD27 and CD127 (**Figure 1A**). A higher frequency of $T\text{-}bet^{+}$ but $CD57^{-}$ was observed in EM CD8 subsets, a phenotype that confirmed their memory status. TEMRA, naive and EM cells were then purified by flow cytometry on the basis of CD3, CD8, CD45RA and CD28 markers and were subjected to qPCR analysis. Transcriptional factors T-bet and EOMES have been ascribed with the CD8 T cell differentiation process with a relative balance between effector and memory features for T-bet EOMES respectively. Nonetheless, both transcriptional factors are partially redundant and triggers the expression of IFN-γ, PERF and GZM B(14, 15). As expected, and in contrast to naive CD8 T cells, unstimulated TEMRA CD8 T cells expressed high amount of T-bet and EOMES (**Figure 1B**) and high expression of transcripts encoding for pro-inflammatory cytokines (IFN-γ and TNF-α) and cytotoxic molecules (GZM-b and PERF) (**Figure 1B**).BLIMP-1 (B lymphocyte-induced maturation protein 1) is a transcriptional repressor that is robustly expressed by effector CD8 T cells(16) and its expression has been shown to be inversely correlated to BCL-6 expression in effector and memory CD8 T cells (17). In contrast, BCL-6 progressively accumulates in virus-specific memory CD8 T cells (17). No

difference in the level of expression of Bcl-6 was observed for naïve, TEMRA and EM CD8 T cells whereas both TEMRA and EM CD8 T cells expressed high level of BLIMP-1 (**Figure 1B**). It has been recently identified that mouse CD8 T cells constitutively express GATA-3 and its expression was upregulated by activation through TCR and cytokine stimulation(18). Moreover, the absence of GATA-3 precludes the proliferation of CD8 T cells induced by TCR and cytokine stimulation. GATA-3 was detected in all CD8 subsets (**Figure 1B**). Of note, a higher expression of GATA-3 was found in TEMRA and EM subsets that could be linked with a sustained stimulation through TCR and cytokines. Finally the expression of anti-apoptotic molecule Bcl-2 was similar in the 3 CD8 subsets (**Figure 1B**). Altogether, these data showed that TEMRA cells characterized by a $CD45RA^{+}CD28^{-}CD27^{-}CD127^{lo}$T-bet^{+}GZM-b^{+}phenotype express high level of T-bet, EOMES, BLIMP-1, cytotoxic molecules and pro-inflammatory cytokines. Thus TEMRA CD8 T cells exhibit the hallmarks of highly differentiated and effector memory cells.

TCR stimulation of TEMRA CD8 T cells induces T cell proliferation and pro-inflammatory cytokine secretion

Given the contradictory published reports describing the functionality of TEMRA CD8 T cells, we aimed to sequentially investigate the ability of TCR stimulation and then common-gamma chain cytokine stimulation to activate TEMRA CD8 T cells. We first assessed the proliferation induced by TCR stimulation of highly purified CD8 subsets. TCR stimulation alone was sufficient to induce T cell proliferation of TEMRA CD8 T cells (mean±sem 34.4%±7.5 vs 18.9%±3.4 for TEMRA and NAÏVE cells respectively; p=0.0465; **Figure 2A**). The proliferation was not associated with an increase of apoptosis as shown by a similar frequency of annexin-V expressing cells between TEMRA and NAÏVE CD8 T cells (mean±sem 43.5%±8.2 and 32.8%±9.2 TEMRA and NAÏVE respectively; ns; **Figure 2A**).These results suggest that TCR stimulation induces a potent proliferation which is not

associated with cell death in TEMRA CD8 T cells. Proliferation of TEMRA CD8 T cells was associated with an increase of transcripts encoding for pro-inflammatory cytokines IFN-γ (x14.8 as compared to basal level; p=0.018; **Table 2**) and TNF-α (x10.9 as compared to basal level; p=0.011; **Table 2**). Expression of cytotoxic molecules GZM-b remained unchanged (i-e high expression) after culture and the expression level of PERF was decreased after TCR stimulation in TEMRA CD8 T cells (x0.3; p=0.002; **Table 2**). After TCR stimulation, TEMRA CD8 T cells expressed an increase of Bcl-2 (x5.9 as compared to basal level; p=0.038; **Table 2**).Altogether, our data show that TCR stimulation of TEMRA CD8 T cells results in a potent proliferation and an up-regulation of pro-inflammatory cytokines, features of potent effector T cells.

TEMRA CD8 T cell are susceptible to common-γ chain stimulation

Given the low expression of costimulatory molecules CD27 and CD28 by TEMRA CD8 T cells, we questioned the susceptibility of TEMRA CD8 T cells to various cytokines including common-γ chain cytokines. We first screened the susceptibility of freshly and unstimulated CD8 subsets to respond to short-term stimulation with various cytokines involved in CD8 homeostatic process by monitoring the phosphorylation of STAT3 and STAT5 after 15' of stimulation. Binding of IL-2, IL-7 and IL-15 to its cognate receptor should result in the phosphorylation of STAT5 whereas stimulation with IL-10 and IL-21 should preferentially result in STAT3 phosphorylation. TEMRA CD8 cells exhibited a potent response to IL-2 and IL-15 (mean±sem 74.3%±12.0 and 81.8%±4.7 respectively; **Figure 3A**) and with similar magnitude as those of naïve and EM CD8 T cells. Response of TEMRA CD8 T cells to IL-7 was of lower magnitude (56.7%±7.1; p<0.02; **Figure 3A**) as compared to those of naïve and EM CD8 T cells. The lower response of TEMRA CD8 T cells to IL-7 stimulation can be explained by lower expression of CD127 rather than by a different affinity of CD127 for IL-7 as a saturating response was obtained for all CD8 T cell subsets with 1ng/mL of IL-7

(**Supplementary Figure 1**). TEMRA CD8 T cells were as responsive as naïve and EM CD8 T cells to IL-10 and IL-21 stimulation as exemplified by the similar frequency of pSTAT3 (**Figure 3A**). TEMRA CD8 T cells in steady-state were thus able to respond to common-γ chain cytokine stimulation. Highly purified CD8 subsets were then simultaneously stimulated with common-γ chain cytokine and anti-CD3 mAb. After 5 days of culture, addition of IL-2, IL-7 or IL-15 enhanced the proliferation of TEMRA CD8 T cells induced by TCR stimulation (**Figure 3B**) and triggered anti-apoptotic effects as exemplified by the decrease of Annexin V^+ cells in proliferating cells (**Figure 3C**).Collectively, these data show that TEMRA CD8 T cell are susceptible to common-γ chain stimulation and that stimulation of TCR in combination with cytokine stimulation results in the proliferation of TEMRA CD8 T cells while the magnitude of apoptosis is similar to those of naïve and EM CD8 cells.

Resting TEMRA CD8 T cells exhibit a greater amount of ATP that can be efficiently and rapidly mobilized upon stimulation

Proliferation of TEMRA CD8 T cells can be achieved through TCR stimulation in combination or not with common-gamma chain cytokine and was more vigorous than those of naïve CD8 T cells (**Figure 2**). In order to determine if the metabolic state of TEMRA CD8 T cells could explain this difference, ATP was quantified in purified CD8 T cell subsets in resting state and after 2h and 24h of stimulation with PMA and Ionomycin. In resting state, TEMRA CD8 T cells exhibited a 2.5-fold increased in ATP level as compared to naïve CD8 T cells (mean±sem 0.48±0.05 vs.0.18±0.04 respectively; **Figure 4A**). As expected, EM CD8 T cells also exhibit greater amount of ATP as compared to naïve CD8 T cells (mean±sem 0.36±0.05 vs. 0.18±0.04 respectively; **Figure 4A**). Upon polyclonal stimulation, in EM CD8 T cells the ATP level was increased by 2-fold 24 hours after stimulation after a rapid and transient decrease (**Figure 4B**). In contrast, the level of ATP in naïve T cells increased progressively along this 24-hours time period, probably reflecting their slower immunogenic

response. In TEMRA cells, the ATP level decreased greatly and significantly 2 hours after stimulation in TEMRA before being fully reconstituted after 24 hours(**Figure 4B**). Altogether, these results highlight the different bioenergetic requirements of naïve and memory T cells upon stimulation. The slow immune response of naïve T cells to polyclonal stimulation probably allow these cells to switch their metabolism in time to fully fulfill the energetic demand. In contrast, EM and TEMRA CD8 T cells ability to rapidly respond to polyclonal stimulation the rapid immune response required a high bioenergetic demand that exceeds the ATP level available. As a consequence, even if these cells exhibit a larger energy supply than naïve cells in resting state, this ATP pool is not sufficient to fulfill the rapid ATP mobilization required upon stimulation. However, both EM and TEMRA cells are able to reconstitute at least their initial energy supply within 24 hours. These results suggest that EM and TEMRA cells undergo similar metabolic changes.

TEMRA CD8 T cells exhibit polarized and functional mitochondria

The functionality of TEMRA CD8 T cells mitochondria has recently been questioned since TEMRA CD8 T cells have been described as senescent cells(7-9).We thus investigated different mitochondrial parameters. Using the Seahorse XF Analyzer, mitochondrial respiration was assessed under steady-state in purified naïve, TEMRA and EM CD8 T cells. Basal respiration was similar across the different CD8 subsets (**Figure 5A and B**). Mitochondrial coupling efficiency was then measured using olygomycin, an inhibitor of ATP synthase. Mitochondria of all cell subsets, including TEMRA cells, exhibit well-coupled mitochondria with roughly 70% of mitochondrial respiration devoted to ATP production (**Figure 5C**).Finally, mitochondrial membrane potential and mitochondrial mass were assessed by FACS analysis using the fluorescent probes JC-1 and Mitotracker respectively in freshly isolated CD8 subsets. In depolarized mitochondria, JC1 accumulated as a monomer, resulting in green fluorescence whereas JC1 aggregates are formed in polarized mitochondria

membrane resulting in a broad fluorescence emission (green to red). TEMRA CD8 T cells exhibit well-polarized mitochondria as shown by a JC1 red/green ratio similar to those of naïve and EM CD8 T cells (**Figure 5D).** Finally, mitochondrial mass was not different across the 3 CD8 subsets (**Figure 5E**). Collectively, these results show that TEMRA CD8 T cells exhibit well-functioning mitochondria under steady-state settings.

We next assess whereas mitochondria were involved in the T cells metabolic response upon polyclonal stimulation. To by-pass differences in TCR and costimulation signaling, purified CD8 subsets were stimulated with PMA and ionomycin (iono). Polyclonal stimulation of TEMRA CD8 T cells results in an immediate and rapid increase of OCR (**Figure 5F and G**), with a similar magnitude to those of naïve and EM CD8 T cells. However, as EM CD8 T cells, TEMRA CD8 T cells exhibited a sustained OCR overtime whereas mitochondrial respiration of naïve CD8 T cells return to basal level ($p<0.01$; **Figure 5H**). Collectively, TEMRA CD8 T cells exhibit functional mitochondria with similar characteristics as those of EM cells.

TEMRA CD8 T cells switch rapidly to glycolysis upon activation

It has been shown that, unlike naïve CD8 T cells, EM CD8 T cells are able to activate and to sustain aerobic glycolysis upon stimulation (2) and this feature was required for efficient immediate-early IFN-γ recall responses. Extracellular acidification rate (ECAR) was used as an indicator of glycolysis in TEMRA under steady-state and upon polyclonal stimulation. Glycolysis ofallCD8 T cell subsets were similar in resting state and greatly increased within minutes following polyclonal stimulation (**Figure 6A, B and C**). However, similarly to mitochondrial respiration, increased ECAR was sustained up to several hours after polyclonal stimulation in TEMRA and EM CD8 T cells but not in naïve cells ($p=0.0002$; **Figure 6B and 6D**), a phenomenon previously reported for EM CD8 T cells (2). Addition of 2-deoxyglucose

(2-DG), that blocks the first step of the glycolytic pathway, prevented both the immediate and sustained increase of ECAR confirming that increase of ECAR truly reflected increase of glycolysis(**Figure 6E and 6F**). Collectively, these data showed that TEMRA CD8 T cells behave as EM CD8 T cells for their ability to rapidly engage glycolysis for a prolong period of time upon polyclonal stimulation. Furthermore, this sustained higher metabolism, whether it is at the glycolytic level and at the mitochondrial level, in TEMRA and EM CD8 T cells is in agreement with the ability of these cells to reconstitute their ATP supply in the hours following polyclonal stimulation.

Glycolysis and not mitochondrial respiration is necessary for pro-inflammatory cytokine secretion by TEMRA CD8 T cells in healthy volunteers

Given the shared immune-metabolic features between TEMRA and EM CD8 T cells, we finally asked how glycolysis and mitochondrial respiration could regulate the immune function of TEMRA CD8 T cells. CD8 T cell subsets were polyclonally stimulated for 48h in the presence of 2-DG or metformin (MET) to inhibit respectively glycolysis and mitochondrial respiration(19, 20), and the secretion of pro-inflammatory cytokines (IL-2, IFN-γ and TNF-α) was assessed. Addition of glucose inhibitor 2-DG resulted in an inhibition of IL-2, IFN-γ and TNF-α secretion in all CD8 subsets (**Figure 7A**) whereas inhibition of mitochondrial respiration by metformin had no effect on their secretion (**Figure 7B**). Altogether, these data show the critical role of glycolysis in the secretion of the key pro-inflammatory cytokines secreted by CD8 T cells.

TEMRA CD8 T cells from patients with low or high immune stimulation rely on glycolysis to rapidly secrete pro-inflammatory cytokine

We have previously shown that the phenotype and the function of CD8 T cells and especially TEMRA CD8 T cells are modified in patients with multiple sclerosis (low-grade chronic

immune-stimulation) and in recipients of allogeneic kidney transplantation (high-grade chronic immune-stimulation)(10, 21).We then questioned whether prolong *in vivo* stimulation of CD8 T cells could affect their metabolic profiles. The amount and the functionality of mitochondria in CD8 T cells purified from MS and TX patients were first assessed as described previously (**Figure 8A and B**). Interestingly, patients with chronic low-grade immune-stimulation (MS) and in particular with chronic high-grade immune-stimulation (TX) exhibit a higher number of mitochondria as compared to HV ($p<0.05$; **Figure 8A**). Of note, no difference in mitochondrial polarization was observed between different CD8 subsets (**Figure 8B**).

We next assessed how TEMRA CD8 T cells rely on glycolysis and mitochondrial respiration to secrete pro-inflammatory cytokines. As expected, TEMRA and EM CD8 T cells secreted higher amount of IL-2, IFN-γ and TNF-α in MS and TX patients as compared to HV (**Figure 8C**). Addition of glucose inhibitor 2-DG blunted the secretion of IL-2, IFN-γ and TNF-α in all CD8 subsets in both HV and patients with chronic low or high-grade immune stimulation (**Figure 8C**). Finally, similar experiments were performed in the presence of metformin. For most cytokines secretion, metformin had no effect independently of CD8 T cells and chronic inflammation (**Figure 8C**). However, metformin enhanced IL2 secretion by TEMRA CD8 T cells in MS patients ($p=0.0159$; **Figure 8D**).

Collectively, our results show TEMRA CD8 T cells exhibit immune-metabolic profiles similar to those from EM CD8 T cells and that interfering with glycolysis is a potent way to inhibit pro-inflammatory cytokine secretion in patients with chronic immune-stimulation ranging from low to high grade.

Discussion

We report that TEMRA CD8 T cells rapidly respond to stimulation by using the large pre-existing pool of ATP and switch their metabolism toward glycolysis that is sustained in time and required for the secretion of pro-inflammatory cytokines. Thus, TEMRA CD8 T cells exhibit not only high amount of pre-formed cytotoxic molecules and pro-inflammatory cytokines but also metabolic features that confers them an advantage for a rapid response to stimulation. Thus, these data show that, in contrast with other report(22), TEMRA CD8 T subsets are not immune-senescent cells and that their cytopathogenic function are sustained by bioenergetics features shared with EM CD8 T cells and that may be differentially involved according to the immunological situation. Even more, whereas early EM CD8 T cells exhibit high amount of IFN-γ and TNF-α but lack of pre-formed cytotoxic molecules, TEMRA CD8 T cells exhibit both and represent memory CD8 T cells at the frontline with new antigen encounter. Collectively, our results strengthen the need to control these pathogenic CD8 T cells in a wide range of chronic disease ranging from auto-immune diseases like multiple sclerosis to allogeneic kidney graft rejection.

In contrast to published report (22), we provide evidences that TEMRA cells possess well-polarized and functional mitochondria which was assessed by various means. First, we demonstrated that the mitochondrial membrane potential was similar to the ones of naïve and EM cells using a fluorescent mitochondria specific probe. Under resting state, mitochondrial respiration of TEMRA cells was similar to the ones of naïve and EM cells. We also show that TEMRA CD8 T cells exhibit higher amount of ATP as compared to naïve CD8 T cells in resting state. Polyclonal stimulation not only leads to a rapid rise of both mitochondrial respiration and glycolysis in all CD8 T cell subsets but increase of both metabolic pathways after stimulation is sustained with time specifically in TEMRA and EM CD8 T cells. These results are in agreement with previous reports stating that the strength of the stimulation

influences the glycolysis response of naïve and memory CD8 T cells (1). Whereas both naïve and EM CD8 T cells increased their glycolysis upon anti-CD3/28 stimulation, only EM CD8 T cells were able to do so when stronger stimulation was used (i-e PMA/Ionomycin stimulation) (1). We show in this report that TEMRA CD8 T cells were able to engage a sustained and strong glycolysis activity in response to potent stimulation. We also show that TEMRA CD8 T cells as EM CD8 T cells rapidly engage glycolysis for a prolong period of time upon polyclonal stimulation. Taking into account the higher ATP supply under resting state combined with their ability to immediately upregulate and sustained both mitochondrial respiration and glycolysis, similarly to EM CD8 T cells, TEMRA CD8 T cells are able to provide the energy required for an immediate engagement after immunogenic activation. TEMRA CD8 T cells are thus in a ready-to-respond state that could engage rapidly and efficiently their bioenergetic machinery to sustain their proliferation and their effector functions (cytotoxicity and secretion of pro-inflammatory cytokines). The presence of pre-formed cytotoxic molecules in TEMRA CD8 T cells may represent a complementary role with the surveillance role of EM CD8 T cells that lack the expression of these molecules in resting state.

We also provide evidence that metabolic function is tightly linked to immune function, in particular at the level of pro-inflammatory cytokines secretion. Glycolysis is necessary for the secretion of the three main pro-inflammatory cytokines (IL-2, IFN-γ and TNF-α) by TEMRA CD8 T cells as well as by other CD8 subsets (naïve and EM). Furthermore, this statement stands for healthy volunteers and more importantly for patients who had undergone a modest or a strong immune challenge. It has been shown in mice that glucose metabolism was necessary for IFN-γ transcription when naïve CD8 T cells were stimulated (6, 23) whereas IL-2 transcription was less sensitive to glucose deprivation. IFN-γ production by human EBV-specific CD8 T cells is also diminished in the presence of 2-DG (2). Little information is

available regarding the link between glycolysis and TNF-α secretion, especially in CD8 T cell biology. Provision of 2-DG has been shown to inhibit TNF-α secretion by monocyte (24).We now show that *in vivo* primed CD8 T cells as well as CD8 T cells (EM and TEMRA cells) from healthy controls relies primarily on glycolysis and not on mitochondrial respiration to elicit pro-inflammatory cytokines. Targeting the glucose metabolism has been shown to inhibit T_H17 (25), to promote T_{REG} differentiation(25), to enhance memory generation (6) and thus could be useful in the context of vaccine strategy and anti-tumor therapy (6, 26, 27). Our data obtained from kidney transplant recipients and patients with multiple sclerosis support the need to attempt to regulate immune function through metabolic pathway interferences. A great technical challenge would be to target only one CD8 subset (i-e TEMRA CD8 T cells) while preserving the naïve and the EM CD8 T cells.

Freshly purified TEMRA CD8 T cells as defined in the present report are lacking the expression of CCR7, express low levels of IL-7R and CD27, but express the hallmarks of effector memory cells such as high amount of transcriptional factors T-bet and Eomes associated with a high expression of cytotoxic molecules (granzyme B and Perforin) and pro-inflammatory cytokines (IFN-γ and TNF-α). In contrast, early EM CD8 T cells are $CD28^+CD27^+$, with intermediate expression of T-bet and Eomes, pre-formed pro-inflammatory cytokines but without cytotoxic molecules. Using freshly purified TEMRA CD8 T cells from HV, we show that this population has the potential to respond to common γ-chain cytokine (IL-2, IL-7 and IL-15) as exemplified by the phosphorylation of STAT5 as soon as 15' of stimulation. The response to cytokine stimulation was effective as an enhancement of cell proliferation combined with a decrease in apoptotic levels were observed when purified TEMRA CD8 T cells were stimulated through their TCR and common γ-chain cytokine. Cytokine-based signals and not CD28-related signals seemed thus to sustain the survival of TEMRA CD8 T cells as well as their ability to be activated. Maintenance of the

polyclonality of naïve CD8 T cells relies on alternative stimulations between IL-7 and TCR (28) whereas IL-2 or IL-15 sustains the survival of memory CD8 T cells (29). The ability of TEMRA CD8 T cells to efficiently respond to IL-2 and IL-15 strengthen their relationship with EM CD8 T cells. Moreover, the ability of $CD28^{-}CD8^{+}$ T cells to respond to IL-15 + TCR stimulation has been recently identified in the context of allo-immune response (30) and proposed to confer memory CD8 T cell resistance to blockade of CD28-CD80/86 pathway using either CTLA-4Ig-mediated costimulation blockade (31) or anti-CD28 mAb (32). Thus targeting specific cytokine response on TEMRA CD8 T cells will constitute an innovative mean to control this cell population that has been involved in various pathogenic processes, including allogeneic kidney transplantation (10) and bone regeneration (11).

In conclusion, we show in this paper that TEMRA CD8 T cells are metabolically close to CD8 EM T cells since both subsets were able to rapidly switch to aerobic glycolysis and to sustain a high rate of glycolysis over-time. With their well-functioning mitochondria combined with a great pool of ATP under resting state and their rapid metabolic adaptation, TEMRA CD8 T cells are able to engage in a timely and effective manner their effector functions through the secretion of pro-inflammatory cytokines and cytotoxic molecules to elicit an immune response at the frontline of the antigen encounter. Our findings indicate that drugs that can alter glycolysis could hold promise to improve the treatment of kidney transplant recipients and of patients with multiple sclerosis.

References

1. van der Windt GJW et al. CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. *Proc Natl Acad Sci USA*. 2013;110(35):14336–14341.

2. Gubser PM et al. Rapid effector function of memory CD8. *Nat Immunol*. 2013;14(10):1–11.

3. Fox CJ, Hammerman PS, Thompson CB. Fuel feeds function: energy metabolism and the T-cell response. *Nat Rev Immunol*. 2005;5(11):844–852.

4. Pearce EL et al. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature*. 2009;460(7251):103–107.

5. O'Sullivan D et al. Memory CD8+ T Cells Use Cell-Intrinsic Lipolysis to Support the Metabolic Programming Necessary for Development. *Immunity*. 2014;41(1):75–88.

6. Sukumar M et al. Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function. *J Clin Invest*. 2013;123(10):4479–4488.

7. Khan N et al. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. *J Immunol*. 2002;169(4):1984–1992.

8. Pawelec G et al. Is immunosenescence infectious? *Trends in Immunology*. 2004;25(8):406–410.

9. Wertheimer AM et al. Aging and Cytomegalovirus Infection Differentially and Jointly Affect Distinct Circulating T Cell Subsets in Humans. *J Immunol*. 2014;192(5):2143–2155.

10. Yap M et al. Expansion of highly differentiated cytotoxic terminally differentiated effector memory CD8+ T cells in a subset of clinically stable kidney transplant recipients: a potential marker for late graft dysfunction. *J Am Soc Nephrol*. 2014;25(8):1856–1868.

11. Reinke S et al. Terminally differentiated CD8⁺ T cells negatively affect bone regeneration in humans. *Science Translational Medicine*. 2013;5(177):177ra36–177ra36.

12. Goldeck D et al. Multi-parametric phospho-flow cytometry: A crucial tool for T lymphocyte signaling studies. *Cytometry*. 2013;83A(3):265–272.

13. Appay V, van Lier RAW, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: Consensus and issues. *Cytometry*. 2008;73A(11):975–983.

14. Intlekofer AM et al. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat Immunol*. 2005;6(12):1236–1244.

15. Pearce EL et al. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science*. 2003;302(5647):1041–1043.

16. Kaech SM, Cui W. Transcriptional control of effector and memory CD8+ T cell differentiation. *Nat Rev Immunol*. 2012;12(11):1–13.

17. Cui W, Liu Y, Weinstein JS, Craft J, Kaech SM. An Interleukin-21- Interleukin-10-STAT3 Pathway Is Critical for Functional Maturation of Memory CD8+ T Cells. *Immunity*. 2011;35(5):792–805.

18. Wang Y et al. GATA-3 controls the maintenance and proliferation of T cells downstream of TCR and cytokine signaling. *Nat Immunol*. 2013;14(7):1–10.

19. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J*. 2000;348 Pt 3:607–614.

20. El-Mir MY et al. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem*. 2000;275(1):223–228.

21. Laplaud DA. Blood T-cell receptor   chain transcriptome in multiple sclerosis. Characterization of the T cells with altered CDR3 length distribution. *Brain*. 2004;127(5):981–995.

22. Henson SM et al. p38 signaling inhibits mTORC1-independent autophagy in senescent human CD8+ T cells. *J Clin Invest*. 2014;124(9):4004–4016.

23. Cham CM, Gajewski TF. Glucose availability regulates IFN-gamma production and p70S6 kinase activation in CD8+ effector T cells. *J Immunol*. 2005;174(8):4670–4677.

24. Dietl K et al. Lactic acid and acidification inhibit TNF secretion and glycolysis of human monocytes. *J Immunol*. 2010;184(3):1200–1209.

25. Shi LZ et al. HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med*. 2011;208(7):1367–1376.

26. Bénéteau M et al. Combination of glycolysis inhibition with chemotherapy results in an antitumor immune response. *Proc Natl Acad Sci USA*. 2012;109(49):20071–20076.

27. Gattinoni L, Klebanoff CA, Restifo NP. Pharmacologic induction of CD8+ T cell memory: better living through chemistry. *Science Translational Medicine*. 2009;1(11):11ps12–11ps12.

28. Kimura MY et al. IL-7 signaling must be intermittent, not continuous, during $CD8^{+}$ T cell homeostasis to promote cell survival instead of cell death. *Nat Immunol*. 2013;14(2):143–151.

29. Rubinstein MP et al. IL-7 and IL-15 differentially regulate CD8+ T-cell subsets during contraction of the immune response. *Blood*. 2008;112(9):3704–3712.

30. Traitanon O et al. IL-15 Induces Alloreactive CD28 −Memory CD8 T Cell Proliferation and CTLA4-Ig Resistant Memory CD8 T Cell Activation. *Am J Transplant*. 2014;14(6):1277–1289.

31. Vincenti F et al. Costimulation blockade with belatacept in renal transplantation. *N Engl J Med*. 2005;353(8):770–781.

32. Poirier N et al. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. *Science Translational Medicine*. 2010;2(17):17ra10–17ra10.

Figures Legends

Figure 1. Characterization of naïve, TEMRA and EM CD8 T cells identified using CD45RA and CD28. (**A**)Expression of CCR7, CD27, CD127, CD57, T-bet, GZM-b and PERF by naïve ($CD45RA^{+}CD28^{+}$), TEMRA ($CD45RA^{+}CD28^{-}$) and EM ($CD45RA^{-}CD28^{+}$) CD8 T cells was measured by multi-color flow cytometry in PBMC from 15 healthy donors. Each dot represents one donor, and the mean and the SEM are displayed.(**B**) Naïve, TEMRA and EM CD8 T cells were FACS-sorted from 7 healthy donors and expression of the mentioned transcripts was determined by qPCR. Data are shown in arbitrary unit and the mean and the SEM are displayed. Comparison between the 3 CD8 subsets was performed using a Kruskal-Wallis test followed by a Dunn's Multiple Comparison Test.

Figure 2. TCR stimulation alone elicits proliferation of TEMRA CD8 T cells. CPD eFluor450 purified CD8 T cell subsets were stimulated with 1ug/mL anti-CD3 for 5 days. Proliferation was assessed according to the frequency of CDP^{low} cells and the expression of annexin-V was used to evaluate the apoptosis level. The mean and the SEM of 7 healthy volunteers are displayed. Comparison between the 3 CD8 subsets was performed using a Kruskal-Wallis test followed by a Dunn's Multiple Comparison Test.

Figure 3. TEMRA CD8 T cell are susceptible to common-γ chain stimulation. (A) PBMC were stimulated for 15' with various cytokines (IL-2, IL-7, IL-15, IL-6, Il-10 and IL-21) and phosphorylation of STAT5 and STAT3 was analyzed within naïve, TEMRA and EM CD8 T cells. One representative experiment is shown and the mean and the SEM of 6 to 11 individuals are shown. Comparison between the 3 CD8 subsets was performed using a Kruskal-Wallis test followed by a Dunn's Multiple Comparison Test. CPD eFluor450 purified CD8 T cell subsets were stimulated with 1ug/mL anti-CD3 for 5 days in the presence of the indicated cytokines. Proliferation was assessed according to the frequency of CDP^{low} cells (**B**)

and the expression of annexin-V was used to evaluate the apoptosis level (**C**). The mean and the SEM of 7 healthy volunteers are displayed.

Figure 4. Resting TEMRA CD8 T cells exhibit a greater amount of ATP that can be efficiently and rapidly mobilized upon stimulation. ATP was quantified in freshly purified CD8 T cell subsets (**A**) or after 2h and 24h of stimulation with PMA/Ionomycin (**B**). Each dot represents one HV and the mean and the SEM of 6-10 healthy volunteers are displayed. Comparison between the 3 CD8 subsets in resting state was performed using a Kruskal-Wallis test followed by a Dunn's Multiple Comparison Test.

Figure 5. TEMRA CD8 T cells exhibit polarized and functional mitochondria. OCR of purified CD8 T cell subsets (naïve, black; TEMRA, green; EM, red) was measured before and after sequential addition of metabolic stress drugs. OCR profile of one out of 5 HV is shown (**A**) and the mean and the SEM of 5 healthy volunteers are displayed (**B**). Oxygen consumption devoted to ATP production by mitochondria was assessed by comparing OCR before and after addition of oligomycin (n=5; **C**). Mitochondrial Membrane Potential (**D**) and mitochondrial load (**E**) of each CD8 T cell subsets were assessed according to the JC1 red / JC1 green ratio (n=4; **D**) and the mean fluorescence intensity of Mitotracker Dye (n=19; **E**) respectively. OCR of purified CD8 T cell subsets (naïve, black; TEMRA, green; EM, red) upon PMA/Iono stimulation was assessed and one of 14 HV is shown (**F**). OCR modification within each CD8 subset induced by PMA/Iono stimulation was assessed according to the ratio of OCR after/before drug injection (n=14; **G**) and 60' after/before drug injection (n=14; **H**). Values for each HV or mean and SEM are displayed. Comparison between the 3 CD8 subsets was performed using a Kruskal-Wallis test followed by a Dunn's Multiple Comparison Test. Mann-Whitney test was used to investigate the effect of oligomycin on OCR before and after stimulation.

Figure 6. TEMRA CD8 T cells switch rapidly to glycolysis upon activation. ECAR of purified CD8 T cell subsets was measured in resting state (n=14; **A**) or after PMA/Iono stimulation (**B**). ECAR profile of one out of 14 HV is shown (**B**; naïve, black; TEMRA, green; EM, red). ECAR modification within each CD8 subset induced by PMA/Iono stimulation was assessed according to the ratio of ECAR after/before drug injection (n=14; **C**) and 60' after/before drug injection (n=14; **D**). Glycolysis was inhibited by provision of 2-DG before PMA/Iono stimulation and similar readout was used (n=11-14; **E** and **F**).Values for each HV or mean and SEM are displayed. Comparison between the 3 CD8 subsets was performed using a Kruskal-Wallis test followed by a Dunn's Multiple Comparison Test. Mann-Whitney test was used to investigate the effect of 2-DG on ECAR before and after stimulation.

Figure 7. Glycolysis and not mitochondrial respiration is necessary for pro-inflammatory cytokine secretion by TEMRA CD8 T cells. Secretion of IL-2, IFN-γ and TNF-α by each CD8 subsets purified from HV (n=7-13) was assessed 48h after stimulation with anti-CD3 anti-CD28 in the presence of 2-DG (**A**) or Metformin (MET; **B**).Mean and SEM are displayed.

Figure 8. TEMRA CD8 T cells from patients with low or high immune stimulation rely on glycolysis to secrete pro-inflammatory cytokine. Mitochondrial load (**A**) and Mitochondrial Membrane Potential (**B**) of each CD8 T cell subsets were assessed according to the mean fluorescence intensity of Mitotracker Dye (HV, n=19; MS, n=17; TX, n=8; **A**) and to the JC1 red / JC1 green ratio (HV, n=10; MS, n=7; TX, n=5; **B**) respectively. Secretion of IL-2, IFN-γ and TNF-α by each CD8 subsets purified from HV (n=7-13), MS (n=4-7) and TX (n=4-10) was assessed 48h after stimulation with anti-CD3 anti-CD28 in the presence of 2-DG (**C**) or Metformin (MET; **D**). Values for each HV or mean and SEM are displayed.

Supplementary Figure 1. Dose-response to IL-7 stimulation is similar between TEMRA, Naïve and EM CD8 T cells. PBMC were stimulated for 15' with various dose of IL-7 and phosphorylation of STAT5 was analyzed within naïve, TEMRA and EM CD8 T cells. One of 3 representative experiments is shown.

	Mean or Number	**SD or Percentage**
Recipient age (years)	48.45	9.48
Donor age (years)	53.14	14.74
Woman recipient	7	50.0%
Woman donor	10	71.4%
Incompatibility HLA-A, -B, -DR, DP ≥4	13	92.8%
At blood sampling		
Time-post-transplantation (yr)	3.87	1.23
Creatinemia (μmol/L)	132	34
Induction Therapy		
Monoclonal Ab	7	50%
Polyclonal Ab	7	50%
None	0	0%
Other	0	0%
Maitenance therapy		
MMF	9	64%
CSA	0	0%
AZA	3	21%
FK	12	86%
Corticotherapy	6	43%

Table 1A

	Mean or Number	**SD or Percentage**
Age (years)	39,40	9.83
Gender (Woman)	17	70.8%
Disease duration (years)	6.96	5.97
Disease type (RR)	24	100%
EDSS		
0 – 1	6	25%
1 – 2	10	42%
2 – 3	3	13%
≥3	4	17%

Table 1B

	Fold change aCD3 vs. unstimulated[1]		
Transcript	NAIVE	TEMRA	EM
T-bet		0.3±0±0 (p=0.002)	
EOMES			
BLIMP-1			
GATA-3			
PERF		0.3±0.1 (p=0.002)	
GZMb	60.3±21±3 (p=0.001)		38.4±7.6 (p=0.001)
IFN-γ	29.8±9±8 (p=0.004)	14.8±7.5 (p=0.018)	32.0±14.0 (p=0.004)
TNF-α	24.8±9.1 (p=0.001)	10.9±3.5 (p=0.011)	11.7±5.9 (p=0.001)
Bcl-2	4.4±1.2 (p=0.012)	5.9±2.2 (p=0.038)	
Bcl-6	0.5±0.1 (p=0.012)		

[1]changes in transcript expression are expressed in fold change using FACS-sorted level of expression as reference

Table 2.

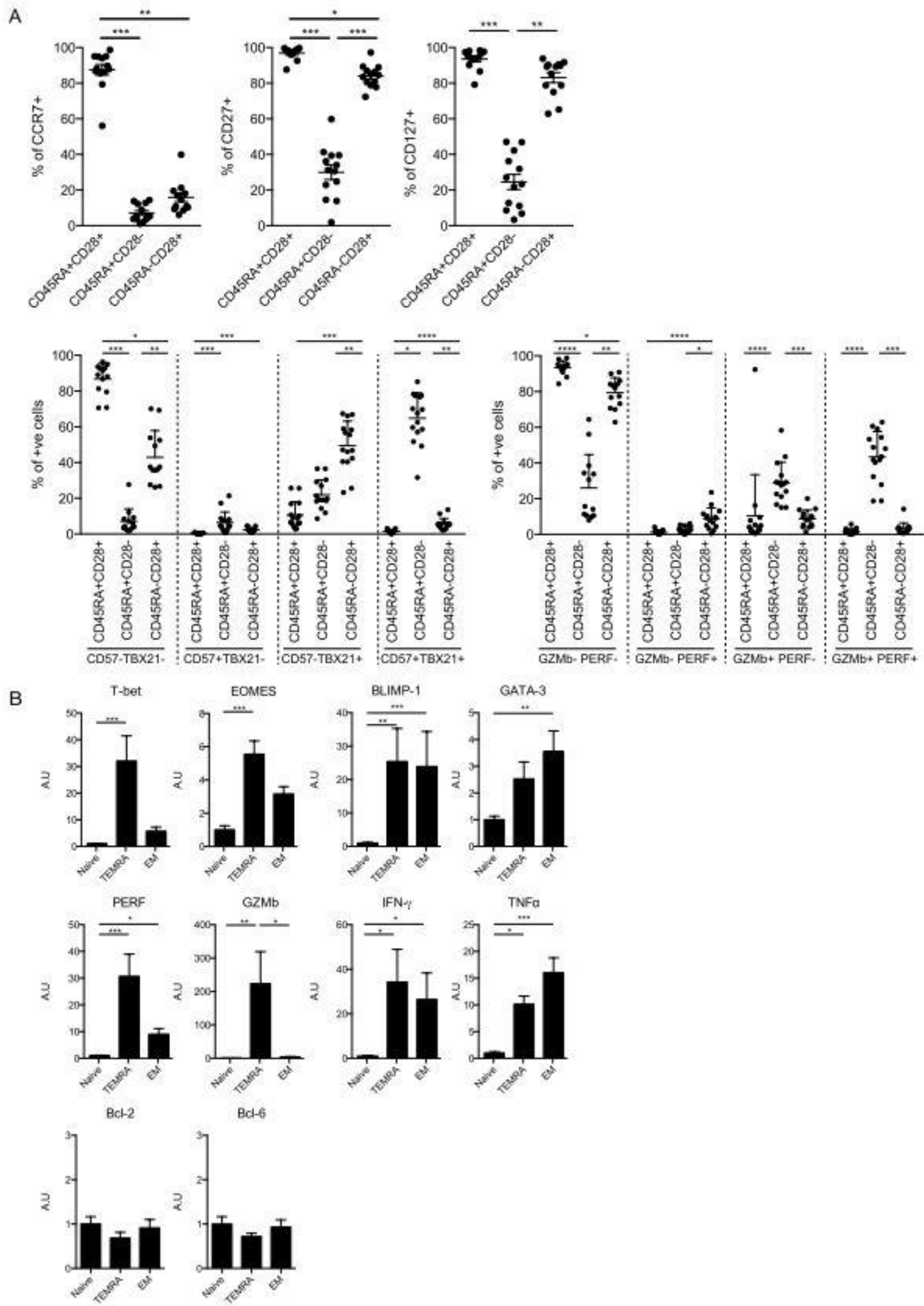


Figure 1.

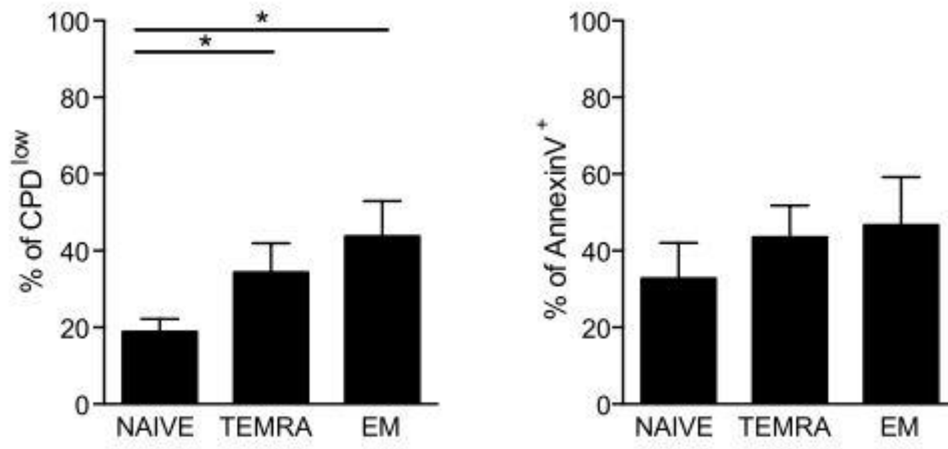


Figure 2.

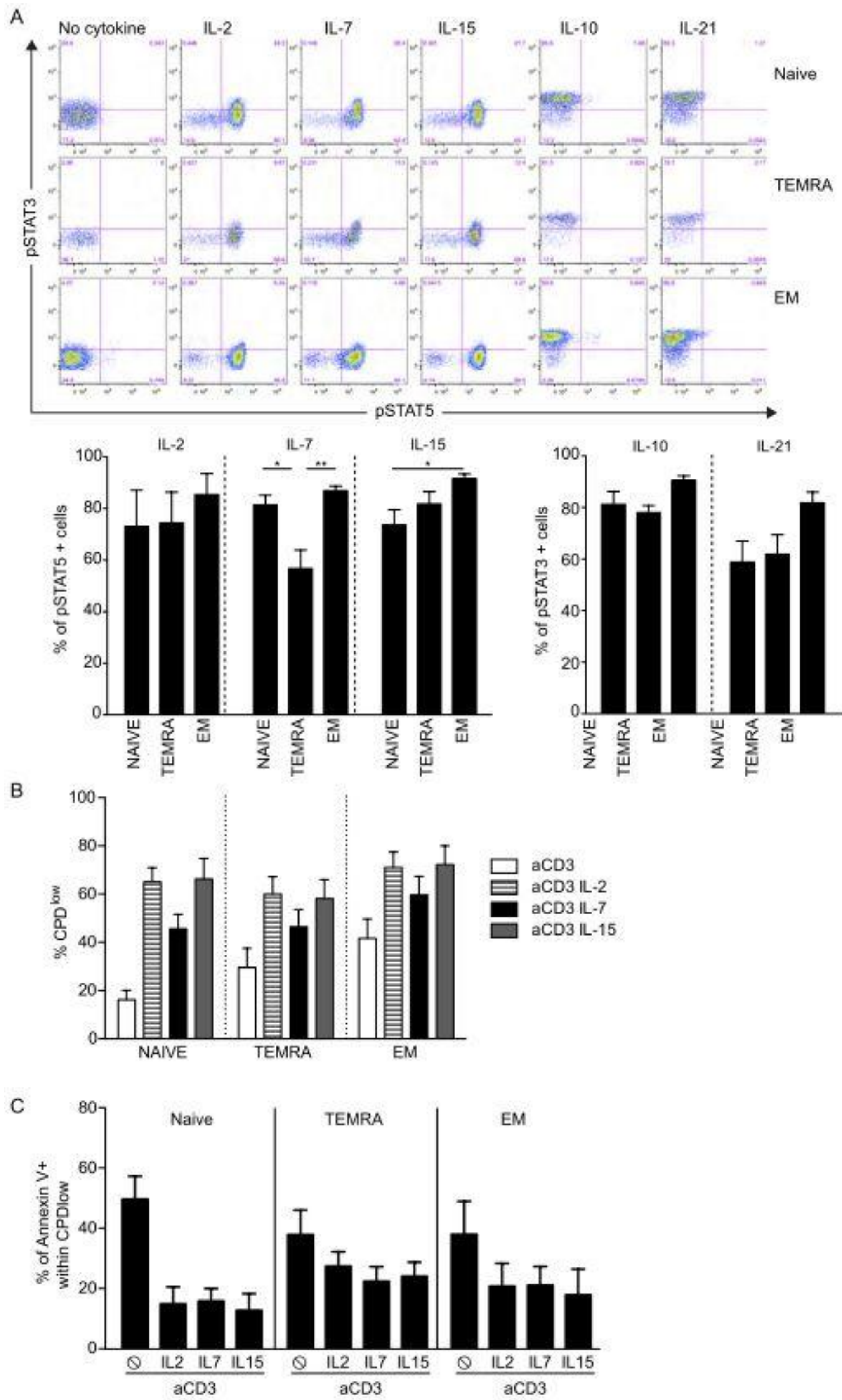


Figure 3.

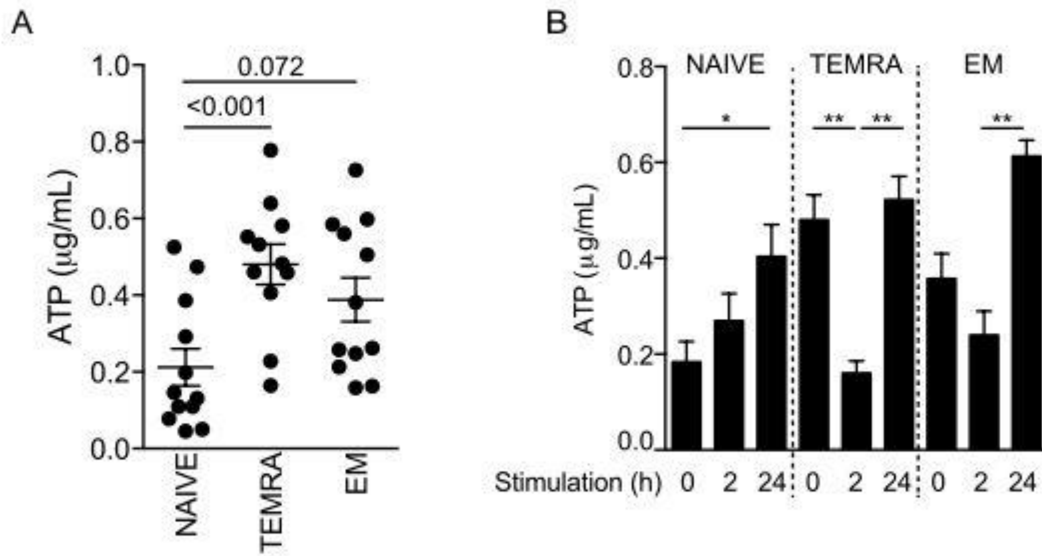


Figure 4.

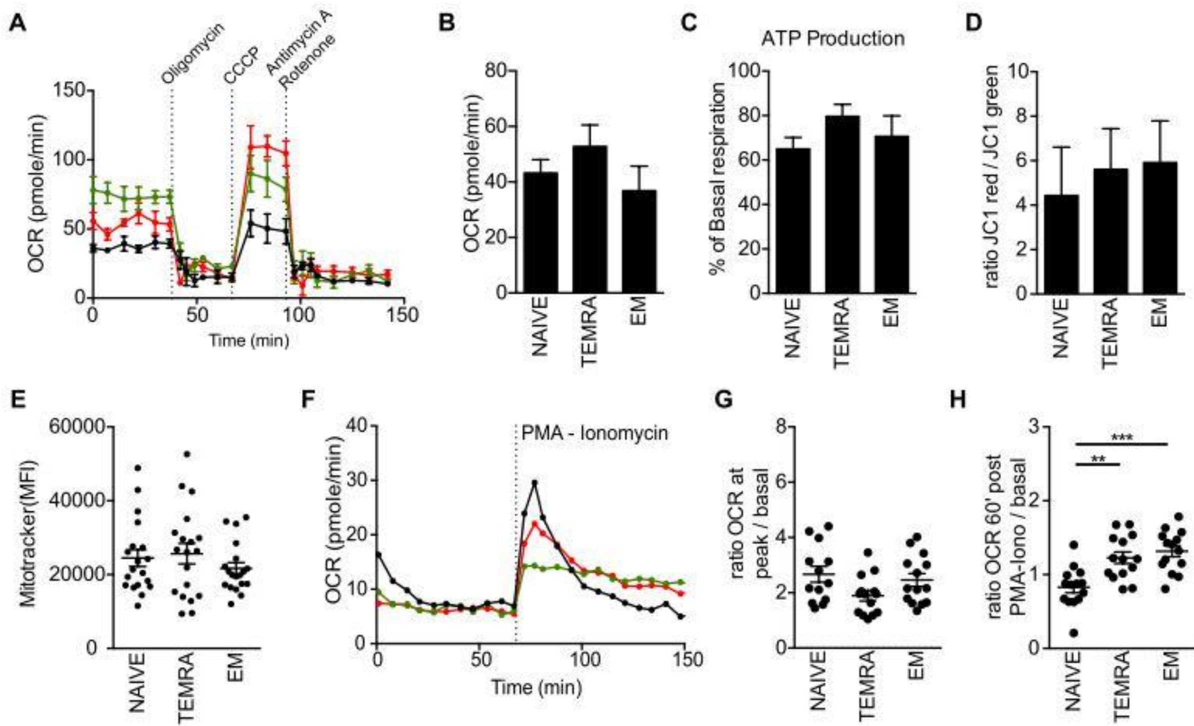


Figure 5.

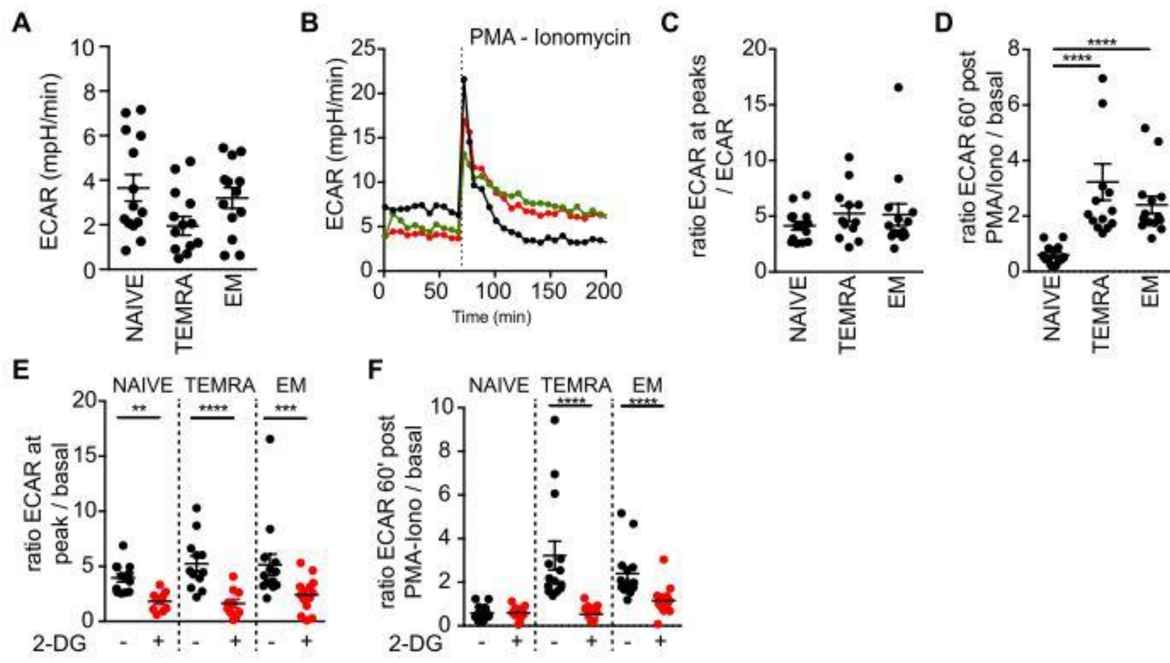


Figure 6.

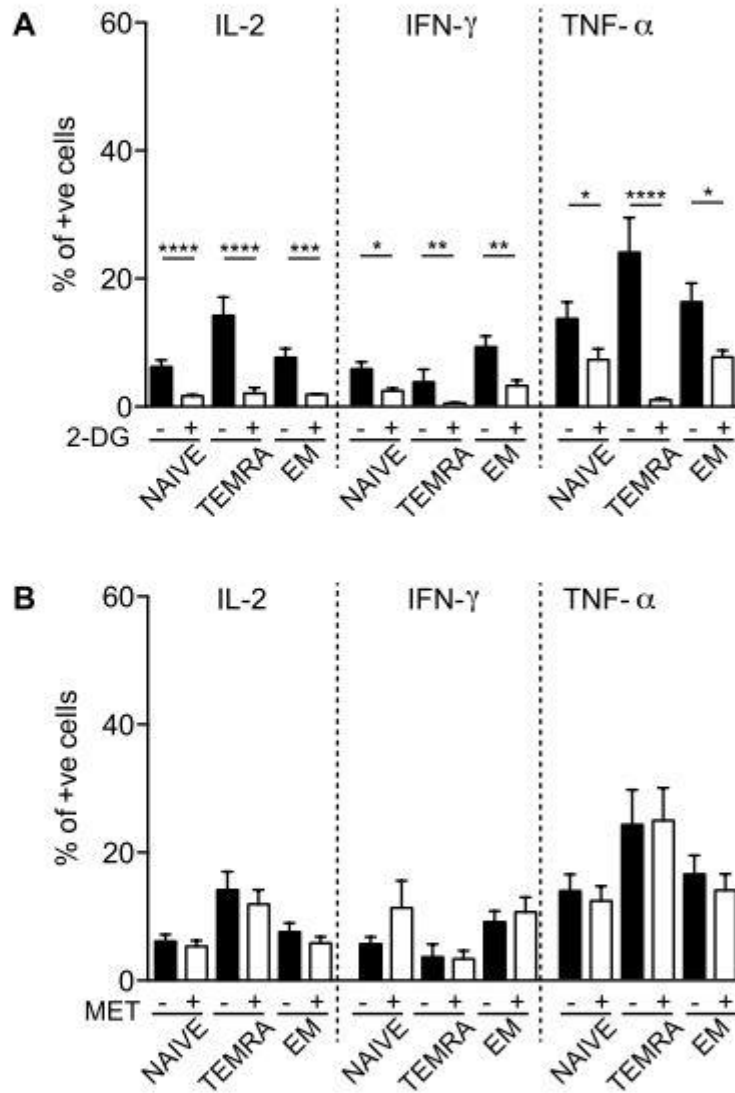


Figure 7.

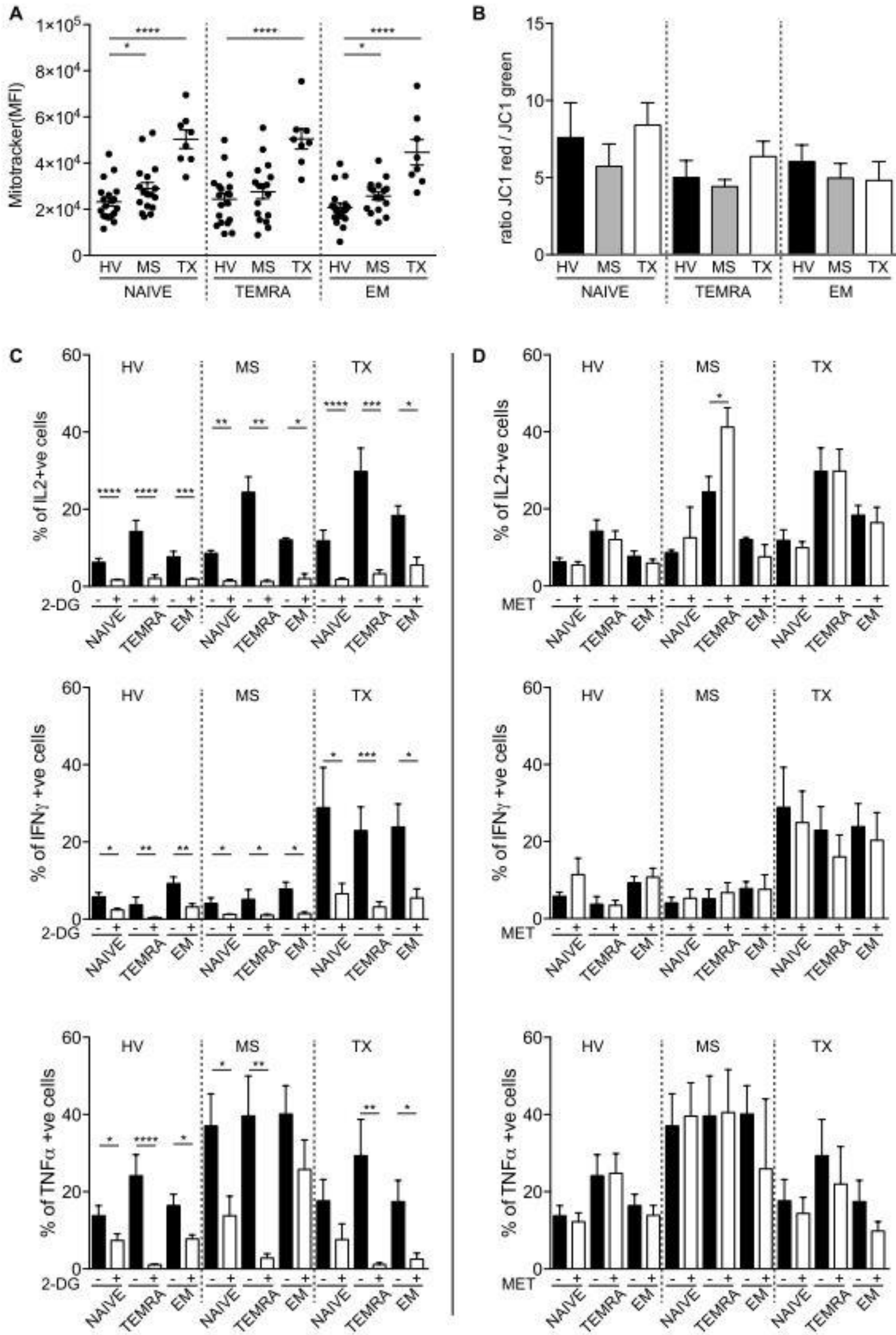
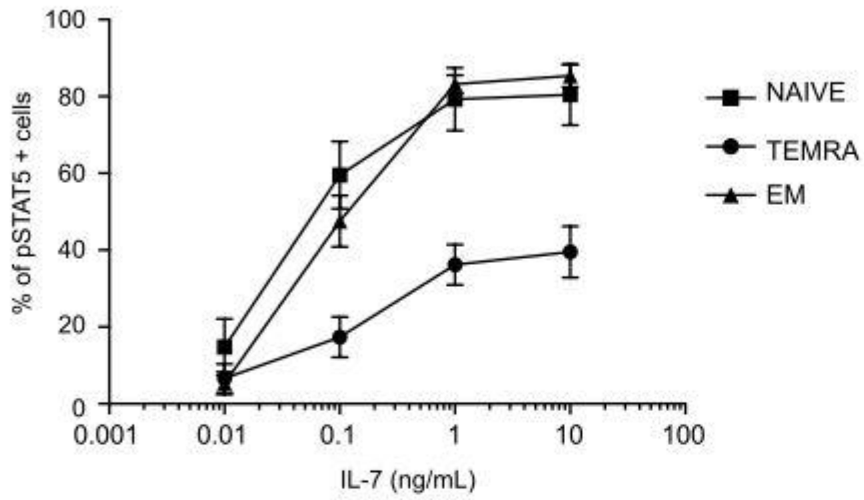


Figure 8.



Supplemental Figure 1.

4 Discussion

4.1 CD8 T Cell Biomarker Research in Transplantation

Kidney transplantation is the best treatment for end-stage renal disease. The goal of biomarker research in transplantation is to find markers that are sensitive and predictive of allograft loss, and can help guide physicians decisions regarding patient care. The involvement of CD8 T cells in allograft rejection is a well-researched topic in the transplant field. Therefore, it is a logical leap to evaluate the use of CD8 cells as potential biomarkers for allograft rejection. Betjes et al. published that an increased frequency of TEMRA CD8 cells in the periphery pre-transplant was an indicator of graft rejection post-transplantation (Betjes et al. 2012). We found that higher frequencies of TEMRA CD8 cells with strong cytotoxic characteristics were associated with altered TCR Vβ repertoire and an almost 2-fold higher risk of long term allograft dysfunction in long term stable kidney transplant patients (Article 1) (Yap et al. 2014). In order to validate the usefulness of using CD8 T cells as biomarkers of long term rejection, we decided to evaluate the value of including phenotypic data about CD8 T cells into the KTFS. The aim of the KTFS is to use current clinical parameters to calculate a patient’s risk of renal allograft rejection. When we included immunological parameters into the calculations, we were able to improve the AUC of the KTFS. By including immunological markers into the KTFS, 25% of patients were better classified into the low- and high-risk groups, which will lead to improved care for the patients (Article 3).

TEMRA CD8 T cells, in the context of acting as an immunological component of the KTFS, would be a potentially powerful tool for determining patient follow up care. The TELEGRAFT study carried out by nephrologists at several transplant centers in France used the KTFS to divide patients into low-risk and high-risk groups and includes new videoconferencing tools to improve patient care (Foucher et al. 2015). Patients in the low-risk group will follow a more relaxed follow-up schedule with 4 hospital visits per year as

opposed to the 6 visits per year scheduled for the high risk group. The reason for arranging the follow-up schedule as such is to reduce the number of hospital visits for low-risk patients, thus reduce their overall health care cost, and to increase the number of consultations for high-risk patients in order to minimize the risk or allograft dysfunction. A key aim of this study is evaluating if the quality of life of low-risk patients can be improved by reducing their hospital visits and if the increased clinical schedule of high-risk patients will reduce the occurrence of allograft rejection. In order to thoroughly address these concerns, a secondary objective of the TELEGRAFT study is to use a video conferencing system to replace (in the case of low-risk patients) or supplement (in the case of high-risk patients) hospital visits. Low-risk patients will have one hospital visit and 3 video conference consultations, while high-risk patients will have 6 video conference consultations in addition to their 6 hospital visits. The aim of this arm of the TELEGRAFT study, called eHealth, is to reduce the number of hospital visits of low-risk patients without impacting their allograft health and to increase the number of consultations for high-risk patients in hopes of preventing allograft dysfunction without causing major disruption to the patients' everyday life (Foucher et al. 2015). While the study is currently ongoing, it will be interesting to see how new biomarkers and new technology can improve patient health and allograft survival.

While the inclusion of TEMRA cells into the statistical calculation was able to improve the KTFS, there are still logistical problems that prevent TEMRA CD8 cells from becoming a common clinical biomarker of renal allograft dysfunction. Clinical flow cytometry currently lacks standardization in protocol, reagents, and data analysis, and therefore it is difficult to compare studies from different centers. Before clinical flow cytometry can be used as a biomarker of kidney transplantation, protocol needs to be standardized. Villanova et al. demonstrated how to use of lyophilized reagent plates as a means to address the issue faced with sample preparation and assay reagent and how this could be translated into a flow

cytometric platform for clinical biomarker detection. In this study, lyophilized plates for stimulation and for antibody staining were compared to standard liquid flow cytometry techniques to assess the expression of cytokine secretion and activation markers. They showed that using lyophilized plates resulted in higher sensitivity for IFNγ and IL10 detection and higher antibody staining indexes compared to traditional flow cytometry techniques. This study shows that lyophilized antibodies would solve the standardization issues of clinical flow cytometry. Furthermore, they showed that automated computational analysis could be used to reliably analyze the flow cytometric data and that their computational program could identify populations that were difficult to identify using manual analysis (Villanova et al. 2013). Currently, clinical flow cytometry is largely used in oncology. In the field of transplantation, flow cytometers have been used for HLA typing and in hematopoietic stem cell transplants (Jaye et al. 2012). However, with the considerable amount of effort going into biomarker studies in the transplantation field, it is expected that soon new clinical biomarkers will be available for medical use and it will be accompanied by new flow cytometric protocols. Lyophilized plates have been available for 'research use only' and it is only a matter of time before this technique is translated and validated for clinical use.

As our research was focused on exploring biomarkers from peripheral blood, it would also be interesting to investigate the potential of using CD8 T cells as urinary biomarkers. Urine cytology studies have shown that the increased presence of lymphocytes in the urine sediment was associated with acute rejection and BK polyoma virus infection (BKV) (Roberti et al. 1995; Corey et al. 1997; Chatterjee et al. 2012; van Doesum et al. 2014; Ringsrud 2001). Van Doesum et al. found an increase in the frequency of urinary EM and TEMRA CD8 cells in acute rejection patients, thus furthering the argument of using TEMRA CD8 cells as biomarker of allograft rejection (van Doesum et al. 2014). One interesting point to note is that in the study of van Doesum et al., urinary CD8 T cells were used as a monitoring tool, where

they observed an increase of CD8 cells correlated with an increased serum creatinine and a graft rejection episode, and successful treatment of the rejection episode saw an associated drop in urinary CD8 T cells. In this study, there was no noticeable increase in the absolute number of CD8 T cells in the periphery nor was there a difference the frequency of the CD8 T cell subsets in the rejecting patients compared to the control patients (van Doesum et al. 2014). Conversely, our work on peripheral CD8 T cell subsets as biomarkers has been more focused on using TEMRA CD8 cells as predictive biomarkers of long-term outcomes. The contrasting endpoints (where van Doesum et al. followed patients for 2 months after inclusion into the study whereas we followed patients for several years after study inclusion) highlight the utility and diverse applications that CD8 T cells can fulfill as biomarkers. Peripheral CD8 T cell subset frequencies can act as predictive biomarkers of long-term allograft survival and urinary CD8 T cell subsets can function as diagnostic biomarkers which indicate the immediate health of the allograft. The usefulness of CD8 T cells as biomarkers is not just limited to CD8 subset analysis; the cytotoxic molecules secreted by CD8 T cells can also act as biomarkers of allograft dysfunction. We have showed that including the frequencies of $CD27^{+}CD28^{-}$, $GZMb^{+}PERF^{+}$ and $GZMb^{-}PERF^{+}$ CD8 T cells into the calculation of KTFS can also serve as a predictive biomarker of long term allograft dysfunction (Article 3). This is in agreement with work from others who have shown that transcripts of perforin and granzyme-b can be used as serum and urinary biomarkers of acute rejection (B. Li et al. 2001; Simon et al. 2003). However, Heng et al. recently publish a meta-analysis study evaluating the clinical value of using perforin and granzyme-b as biomarkers of acute rejection. They included 16 studies into their analysis and found that perforin or granzyme-b as single predictors of allograft rejection were not convincing diagnostic markers. However, using both perforin and granzyme-b as combine markers resulted in a better predictor of acute rejection (Heng et al. 2015). Overall, CD8 T cells and their cytotoxic molecules play an important role

in the pathology of kidney transplant patients and they could potential be strong biomarkers of allograft dysfunction.

The Immunknow assay is a bridge linking the fields of immunometabolics and biomarker research. It is a T cell immune function assay which measures the amount of adenosine triphosphate in CD4 T cells as a biomarker of the immunoreactivity in transplant recipients. However, there appears to be a lack of consensus among transplant centers who have tested the assay regarding which conditions, viral infection or acute rejection, would best benefit from this assay (De Paolis et al. 2011; Quaglia et al. 2014; Cadillo-Chávez et al. 2006; Pérez-Flores et al. 2009). This leads to the question if CD4 ATP concentration is sufficient for this assay, or if adding the concentration of ATP in CD8 cells would improve the predictive abilities of this assay. We have shown that EM and TEMRA have greater concentrations of ATP in basal conditions and that when activated all CD8 subsets increase their concentration of ATP 24 hours after stimulation (Article 4). It would be interesting to see if the ATP in CD8 cells could serve as a predictive biomarker of immunoreactivity in a similar fashion to the Immunknow assay, and if a combined assay of CD4 and CD8 T cells would eliminate the inter-center variability that is currently documented in the literature.

Cumulatively, this suggests that TEMRA CD8 T cells are important to renal allograft health and survival and are a potentially powerful biomarker of cellular-mediated rejection. Furthermore, examining the relationship between the immune response and the metabolic pathways involved could yield new biomarkers for solid organ transplantation.

4.2 *Metabolic Pathways in CD8 Cell subsets*

In the recent years, there has been a renewed interest in the immunometabolic programming of CD8 lymphocytes and due to this resurgence of interests, our understanding of the importance of metabolism in differentiation and function of CD8 T cell subsets has been greatly expanded. However, most of the research has been focused in non-pathological or oncological setting and not other immunological pathologies. The work performed in this thesis has begun to fill this gap of knowledge, by showing that in kidney transplant recipients and in multiple sclerosis patients, glycolysis is important for its effector functions. These results are in accordance to what is known in other studies (Chang et al. 2013; Cham and Gajewski 2005), suggesting that immunometablic mechanisms are highly conserved in CD8 lymphocytes and remains unchanged in different pathologies. It would be interesting to see if the involvement of the different metabolic pathways in immune function is conserved in all respects. We also observed that there are discrepancies in the basal levels of stored ATP in the different CD8 subsets. Perhaps in certain CD8 subsets or in certain patient sets, such as tolerant transplant patients, there are changes in the metabolic programming which drastically affect the immune response of CD8 T cells.

Additionally, it would also be interesting to know if the differences observed in the immunometabolic programming in the different CD8 subsets are also conserved in patients with different immune pathologies. We found that activation of CD8 T cells triggers a rapid glycolytic switch in the immunometabolic programming, resulting in a swift upregulation of glycolysis in all CD8 subsets. However, only TEMRA and EM CD 8 T cells were able to maintain high glycolytic activity an hour after stimulation while naïve cells return to basal levels of glycolysis. Furthermore, we found that TEMRA and EM CD8 T cells have higher basal levels of stored ATP compared to naïve CD8 cells, suggesting that the discrepancy in the amount of ATP is due to the more energetically demanding immune functions of TEMRA

and effector cells (Article 4). However, the reason for the upregulation of glycolysis is support the proliferation of the stimulated CD8 T cells by providing intermediates for biosynthesis (Vander Heiden, M. G., Cantley, L.C., Thompson 2009; Lunt and Vander Heiden 2011). Therefore, it is possible that the increased basal levels of ATP in TEMRA and EM cells compared to naive CD8 cells, as well as their ability to rapidly utilize and recover these stores after stimulation, maybe a strategy to have sufficient energy to carry out both the cytotoxic effector functions while simultaneously using the increased glycolytic activity to proliferate.

4.2.1 Metabolic Targets for Immunomodulating CD8 T Cells

As we learn more about the importance of immunometabolics in CD8 T cells in transplantation, it also opens the possibility of finding new targets for immunosuppression therapy.

There are two possible approaches to manipulating the metabolic programming of CD8 cells as a mean of immunosuppression for transplantation. The first would be to target metabolic pathways that are involved in cytokine production and cytotoxic activity of effector and effector memory cells, in order to shut down the ability of CD8 T cells to cause damage to allografts (Figure 2). The other method would be to target metabolic pathways to enhance and promote the proliferation of CD8 Tregs, which will in turn cause a suppressive effect on the CD8 effector population, thus prolonging allograft half life and possibly favoring tolerance induction.

4.2.1.1 Shutting down the Warburg effect in effector CD8 T cells

As previously discussed, glycolysis and glutaminolysis are two key metabolic pathways which are imperative for proper CD8 effector function. Finding methods to interrupt these

pathways in allograft infiltrating effector cells would be a mean stopping allograft rejection. 2-Deoxy-D-glucose (2-DG) is a glucose analog that inhibits glycolysis by blocking hexokinase function. Many studies showed the potent effect of 2-DG in inhibiting the cytotoxic function of effector CD8 cells (O'Sullivan and Pearce 2015; Cham and Gajewski 2005; Gubser et al. 2013; Sukumar et al. 2013). Interestingly, Cham et al. showed that 2-DG inhibited the production of IFNγ but not IL-2 in CD8 T cells, suggesting that effector cytokine production was glucose-dependent, and that the inhibitory effect of 2-DG is at the transcriptional level (Cham and Gajewski 2005; Cham et al. 2008). In a similar fashion, blocking glutaminolysis with a glutamine antagonist such as 6-diazo-5-oxo-l-norleucine (DON) inhibits lymphocyte proliferation (R. Wang et al. 2011). Additionally, blocking glucose and glutamine transporters disrupt lymphocyte activation and affect memory differentiation (Sinclair et al. 2013; Singer et al. 2011; Anastasiou et al. 2011; Macintyre et al. 2014).

Targeting major bioenergetic pathways seems at first glance a rather dangerous mean to control an immune response and major side effects such as a massive toxicity to normal tissue is expected. Animal models and ongoing therapeutic use of metabolic interferences therapies prove that such strategy is safe and feasible. For instance, *leflunomide*, a molecule from which teriflunomide is the active metabolite, prevents *de novo* biosynthesis of pyrimidine and efficiently inhibit the development of experimental autoimmune encephalomyelitis (EAE) (Korn et al. 2004). In Multiple Sclerosis patients, teriflunomide, compared to a placebo, significantly reduces relapse rates, disability progression (at the higher dose), and MRI evidence of disease activity (O'Connor et al. 2011). Interestingly, whereas a link with metabolism has not been established, Leflunomide or analogues have also been shown to be effective in prolonging graft survival and even to induce tolerance in a model of heart allograft transplantation (Le Texier et al. 2011). Metabolic interferences have been mainly

studied in the cancer field. Given the similarity of metabolic adaptation between cancer cells and activated T cells, immunometabolic regulation of CD8 T cells could be used as a mean to manipulate the CD8 T cell immune function for effective immunosuppression. However, the doses required to eradicate all malignant cells and those required to control autoreactive or alloreactive T cells might be radically different. Whereas, cancer therapy aims to eradicate all cancer cells, transplant therapy aims to control alloreactive T cells. Given their effector nature, alloreactive T cells are likely to be characterized by a higher use of glycolysis as compared to quiescent T cells. The selective targeting of glycolysis processes will thus focus preferentially alloreactive and not quiescent (naïve or memory) T cells.

4.2.1.2 Targeting transcriptional regulators of immunometabolism

Since metabolic adaptation is required to support T cells activation and function, nutrient availability or limitation will affect these processes. A recent publication has shown that adenosine monophosphate-activated protein kinase (AMPK) couples T cell function to nutrient availability (Blagih et al. 2015). AMPK is a serine-threonine kinase that is sensitive to energy levels and is activated during cellular stress. By sensing the AMP/ATP ratio, AMPK senses energy deficiency and favors pathways leading to ATP production while inhibiting ATP-consuming pathway. It increases catabolic processes and inhibits anabolic processes to increase ATP production when activated. AMPK upregulates fatty acid β oxidation by promoting the transfer of long chain fatty acids into the mitochondria via carnitine palmitoyltransferase 1 (CPT1) (MacIver, Michalek, and Rathmell 2013). Metformin, a drug commonly used in diabetes treatment, blocks mitochondrial complex I, which has the downstream effect of promoting AMPK activity. Interestingly, Metformin fosters memory CD8 T cell differentiation in mice (Pearce et al. 2009). In agreement with these results, it has been shown that autoreactive T cells can be efficiently control by the co-administration of 2-DG and Metformin in a mouse model of systemic lupus erythematosus (SLE) (Yin et al.

2015). However, as Meformin also inhibits OXPHOS, in vitro or in vivo administration is likely to have a broader target than solely the memory compartment. As Metformin is used in diabetes treatment, it has been shown to have a large range of effect as it is involve in mediating blood glucose levels and can cause rare cases of lactic acidosis (Jones, Macklin, and Alexander 2003; Squibb 2008; Eurich et al. 2007). In fact, all treatments using drugs which intervene in the metabolic pathways should be approached with caution as they will affect more than the targeted cells.

The PI3K/AKT pathway is another key pathway that integrates immune stimulation and nutrient uptake (Pollizzi and Powell 2014). Blocking PI3K/AKT pathway would therefore be another way to suppress the effector function of CD8 cells. The core kinases of this pathway are AKT, AMPK and mTOR. An intimate positive and negative cross-regulation of these protein kinases has been shown this topic has been covered in-depth (Gaber et al. 2015; MacIver, Michalek, and Rathmell 2013; Pollizzi and Powell 2014). AKT pathway is optimally activated by the co-ligation of TCR and CD28 and leads to the increase of glycolysis, via an increase of glucose uptake and the enhancement of rate-limiting glycolytic enzymes hexokinase and phosphofructokinase (Frauwirth and Thompson 2004). Preventing activation of this pathway could be accomplished by inhibiting the costimulation signaling provided by CD28 or by directly inhibiting AKT activity. FR104, an anti-CD28 antagonist antibody, has been shown to prevent lymphocyte activation and proliferation in a murine model (Mary et al. 2013). Furthermore, this drug has been shown to be effective in reducing allograft rejection in both murine and non-human primate models of transplantation (Poirier et al. 2012; Poirier et al. 2015). Another possible strategy would be to target AKT directly through the use of one of the AKT inhibitors currently in development (Alexander 2011). For example, AKT inhibitor MK-2206 treatment in a murine model increased the differentiation of naïve CD8 cells into central memory CD8 cells and diminished terminal differentiation in

the CD8 population (Abu Eid et al. 2015). While clinical research on these developmental AKT inhibitors has been mainly focused on the field of oncology, there could be possible use for them in the transplantation field.

4.2.1.3 Boosting Treg populations with metabolic programming

In the field of CD4 Tregs, there has been a lot of progress in finding viable ways to use them in clinical practices. Several clinical trials are currently in progress to evaluate the safety and efficacy of adoptive transfer of CD4 Tregs in allograft transplant recipients (Edozie et al. 2014). However, studies concerning CD8 Tregs are at more exploratory stages.

Rapamycin is an anti-fungal macrolide that is produced by bacteria discovered on Easter Island which targets and inhibits mTOR and is already used as a part of immunosuppressive regimens in transplantation. mTOR is a serine/threonine kinase that regulates cell survival, growth and energy metabolism. mTOR can form two distinct complexes, mTOR complex 1 (mTORC1), which is Rapamycin sensitive, and mTOR complex 2 (mTORC2), which is Rapamycin insensitive and much less studied compared to mTORC1 (Heitman, Movva, and Hall 1991; Brown et al. 1994; Xu et al. 2012; Laplante and Sabatini 2009; Laplante and Sabatini 2013). mTORC1 is the master regulator of cell growth and metabolism and can be activated through either PI3K/AKT signaling. Upon activation, mTORC1 promotes ribosome biogenesis and increase protein translation and synthesis (Gaber et al. 2015; Laplante and Sabatini 2013; Laplante and Sabatini 2009). mTORC1 also promotes lipid biosynthesis and regulates mitochondrial metabolism and biogenesis through sterol regulatory element binding proteins (SREBP) which are involved in positively regulating lipid homeostasis (DeBerardinis, Lum, and Thompson 2006; Porstmann et al. 2008). Rapamycin targets and inhibits mTOR activity and has been shown to induce the preferential growth of $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs *in vitro* (Battaglia et al. 2006). Evidence is emerging that

Rapamycin treatment *in vitro* can also induce $CD8^+CD28^-$Tregs in murine models (El Essawy et al. 2011), indicating that adoptive transfer strategies to induce allograft tolerance that are currently in under investigation for CD4 Tregs could potentially be adjusted for CD8 Tregs.

Another immunosuppressive drug that has been shown to be able to induce preferential expansion of CD8 Tregs *in vitro* is CTLA4-Ig Belatacept, a recently approved drug for transplant immunosuppression treatment (Barbon et al. 2014). A CTLA-4 antagonist would affect the metabolic function of effectors CD8 cells by blocking CD28 costimulation signaling, which has the downstream effects of impeding AKT activation and downregulating glucose and glutamine uptake. Additionally, Barbon et al. showed that by first alloanergizing human PBMC with Belatacept then allostimulating the cells caused an increase in the frequency of $CD8^+CD28^-$ T cells. Furthermore, repeated rounds of allorestimulation after alloanergization would continue to expand the $CD8^+CD28^-$Tregs, which were able to suppress the proliferation of CD4 T cells in a dose-dependent manner (Barbon et al. 2014). Additionally, Barbon et al. reported on 3 hematopoietic stem cell transplant recipients with a marked increase in blood circulating $CD8^+CD28^-$ T cells between days 20 to 40 post-transplantation (Barbon et al. 2014). While these findings are still preliminary, it is promising evidence indicating that CD8 Tregs could play a future role in controlling allograft rejection.

5 References

Abu Eid, Rasha, Kevin M Friedman, Mikayel Mkrtichyan, Andrea Walens, William King, John Janik, and Samir N Khleif. 2015. "Akt1 and -2 Inhibition Diminishes Terminal Differentiation and Enhances Central Memory CD8 + T-Cell Proliferation and Survival." *OncoImmunology* 4 (5): e1005448. doi:10.1080/2162402X.2015.1005448.

Afzali, B., R. I. Lechler, and M. P. Hernandez-Fuentes. 2007. "Allorecognition and the Alloresponse: Clinical Implications." *Tissue Antigens* 69 (6): 545–56. doi:10.1111/j.1399-0039.2007.00834.x.

Alexander, Walter. 2011. "Inhibiting the Akt Pathway in Cancer Treatment Three Leading Candidates Meeting Highlights : Inhibiting the Akt Pathway in Cancer Treatment." *Pharmacy & Therapeutics* 36 (4): 225–27.

Anastasiou, Dimitrios, George Poulogiannis, John M Asara, Matthew B Boxer, Jian-kang Jiang, Min Shen, Gary Bellinger, et al. 2011. "Inhibition of Pyruvate Kinase M2 by Reactive Oxygen Species Contributes to Cellular Antioxidant Responses." *Science* 334 (6060): 1278–83. doi:10.1126/science.1211485.Inhibition.

Appay, Victor, P Rod Dunbar, Margaret Callan, Paul Klenerman, Geraldine M a Gillespie, Laura Papagno, Graham S Ogg, et al. 2002. "Memory CD8+ T Cells Vary in Differentiation Phenotype in Different Persistent Virus Infections." *Nature Medicine* 8: 379–85. doi:10.1038/nm0402-379.

Appay, Victor, Rene a W Van Lier, Federica Sallusto, and Mario Roederer. 2008. "Phenotype and Function of Human T Lymphocyte Subsets: Consensus and Issues." *Cytometry Part A* 73: 975–83. doi:10.1002/cyto.a.20643.

Araki, Koichi, Alexandra P Turner, Virginia Oliva Shaffer, Shivaprakash Gangappa, Susanne A Keller, Martin F Bachmann, Christian P Larsen, and Rafi Ahmed. 2009. "mTOR Regulates Memory CD8 T-Cell Differentiation." *Nature* 460 (7251): 108–12. doi:10.1038/nature08155.

Araki, Koichi, Ben Youngblood, and Rafi Ahmed. 2010. "The Role of mTOR in Memory CD8+ T-Cell Differentiation." *Immunological Reviews, Immunological Reviews* 235 (1): 234–43. doi:10.1111/j.0105-2896.2010.00898.x, 10.1111/j.0105-2896.2010.00898.x.

Arosa, Fernando a. 2002. "CD8+CD28- T Cells: Certainties and Uncertainties of a Prevalent Human T-Cell Subset." *Immunology and Cell Biology* 80 (1): 1–13. doi:10.1046/j.1440-1711.2002.01057.x.

Auchincloss, H, R Lee, S Shea, J S Markowitz, M J Grusby, and L H Glimcher. 1993. "The Role of 'Indirect' Recognition in Initiating Rejection of Skin Grafts from Major Histocompatibility Complex Class II-Deficient Mice." *Proceedings of the National Academy of Sciences of the United States of America* 90 (8): 3373–77. doi:10.1073/pnas.90.8.3373.

Baeten, Dominique, Stéphanie Louis, Christophe Braud, Cécile Braudeau, Caroline Ballet, Frédéric Moizant, Annaik Pallier, Magali Giral, Sophie Brouard, and Jean-Paul Soulillou. 2006. "Phenotypically and Functionally Distinct CD8+ Lymphocyte

Populations in Long-Term Drug-Free Tolerance and Chronic Rejection in Human Kidney Graft Recipients." *Journal of the American Society of Nephrology : JASN* 17: 294–304. doi:10.1681/ASN.2005020178.

Ballet, C., K. Renaudin, N. Degauque, H. L. Mai, F. Boëffard, D. Lair, L. Berthelot, et al. 2009. "Indirect CD4+ TH1 Response, Antidonor Antibodies and Diffuse C4d Graft Deposits in Long-Term Recipients Conditioned by Donor Antigens Priming." *American Journal of Transplantation* 9: 697–708. doi:10.1111/j.1600-6143.2009.02556.x.

Barbon, C M, J K Davies, a Voskertchian, R H Kelner, L L Brennan, L M Nadler, and E C Guinan. 2014. "Alloanergization of Human T Cells Results in Expansion of Alloantigen-Specific CD8(+) CD28(-) Suppressor Cells." *American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 14: 305–18. doi:10.1111/ajt.12575.

Battaglia, Manuela, Angela Stabilini, Barbara Migliavacca, Jutta Horejs-Hoeck, Thomas Kaupper, and Maria-Grazia Roncarolo. 2006. "Rapamycin Promotes Expansion of Functional CD4+CD25+FOXP3+ Regulatory T Cells of Both Healthy Subjects and Type 1 Diabetic Patients." *Journal of Immunology (Baltimore, Md. : 1950)* 177 (12): 8338–47. doi:10.4049/jimmunol.177.12.8338.

Benichou, Gilles, Yohei Yamada, Seok-Hyun Yun, Charles Lin, Michael Fray, and Georges Tocco. 2011. "Immune Recognition and Rejection of Allogeneic Skin Grafts." *Immunotherapy* 3 (6): 757–70. doi:10.2217/imt.11.2.

Bental, M, and C Deutsch. 1993. "Metabolic Changes in Activated T Cells: An NMR Study of Human Peripheral Blood Lymphocytes." *Magnetic Resonance in Medicine : Official Journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine* 29 (3): 317–26. doi:10.1002/mrm.1910290307.

Berg, J M, J L Tymoczko, and L Stryer. 2002. "Biochemistry." *W H Freeman* New York. : 1. papers2://publication/uuid/7EB6183C-F1A3-4903-A72F-B1A659CECF68.

Bestard, Oriol, Peter Nickel, Josep M Cruzado, Constanze Schoenemann, Olaf Boenisch, Anett Sefrin, Josep M Grinyó, Hans-Dieter Volk, and Petra Reinke. 2008. "Circulating Alloreactive T Cells Correlate with Graft Function in Longstanding Renal Transplant Recipients." *Journal of the American Society of Nephrology : JASN* 19 (7): 1419–29. doi:10.1681/ASN.2007050539.

Betjes, Michiel G.H., Ruud W.J. Meijers, Elly a. de Wit, Willem Weimar, and Nicolle H.R. Litjens. 2012. "Terminally Differentiated CD8+ Temra Cells Are Associated With the Risk for Acute Kidney Allograft Rejection." *Transplantation Journal* 94 (1): 63–69. doi:10.1097/TP.0b013e31825306ff.

Bhowmik, D M, A K Dinda, P Mahanta, and S K Agarwal. 2010. "The Evolution of the Banff Classification Schema for Diagnosing Renal Allograft Rejection and Its Implications for Clinicians." *Indian Journal of Nephrology* 20 (1): 2–8.

Billingham, R. E., L Brent, and P. B. Medawar. 1953. "'Actively Acquired Tolerance' of Foreign Cells." *Nature* 172: 603–6. doi:10.1038/172603a0.

Billingham, R. E., and P. B. Medawar. 1951. “The Technique of Free Skin Grafting in Mammals.” *J. Exp. Biol.* 28 (3): 385–402. http://jeb.biologists.org/content/28/3/385.

Blagih, Julianna, François Coulombe, Emma E. Vincent, Fanny Dupuy, Gabriela Galicia-Vázquez, Ekaterina Yurchenko, Thomas C. Raissi, et al. 2015. “The Energy Sensor AMPK Regulates T Cell Metabolic Adaptation and Effector Responses In Vivo.” *Immunity* 42 (1): 41–54. doi:10.1016/j.immuni.2014.12.030.

Blancho, G, R Josien, D Douillard, J D Bignon, A Cesbron, and J P Soulillou. 1992. “The Influence of HLA A-B-DR Matching on Cytomegalovirus Disease after Renal Transplantation. Evidence That HLA-DR7-Matched Recipients Are More Susceptible to Cytomegalovirus Disease.” *Transplantation* 54 (5): 871–74.

Böhmig, Georg a., Andreas M. Farkas, Farsad Eskandary, and Thomas Wekerle. 2015. “Strategies to Overcome the ABO Barrier in Kidney Transplantation.” *Nature Reviews Nephrology*. Nature Publishing Group, 1–16. doi:10.1038/nrneph.2015.144.

Brachemi, Soumeya. 2014. “Renal Biopsy Practice: What Is the Gold Standard?” *World Journal of Nephrology* 3 (4): 287. doi:10.5527/wjn.v3.i4.287.

Brenchley, Jason M, Nitin J Karandikar, Michael R Betts, David R Ambrozak, Brenna J Hill, Laura E Crotty, Joseph P Casazza, et al. 2003. “Expression of CD57 Defines Replicative Senescence and Antigen-Induced Apoptotic Death of CD8+ T Cells.” *Blood* 101 (7): 2711–20. doi:10.1182/blood-2002-07-2103.Supported.

Brouard, Sophie, Alexandre Dupont, Magali Giral, Stéphanie Louis, David Lair, Cécile Braudeau, Nicolas Degauque, et al. 2005. “Operationally Tolerant and Minimally Immunosuppressed Kidney Recipients Display Strongly Altered Blood T-Cell Clonal Regulation.” *American Journal of Transplantation* 5 (2): 330–40. doi:10.1111/j.1600-6143.2004.00700.x.

Brouard, Sophie, Elaine Mansfield, Christophe Braud, Li Li, Magali Giral, Szu-chuan Hsieh, Dominique Baeten, et al. 2007. “Identification of a Peripheral Blood Transcriptional Biomarker Panel Associated with Operational Renal Allograft Tolerance.” *Proceedings of the National Academy of Sciences of the United States of America* 104 (39): 15448–53. doi:10.1073/pnas.0705834104.

Brown, E J, M W Albers, T B Shin, K Ichikawa, C T Keith, W S Lane, and S L Schreiber. 1994. “A Mammalian Protein Targeted by G1-Arresting Rapamycin-Receptor Complex.” *Nature* 369 (6483): 756–58. doi:10.1038/369756a0.

Bueno, V, and J.O.M Pestana. 2002. “The Role of CD8 + T Cells during Allograft Rejection” 35: 1247–58.

Burra, Patrizia, and Manuela De Bona. 2007. “Quality of Life Following Organ Transplantation.” *Transplant International* 20 (5): 397–409. doi:10.1111/j.1432-2277.2006.00440.x.

Burrows, Scott R., Rajiv Khanna, Sharon L. Silins, and Denis J. Moss. 1999. "The Influence of Antiviral T-Cell Responses on the Alloreactive Repertoire." *Immunology Today* 20 (5): 203–7. doi:10.1016/S0167-5699(98)01429-7.

Cadillo-Chávez, R., Sally de Echegaray, E. a. Santiago-Delpín, a. T. Rodríguez-Trinidad, B. Camacho-Carrazo, T. Alfaro, M. Saavedra-Pozo, L. Carrasquillo, Z. a. González-Caraballo, and L. a. Morales-Otero. 2006. "Assessing the Risk of Infection and Rejection in Hispanic Renal Transplant Recipients by Means of an Adenosine Triphosphate Release Assay." *Transplantation Proceedings* 38 (3): 918–20. doi:10.1016/j.transproceed.2006.02.051.

Carr, Erikka L, Alina Kelman, Glendon S Wu, Ravindra Gopaul, Emilee Senkevitch, Anahit Aghvanyan, Achmed M Turay, and Kenneth a Frauwirth. 2010. "Glutamine Uptake and Metabolism Are Coordinately Regulated by ERK/MAPK during T Lymphocyte Activation." *Journal of Immunology (Baltimore, Md. : 1950)* 185 (2): 1037–44. doi:10.4049/jimmunol.0903586.

Cham, Candace M, and Thomas F Gajewski. 2005. "Glucose Availability Regulates IFN-Gamma Production and p70S6 Kinase Activation in CD8+ Effector T Cells." *Journal of Immunology (Baltimore, Md. : 1950)* 174 (8): 4670–77. doi:10.4049/jimmunol.174.8.4670.

Cham, Candace M., Gregory Driessens, James P. O'Keefe, and Thomas F. Gajewski. 2008. "Glucose Deprivation Inhibits Multiple Key Gene Expression Events and Effector Functions in CD8+ T Cells." *European Journal of Immunology* 38 (9): 2438–50. doi:10.1002/eji.200838289.

Chang, Chih Hao, Jonathan D. Curtis, Leonard B. Maggi, Brandon Faubert, Alejandro V. Villarino, David O'Sullivan, Stanley Ching Cheng Huang, et al. 2013. "Posttranscriptional Control of T Cell Effector Function by Aerobic Glycolysis." *Cell* 153 (6). Elsevier Inc.: 1239–51. doi:10.1016/j.cell.2013.05.016.

Chatterjee, Priti, Sandeep R. Mathur, Amit K. Dinda, Sandeep Guleria, Sandeep Mahajan, V. K. Iyer, and V. K. Arora. 2012. "Analysis of Urine Sediment for Cytology and Antigen Expression in Acute Renal Allograft Rejection: An Alternative to Renal Biopsy." *American Journal of Clinical Pathology* 137 (5): 816–24. doi:10.1309/AJCPQFZ0GELH5ZPN.

Chen, Xiaozhuo, Yanrong Qian, and Shiyong Wu. 2015. "The Warburg Effect: Evolving Interpretations of an Established Concept." *Free Radical Biology and Medicine* 79. Elsevier: 253–63. doi:10.1016/j.freeradbiomed.2014.08.027.

Chiu, Wai Kan, Monchou Fann, and Nan-ping Weng. 2006. "Generation and Growth of CD28nullCD8+ Memory T Cells Mediated by IL-15 and Its Induced Cytokines." *Journal of Immunology (Baltimore, Md. : 1950)* 177 (11): 7802–10. doi:177/11/7802 [pii].

Chong, Lee K., Rebecca J. Aicheler, Sian Llewellyn-Lacey, Peter Tomasec, Paul Brennan, and Eddie C Y Wang. 2008. "Proliferation and Interleukin 5 Production by

CD8hiCD57+ T Cells." *European Journal of Immunology* 38 (4): 995–1000. doi:10.1002/eji.200737687.

Ciubotariu, Rodica, Rodica Vasilescu, Eric Ho, Paola Cinti, Corrado Cancedda, Luca Poli, Maurizio Late, et al. 2001. "Detection of T Suppressor Cells in Patients with Organ Allografts." *Human Immunology* 62 (00): 15–20. doi:10.1016/S0198-8859(00)00226-3.

Colvin, Robert B, and R Neal Smith. 2005. "Antibody-Mediated Organ-Allograft Rejection." *Nature Reviews. Immunology* 5 (10): 807–17. doi:10.1038/nri1702.

Corey, H E, F Alfonso, D Hamele-Bena, S M Greenstein, R Schechner, V Tellis, P Geva, and L G Koss. 1997. "Urine Cytology and the Diagnosis of Renal Allograft Rejection. I. Studies Using Conventional Staining." *Acta Cytol* 41 (6): 1732–41.

Cornell, Lynn D, R Neal Smith, and Robert B Colvin. 2008. "Kidney Transplantation: Mechanisms of Rejection and Acceptance." *Annual Review of Pathology* 3: 189–220. doi:10.1146/annurev.pathol.3.121806.151508.

Crawley, Angela M, Agatha Vranjkovic, Elliott Faller, Michaeline McGuinty, Aurelia Busca, Stephanie C Burke, Sophie Cousineau, Ashok Kumar, Paul a Macpherson, and Jonathan B Angel. 2014. "Jak/STAT and PI3K Signaling Pathways Have Both Common and Distinct Roles in IL-7-Mediated Activities in Human CD8+ T Cells." *Journal of Leukocyte Biology* 95 (1): 117–27. doi:10.1189/jlb.0313122.

D'Orsogna, L. J a, D. L. Roelen, I. I N Doxiadis, and F. H J Claas. 2010. "Alloreactivity from Human Viral Specific Memory T-Cells." *Transplant Immunology* 23 (4). Elsevier B.V.: 149–55. doi:10.1016/j.trim.2010.06.008.

Dalchau, R, J Fangmann, and J W Fabre. 1992. "Allorecognition of Isolated, Denatured Chains of Class I and Class II Major Histocompatibility Complex Molecules. Evidence for an Important Role for Indirect Allorecognition in Transplantation." *European Journal of Immunology* 22 (3): 669–77. doi:10.1002/eji.1830220309.

De Paolis, P., a. Favarò, a. Piola, F. Martini, G. Cristiana, C. Agrati, M. Iappelli, and S. Di Giulio. 2011. "'Immuknow' to Measurement of Cell-Mediated Immunity in Renal Transplant Recipients Undergoing Short-Term Evaluation." *Transplantation Proceedings* 43 (4). Elsevier Inc.: 1013–16. doi:10.1016/j.transproceed.2011.02.043.

DeBerardinis, Ralph J., Julian J. Lum, and Craig B. Thompson. 2006. "Phosphatidylinositol 3-Kinase-Dependent Modulation of Carnitine Palmitoyltransferase 1A Expression Regulates Lipid Metabolism during Hematopoietic Cell Growth." *Journal of Biological Chemistry* 281 (49): 37372–80. doi:10.1074/jbc.M608372200.

Department of Health and Human Services, Health Resources and Services Administration. 2014. *Organ Procurement and Transplantation Network (OPTN) and Scientific Registry of Transplant Recipients (SRTR). OPTN/SRTR 2012 Annual Data Report. American Journal of Transplantation*. Vol. 14.

Devarajan, Prasad. 2011. "Neutrophil Gelatinase-Associated Lipocalin: A Promising Biomarker for Human Acute Kidney Injury." *Biomarkers in Medicine* 4 (2): 265–80. doi:10.2217/bmm.10.12.Neutrophil.

Dharnidharka, Vikas R. 2005. "Renal Transplantation: The Present and the Future." *Indian Journal of Pediatrics* 72 (9): 785–88.

Dhaun, Neeraj, Christopher O Bellamy, Daniel C Cattran, and David C Kluth. 2014. "Utility of Renal Biopsy in the Clinical Management of Renal Disease." *Kidney International* 85 (5). Nature Publishing Group: 1039–48. doi:10.1038/ki.2013.512.

Diamond, Andrew S., and Ronald G. Gill. 2000. "An Essential Contribution by IFN-Gamma to CD8+ T Cell-Mediated Rejection of Pancreatic Islet Allografts." *Journal of Immunology (Baltimore, Md. : 1950)* 165 (1): 247–55. doi:10.4049/jimmunol.165.1.247.

Dolfi, Douglas V, Kathleen D Mansfield, Antonio M Polley, Susan a Doyle, Gordon J Freeman, Hanspeter Pircher, Kenneth E Schmader, and E John Wherry. 2013. "Increased T-Bet Is Associated with Senescence of Influenza Virus-Specific CD8 T Cells in Aged Humans." *Journal of Leukocyte Biology* 93 (June): 825–36. doi:10.1189/jlb.0912438.

Edozie, Francis C, Estefania a Nova-Lamperti, Giovanni a M Povoleri, Cristiano Scottà, Susan John, Giovanna Lombardi, and Behdad Afzali. 2014. "Regulatory T-Cell Therapy in the Induction of Transplant Tolerance: The Issue of Subpopulations." *Transplantation* 00 (00): 1–10. doi:10.1097/TP.0000000000000243.

El Essawy, Basset, Prabhakar Putheti, Wenda Gao, and Terry B. Strom. 2011. "Rapamycin Generates Graft-Homing Murine Suppressor CD8+ T Cells That Confer Donor-Specific Graft Protection." *Cell Transplantation* 20 (0): 1759–69. doi:10.1016/j.biotechadv.2011.08.021.Secreted.

Eurich, Dean T, Finlay a McAlister, David F Blackburn, Sumit R Majumdar, Ross T Tsuyuki, Janice Varney, and Jeffrey a Johnson. 2007. "Benefits and Harms of Antidiabetic Agents in Patients with Diabetes and Heart Failure: Systematic Review." *BMJ (Clinical Research Ed.)* 335 (7618): 497. doi:10.1136/bmj.39314.620174.80.

Fehr, Thomas, and Clemens D Cohen. 2011. "Predicting an Allograft's Fate." *Kidney International* 80 (12): 1254–55. doi:10.1038/ki.2011.328.

Fluck, N., O. Witzke, P. J. Morris, and K. J. Wood. 1999. "Indirect Allorecognition Is Involved in Both Acute and Chronic Allograft Rejection." *Transplantation Proceedings* 31 (1-2): 842–43. doi:10.1016/S0041-1345(98)01797-7.

Foucher, Yohann, Pascal Daguin, Ahmed Akl, Michèle Kessler, Marc Ladrière, Christophe Legendre, Henri Kreis, et al. 2010. "A Clinical Scoring System Highly Predictive of Long-Term Kidney Graft Survival." *Kidney International* 78: 1288–94. doi:10.1038/ki.2010.232.

Foucher, Yohann, Aurélie Meurette, Pascal Daguin, Angélique Bonnaud-Antignac, Jean-Benoît Hardouin, Sabrina Chailan, Karine Neau, et al. 2015. "A Personalized Follow-up of Kidney Transplant Recipients Using Video Conferencing Based on a 1-Year Scoring

System Predictive of Long Term Graft Failure (TELEGRAFT Study): Protocol for a Randomized Controlled Trial." *BMC Nephrology* 16 (1): 6. doi:10.1186/1471-2369-16-6.

Frauwirth, Kenneth a, and Craig B Thompson. 2004. "Regulation of T Lymphocyte Metabolism." *Journal of Immunology (Baltimore, Md. : 1950)* 172 (8): 4661–65. doi:10.4049/jimmunol.172.8.4661.

Frauwirth, Kenneth a., James L. Riley, Marian H. Harris, Richard V. Parry, Jeffrey C. Rathmell, David R. Plas, Rebecca L. Elstrom, Carl H. June, and Craig B. Thompson. 2002. "The CD28 Signaling Pathway Regulates Glucose Metabolism." *Immunity* 16 (6): 769–77. doi:10.1016/S1074-7613(02)00323-0.

Furness, Peter N, Nicholas Taub, Karel J M Assmann, Giovanni Banfi, Jean-Pierre Cosyns, Anthony M Dorman, Claire M Hill, et al. 2003. "International Variation in Histologic Grading Is Large, and Persistent Feedback Does Not Improve Reproducibility." *The American Journal of Surgical Pathology* 27 (6): 805–10. doi:10.1097/00000478-200402000-00019.

Gaber, Timo, Cindy Strehl, Birgit Sawitzki, Paula Hoff, and Frank Buttgereit. 2015. "Cellular Energy Metabolism in T-Lymphocytes." *International Reviews of Immunology*, no. August: 1–16. doi:10.3109/08830185.2014.956358.

Geissler, Edward K. 2012. "The ONE Study Compares Cell Therapy Products in Organ Transplantation: Introduction to a Review Series on Suppressive Monocyte-Derived Cells." *Transplantation Research* 1 (1): 10. doi:10.1186/2047-1440-1-10.

Gibson, T., and P. B. Medawar. 1943. "The Fate of Skin Homografts in Man." *Journal of Anatomy* 77 (Pt 4): 299–310.4.

Gubser, Patrick M, Glenn R Bantug, Leyla Razik, Marco Fischer, Sarah Dimeloe, Gideon Hoenger, Bojana Durovic, Annaïse Jauch, and Christoph Hess. 2013. "Rapid Effector Function of Memory CD8+ T Cells Requires an Immediate-Early Glycolytic Switch." *Nature Immunology* 14 (10): 1064–72. doi:10.1038/ni.2687.

Guh, Jinn Yuh. 2010. "Proteinuria versus Albuminuria in Chronic Kidney Disease." *Nephrology* 15 (SUPPL. 2): 53–56. doi:10.1111/j.1440-1797.2010.01314.x.

Guppy, M, E Greiner, and K Brand. 1993. "The Role of the Crabtree Effect and an Endogenous Fuel in the Energy Metabolism of Resting and Proliferating Thymocytes." *European Journal of Biochemistry / FEBS* 212 (1): 95–99. doi:10.1111/j.1432-1033.1993.tb17637.x.

Haase, Michael, Rinaldo Bellomo, Prasad Devarajan, Peter Schlattmann, Anja Haase-Fielitz, Sean M. Bagshaw, Richard Bogle, et al. 2009. "Accuracy of Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Diagnosis and Prognosis in Acute Kidney Injury: A Systematic Review and Meta-Analysis." *American Journal of Kidney Diseases* 54 (6). Elsevier Inc.: 1012–24. doi:10.1053/j.ajkd.2009.07.020.

Halloran, P F, J Schlaut, K Solez, and N S Srinivasa. 1992. "The Significance of the Anti-Class I Response. II. Clinical and Pathologic Features of Renal Transplants with Anti-Class I-like Antibody." *Transplantation* 53 (3): 550–55.

Halloran, P F, A Wadgymar, S Ritchie, J Falk, K Solez, and N S Srinivasa. 1990. The significance of the anti-class I antibody response. I. Clinical and pathologic features of anti-class I-mediated rejection., 49 Transplantation 85–91.

Halloran, P. F., a. B. Pereira, J. Chang, a. Matas, M. Picton, D. De Freitas, J. Bromberg, et al. 2013. "Microarray Diagnosis of Antibody-Mediated Rejection in Kidney Transplant Biopsies: An International Prospective Study (INTERCOM)." *American Journal of Transplantation* 13 (11): 2865–74. doi:10.1111/ajt.12465.

Halloran, Philip F. 2004. "Immunosuppressive Drugs for Kidney Transplantation." *The New England Journal of Medicine* 351: 2715–30.

Hamann, D, P a Baars, M H Rep, B Hooibrink, S R Kerkhof-Garde, M R Klein, and R a van Lier. 1997. "Phenotypic and Functional Separation of Memory and Effector Human CD8+ T Cells." *The Journal of Experimental Medicine* 186 (9): 1407–18. doi:10.1084/jem.186.9.1407.

Hamann, Dörte, Marijke Th.L Roos, and René a.W van Lier. 1999. "Faces and Phases of Human CD8+ T-Cell Development." *Immunology Today* 20 (4): 177–80. doi:10.1016/S0167-5699(99)01444-9.

Hara, Shigeo. 2015. "Banff 2013 Update: Pearls and Pitfalls in Transplant Renal Pathology." *Nephrology* 20: 2–8. doi:10.1111/nep.12474.

Hartono, Choli, Thangamani Muthukumar, and Manikkam Suthanthiran. 2010. "Noninvasive Diagnosis of Acute Rejection of Renal Allografts." *Current Opinion in Organ Tranplantation* 15 (1): 35–41. doi:10.1097/MOT.0b013e3283342728.Noninvasive.

Heitman, Joseph, N. Rao Movva, and Michael N. Hall. 1991. "Targets for Cell Cycle Arrest by the Immunosuppressant Rapamycin in Yeast." *Science (New York, N.Y.)* 253 (5022): 905–9. doi:10.1126/science.1715094.

Heng, Baoli, Yunfei Li, Liping Shi, Xinghua Du, Caiyong Lai, Long Cheng, and Zexuan Su. 2015. "A Meta-Analysis of the Significance of Granzyme B and Perforin in Noninvasive Diagnosis of Acute Rejection After Kidney Transplantation." *Transplantation* 99 (7): 1477–86. doi:10.1097/TP.0000000000000567.

Henson, Sian M, Alessio Lanna, Natalie E Riddell, Ornella Franzese, Richard Macaulay, Stephen J Griffiths, Daniel J Puleston, et al. 2014. "p38 Signaling Inhibits mTORC1-Independent Autophagy in Senescent Human CD8+ T Cells." *The Journal of Clinical Investigation*, no. 16: 1–13. doi:10.1172/JCI75051.

Herrera, Osquel Barroso, Dela Golshayan, Rebecca Tibbott, Francisco Salcido Ochoa, Martha J James, Federica M Marelli-Berg, and Robert I Lechler. 2004. "A Novel Pathway of Alloantigen Presentation by Dendritic Cells." *Journal of Immunology (Baltimore, Md. : 1950)* 173 (8): 4828–37. doi:10.4049/jimmunol.173.8.4828.

Hersperger, Adam R, George Makedonas, and Michael R Betts. 2008. "Flow Cytometric Detection of Perforin Upregulation in Human CD8 T Cells." *Cytometry. Part A : The Journal of the International Society for Analytical Cytology* 73 (11): 1050–57. doi:10.1002/cyto.a.20596.

Hollmen, Maria E, Lauri E Kyllönen, Kaija a Inkinen, Martti L T Lalla, and Kaija T Salmela. 2011. "Urine Neutrophil Gelatinase-Associated Lipocalin Is a Marker of Graft Recovery after Kidney Transplantation." *Kidney International* 79 (1): 89–98. doi:10.1038/ki.2010.351.

Issa, Fadi, Alexandru Schiopu, and Kathryn J Wood. 2010. "Role of T Cells in Graft Rejection and Transplantation Tolerance." *Expert Review of Clinical Immunology* 6 (1): 155–69.

Jacobs, Sarah R, Catherine E Herman, Nancie J Maciver, Jessica a Wofford, Heather L Wieman, Jeremy J Hammen, and Jeffrey C Rathmell. 2008. "Glucose Uptake Is Limiting in T Cell Activation and Requires CD28-Mediated Akt-Dependent and Independent Pathways." *Journal of Immunology (Baltimore, Md. : 1950)* 180 (7): 4476–86. doi:180/7/4476.

Jaye, David L, Robert a Bray, Howard M Gebel, Wayne a C Harris, and Edmund K Waller. 2012. "Translational Applications of Flow Cytometry in Clinical Practice." *Journal of Immunology (Baltimore, Md. : 1950)* 188 (10): 4715–19. doi:10.4049/jimmunol.1290017.

Jiang, Shuiping, Osquel Herrera, and Robert I. Lechler. 2004. "New Spectrum of Allorecognition Pathways: Implications for Graft Rejection and Transplantation Tolerance." *Current Opinion in Immunology* 16 (5): 550–57. doi:10.1016/j.coi.2004.07.011.

Jones, G C, J P Macklin, and W D Alexander. 2003. "Contraindications to the Use of Metformin." *BMJ (Clinical Research Ed.)* 326 (7379): 4–5. doi:10.1136/bmj.326.7392.762/a.

Kaech, Susan M., and Weiguo Cui. 2012. "Transcriptional Control of Effector and Memory CD8+ T Cell Differentiation." *Nature Reviews Immunology* 12 (11). Nature Publishing Group: 749–61. doi:10.1038/nri3307.

Kaneko, H, K Saito, H Hashimoto, H Yagita, K Okumura, and M Azuma. 1996. "Preferential Elimination of CD28+ T Cells in Systemic Lupus Erythematosus (SLE) and the Relation with Activation-Induced Apoptosis." *Clinical and Experimental Immunology* 106 (2): 218–29. doi:10.1046/j.1365-2249.1996.d01-849.x.

Kato, Masako, Kunio Morozumi, Oki Takeuchi, Tadashi Oikawa, Katsushi Koyama, Takeshi Usami, Yasunobu Shimano, et al. 2003. "Complement Fragment C4d Deposition in Peritubular Capillaries in Acute Humoral Rejection after ABO Blood Group-Incompatible Human Kidney Transplantation." *Transplantation* 75 (5): 663–65. doi:10.1097/01.TP.0000053402.87256.6B.

Kennedy, Eugene P, and Albert L. Lehninger. 1949. "Oxidation of Fatty Acids and Tricarboxylic Acid Cycle Intermediates by Ioslated Rat Liver Mitochondria." *Journal of Biological Chemistry* 179: 957–72.

Khatri, Purvesh, Silke Roedder, Naoyuki Kimura, Katrien De Vusser, Alexander a Morgan, Yongquan Gong, Michael P Fischbein, et al. 2013. "A Common Rejection Module (CRM) for Acute Rejection across Multiple Organs Identifies Novel Therapeutics for Organ Transplantation." *The Journal of Experimental Medicine* 210 (11): 2205–21. doi:10.1084/jem.20122709.

Kober, Johanna, Judith Leitner, Christoph Klauser, Ramona Woitek, Otto Majdic, Johannes Stöckl, Dietmar Herndler-Brandstetter, et al. 2008. "The Capacity of the TNF Family Members 4-1BBL, OX40L, CD70, GITRL, CD30L and LIGHT to Costimulate Human T Cells." *European Journal of Immunology* 38 (10): 2678–88. doi:10.1002/eji.200838250.

Korn, Thomas, Tim Magnus, Klaus Toyka, and Stefan Jung. 2004. "Modulation of Effector Cell Functions in Experimental Autoimmune Encephalomyelitis by Leflunomide--Mechanisms Independent of Pyrimidine Depletion." *Journal of Leukocyte Biology* 76 (5): 950–60. doi:10.1189/jlb.0504308.

Kowalski, Richard J, Diane R Post, Roslyn B Mannon, Anthony Sebastian, Harlan I Wright, Gary Sigle, James Burdick, et al. 2006. "Assessing Relative Risks of Infection and Rejection: A Meta-Analysis Using an Immune Function Assay." *Transplantation* 82 (5): 663–68. doi:10.1097/01.tp.0000234837.02126.70.

Koyama, I., O. Nadazdin, S. Boskovic, T. Ochiai, R. N. Smith, M. Sykes, H. Sogawa, et al. 2007. "Depletion of CD8 Memory T Cells for Induction of Tolerance of a Previously Transplanted Kidney Allograft." *American Journal of Transplantation* 7: 1055–61. doi:10.1111/j.1600-6143.2006.01703.x.

Kraat, Y J, M H Christiaans, F H Nieman, P M van den Berg-Loonen, J P van Hooff, and C A Bruggeman. 1994. "Risk Factors for Cytomegalovirus Infection and Disease in Renal Transplant Recipients: HLA-DR7 and Triple Therapy." *Transplant International : Official Journal of the European Society for Organ Transplantation* 7 (5): 362–67. doi:10.1007/BF00336713.

Krauss, Stefan, Beth Israel, and Deaconess Medical. 2001. "Mitochondria : Structure and Role in Respiration." *Life Sciences*, 1–6. doi:10.1038/npg.els.0001380.

Laplante, Mathieu, and David M Sabatini. 2013. "Regulation of mTORC1 and Its Impact on Gene Expression at a Glance." *Journal of Cell Science* 126 (Pt 8): 1713–19. doi:10.1242/jcs.125773.

Laplante, Mathieu, and David M. Sabatini. 2009. "mTOR Signaling at a Glance." *Journal of Cell Science* 122 (Pt 20): 3589–94. doi:10.1242/jcs.051011.

Larsen, B Y Christian P, Peter J Morris, and Jonathan M Austyn. 1990. "Migration of Dendritic Leukocytes From Cardiac Allografts Into Host Spleens: A Novel Pathway for Initiation of Rejection." *The Journal of Experimental Medicine* 171 (January): 307–14.

Le Texier, L., P. Thebault, a. Lavault, C. Usal, E. Merieau, T. Quillard, B. Charreau, et al. 2011. "Long-Term Allograft Tolerance Is Characterized by the Accumulation of B Cells Exhibiting an Inhibited Profile." *American Journal of Transplantation* 11 (3): 429–38. doi:10.1111/j.1600-6143.2010.03336.x.

Lechler, B Y R I, and Ji R Batchelor. 1982. "Restoration of Immunogenicity To Passenger Cell-Depleted Kidney Allografts By the Addition of Donor Strain Dendritic Cells." *The Journal of Experimental Medicine* 155 (January): 31–41.

Lee, John R., Thangamani Muthukumar, Darshana Dadhania, Ruchuang Ding, Vijay K. Sharma, Joseph E. Schwartz, and Manikkam Suthanthiran. 2014. "Urinary Cell mRNA Profiles Predictive of Human Kidney Allograft Status." *Immunological Reviews* 258 (1): 218–40. doi:10.1111/imr.12159.

Li, Baogui, Choli Hartono, Ruchuang Ding, Vijay K Sharma, Ravi Ramaswamy, Biao Qian, David Serur, Janet Mouradian, Joseph E Schwartz, and Manikkam Suthanthiran. 2001. "Noninvasive Diagnosis of Renal-Allograft Rejection by Measurement of Messenger RNA for Perforin and Granzyme B in Urine." *The New England Journal of Medicine* 344 (13): 947–54.

Li, Xian Liang, Séverine Ménoret, Séverine Bezie, Lise Caron, Dominique Chabannes, Marcelo Hill, Franck Halary, et al. 2010. "Mechanism and Localization of CD8 Regulatory T Cells in a Heart Transplant Model of Tolerance." *Journal of Immunology (Baltimore, Md. : 1950)* 185 (2): 823–33. doi:10.4049/jimmunol.1000120.

Ligocki, Ann J., and Jerry Y. Niederkorn. 2015. "Advances on Non-CD4 + Foxp3+ T Regulatory Cells." *Transplantation* 99 (8): 1. doi:10.1097/TP.0000000000000813.

Lin, Y. X., L. N. Yan, B. Li, L. L. Wang, T. F. Wen, Y. Zeng, W. T. Wang, et al. 2009. "A Significant Expansion of CD8+ CD28- T-Suppressor Cells in Adult-to-Adult Living Donor Liver Transplant Recipients." *Transplantation Proceedings* 41 (10). Elsevier Inc.: 4229–31. doi:10.1016/j.transproceed.2009.09.072.

Liu, Y., N. Chen, G. Chen, and P. You. 2007. "The Protective Effect of CD8+CD28- T Suppressor Cells On The Acute Rejection Responses in Rat Liver Transplantation." *Transplantation Proceedings* 39 (10): 3396–3403. doi:10.1016/j.transproceed.2007.06.089.

Lochner, Matthias, Luciana Berod, and Tim Sparwasser. 2015. "Fatty Acid Metabolism in the Regulation of T Cell Function." *Trends in Immunology*. Elsevier Ltd, 1–11. doi:10.1016/j.it.2014.12.005.

Loupy, Alexandre, Carmen Lefaucheur, Dewi Vernerey, Jessica Chang, Luis G Hidalgo, Thibaut Beuscart, Jerome Verine, et al. 2014. "Molecular Microscope Strategy to Improve Risk Stratification in Early Antibody-Mediated Kidney Allograft Rejection." *Journal of the American Society of Nephrology : JASN*, 1–11. doi:10.1681/ASN.2013111149.

Loupy, Alexandre, Carmen Lefaucheur, Dewi Vernerey, Christof Prugger, Jean-Paul Duong van Huyen, Nuala Mooney, Caroline Suberbielle, et al. 2013. "Complement-Binding

Anti-HLA Antibodies and Kidney-Allograft Survival." *The New England Journal of Medicine* 369 (13): 1215–26. doi:10.1056/NEJMoa1302506.

Lunt, Sophia Y., and Matthew G. Vander Heiden. 2011. "Aerobic Glycolysis: Meeting the Metabolic Requirements of Cell Proliferation." *Annual Review of Cell and Developmental Biology* 27 (1): 441–64. doi:10.1146/annurev-cellbio-092910-154237.

Macintyre, Andrew N, Valerie A Gerriets, Amanda G Nichols, Ryan D Michalek, Michael C Rudolph, Divino Deoliveira, Steven M Anderson, et al. 2014. "The Glucose Transporter Glut1 Is Selectively Essential for CD4 T Cell Activation and Effector Function." *Cell Metabolism* 20 (1): 61–72. doi:10.1016/j.cmet.2014.05.004.

MacIver, Nancie J, Ryan D Michalek, and Jeffrey C Rathmell. 2013. "Metabolic Regulation of T Lymphocytes." *Annual Review of Immunology* 31: 259–83. doi:10.1146/annurev-immunol-032712-095956.

Mamatov, E., a.F. Kocaay, M.a. Koc, Z.K. Celebi, S. Sengül, K. Keven, H. Tutkak, and a. Tuzuner. 2015. "Predictive Role of Neutrophil Gelatinase–Associated Lipocaline in Donor-Specific Antibody–Positive and Donor-Specific Antibody–Negative Renal Transplant Patients." *Transplantation Proceedings* 47 (6). Elsevier Inc.: 1688–91. doi:10.1016/j.transproceed.2015.06.010.

Mary, Caroline, Flora Coulon, Nicolas Poirier, Nahzli Dilek, Bernard Martinet, Gilles Blancho, and Bernard Vanhove. 2013. "Antagonist Properties of Monoclonal Antibodies Targeting Human CD28." *mAbs* 5 (1): 47–55. doi:10.4161/mabs.22697.

Mauiyyedi, S, P D Pelle, S Saidman, a B Collins, M Pascual, N E Tolkoff-Rubin, W W Williams, a a Cosimi, E E Schneeberger, and R B Colvin. 2001. "Chronic Humoral Rejection: Identification of Antibody-Mediated Chronic Renal Allograft Rejection by C4d Deposits in Peritubular Capillaries." *Journal of the American Society of Nephrology : JASN* 12 (3): 574–82.

Mehra, Narinder K. 2001. "Histocompatibility Antigens." *Encyclopedia of Life Sciences*, 1–6.

Mehra, Narinder K., Jamshaid Siddiqui, Ajay Baranwal, Sanjeev Goswami, and Gurvinder Kaur. 2013. "Clinical Relevance of Antibody Development in Renal Transplantation." *Annals of the New York Academy of Sciences* 1283: 30–42. doi:10.1111/nyas.12034.

Mengel, M., B. Sis, and P. F. Halloran. 2007. "SWOT Analysis of Banff: Strengths, Weaknesses, Opportunities and Threats of the International Banff Consensus Process and Classification System for Renal Allograft Pathology." *American Journal of Transplantation* 7 (10): 2221–26. doi:10.1111/j.1600-6143.2007.01924.x.

Merrill, J P, J E Murray, J H Harrison, and W R Guild. 1956. "Successful Homotransplantation of the Human Kidney between Identical Twins." *Journal of the American Medical Association* 160 (4): 277–82. doi:10.1016/S0022-5347(02)80268-3.

Miqueu, Patrick, Nicolas Degauque, Marina Guillet, Magali Giral, Catherine Ruiz, Annaïck Pallier, Cécile Braudeau, et al. 2010. "Analysis of the Peripheral T-Cell Repertoire in

Kidney Transplant Patients." *European Journal of Immunology* 40: 3280–90. doi:10.1002/eji.201040301.

Monteiro, J, F Batliwalla, H Ostrer, and P K Gregersen. 1996. "Shortened Telomeres in Clonally Expanded CD28-CD8+ T Cells Imply a Replicative History That Is Distinct from Their CD28+CD8+ Counterparts." *Journal of Immunology (Baltimore, Md. : 1950)* 156 (10): 3587–90. doi:IS0219.

Moon, H. H., T. S. Kim, Y. N. Roh, S. Lee, S. Song, M. Shin, J. M. Kim, et al. 2012. "Can Immune Function Assay Predict Infection or Recovery?" *Transplantation Proceedings* 44 (4). Elsevier Inc.: 1048–51. doi:10.1016/j.transproceed.2012.04.001.

Morón, Gabriel, Gilles Dadaglio, and Claude Leclerc. 2004. "New Tools for Antigen Delivery to the MHC Class I Pathway." *Trends in Immunology* 25 (2): 92–97. doi:10.1016/j.it.2003.11.008.

Morris, Peter J. 2004. "Transplantation- A Medical Miracle of the 20th Century." *The New England Journal of Medicine* 351 (26): 2678–80. doi:10.1056/NEJMp048256.

Murphy, Kenneth, Paul Travers, and Mark Walport. 2008. *Janeway's Immunobiology. Garland Science*. Vol. 7. doi:10.1086/596249.

Muthukumar, Thangamani, Darshana Dadhania, Ruchuang Ding, Catherine Snopkowski, Rubina Naqvi, Jun B Lee, Choli Hartono, et al. 2005. "Messenger RNA for FOXP3 in the Urine of Renal-Allograft Recipients." *The New England Journal of Medicine* 353: 2342–51.

Naesens, Maarten, Purvesh Khatri, Li Li, Tara K Sigdel, Matthew J Vitalone, Rong Chen, Atul J Butte, Oscar Salvatierra, and Minnie M Sarwal. 2011. "Progressive Histological Damage in Renal Allografts Is Associated with Expression of Innate and Adaptive Immunity Genes." *Kidney International* 80 (12): 1364–76. doi:10.1038/ki.2011.245.

Nelson, David L, and Michael M Cox. 2005. *Lehninger Principles of Biochemistry. Biological Membranes*. Vol. 4th editio. doi:10.1016/j.jse.2011.03.016.

Nickeleit, Volker, Matthias Zeiler, Fred Gudat, Gilbert Thiel, and M J Mihatsch. 2002. "Detection of the Complement Degradation Product C4d in Renal Allografts: Diagnostic and Therapeutic Implications." *Journal of the American Society of Nephrology : JASN* 13 (1): 242–51.

Nickolas, Thomas L, Jonathan Barasch, and Prasad Devarajan. 2008. "Biomarkers in Acute and Chronic Kidney Disease." *Current Opinion in Nephrology and Hypertension* 17 (2): 127–32. doi:10.1097/MNH.0b013e3282f4e525.

O'Connor, Paul, Jerry S Wolinsky, Christian Confavreux, Giancarlo Comi, Ludwig Kappos, Tomas P Olsson, Hadj Benzerdjeb, et al. 2011. "Randomized Trial of Oral Teriflunomide for Relapsing Multiple Sclerosis." *The New England Journal of Medicine* 365 (14): 1293–1303. doi:10.1056/NEJMoa1014656.

O'Sullivan, David, and Erika L. Pearce. 2015. "Targeting T Cell Metabolism for Therapy." *Trends in Immunology* 36 (2). Elsevier Ltd: 1–10. doi:10.1016/j.it.2014.12.004.

O'Sullivan, David, Gerritje J W van der Windt, Stanley Ching-Cheng Huang, Jonathan D Curtis, Chih-Hao Chang, Michael D Buck, Jing Qiu, et al. 2014. "Memory CD8(+) T Cells Use Cell-Intrinsic Lipolysis to Support the Metabolic Programming Necessary for Development." *Immunity* 41 (1). Elsevier Inc.: 75–88. doi:10.1016/j.immuni.2014.06.005.

Opelz, G. 1988. "Importance of HLA Antigen Splits for Kidney Transplant Matching." *Lancet* 2 (8602): 61–64. doi:10.1016/S0140-6736(88)90672-1.

Ordonez, Laurence, Isabelle Bernard, Marianne Chabod, Jean-François Augusto, Valerie Lauwers-Cances, Christelle Cristini, Maria-Cristina Cuturi, Jean-François Subra, and Abdelhadi Saoudi. 2013. "A Higher Risk of Acute Rejection of Human Kidney Allografts Can Be Predicted from the Level of CD45RC Expressed by the Recipients' CD8 T Cells." *PLoS ONE* 8 (7): e69791. doi:10.1371/journal.pone.0069791.

Pallard, Caroline, Alexander P A Stegmann, Titia Van Kleffens, Fiona Smart, Ashok Venkitaraman, and Hergen Spits. 1999. "Distinct Roles of the Phosphatidylinositol 3-Kinase and STAT5 Pathways in IL-7-Mediated Development of Human Thymocyte Precursors." *Immunity* 10 (5): 525–35. doi:10.1016/S1074-7613(00)80052-7.

Parish, Ian a., and Susan M. Kaech. 2009. "Diversity in CD8+ T Cell Differentiation." *Current Opinion in Immunology* 21 (Figure 1): 291–97. doi:10.1016/j.coi.2009.05.008.

Parry, Richard V., Karin Reif, Graham Smith, David M. Sansom, Brian a. Hemmings, and Stephen G. Ward. 1997. "Ligation of the T Cell Co-Stimulatory Receptor CD28 Activates the Serine-Threonine Protein Kinase Protein Kinase B." *European Journal of Immunology* 27 (10): 2495–2501. doi:10.1002/eji.1830271006.

Pawelec, Graham. 2001. "Hypothesis: Loss of Telomerase Inducibility and Subsequent Replicative Senescence in Cultured Human T Cells Is a Result of Altered Costimulation." *Mechanisms of Ageing and Development* 121 (1-3): 181–85. doi:10.1016/S0047-6374(00)00209-8.

Pawelec, Graham, Medi Adibzadeh, Arnika Rehbein, Karin Hähnel, Wolfgang Wagner, and Andrea Engel. 2000. "In Vitro Senescence Models for Human T Lymphocytes." *Vaccine* 18 (16): 1666–74. doi:10.1016/S0264-410X(99)00504-6.

Pearce, Erika L, Matthew C Walsh, Pedro J Cejas, Gretchen M Harms, Hao Shen, Li-San Wang, Russell G Jones, and Yongwon Choi. 2009. "Enhancing CD8 T-Cell Memory by Modulating Fatty Acid Metabolism." *Nature* 460 (7251). Nature Publishing Group: 103–7. doi:10.1038/nature08097.

Pérez-Flores, I., a. Sánchez-Fructuoso, J. L. Santiago, M. Fernández-Arquero, N. Calvo, E. G. de la Concha, and a. Barrientos. 2009. "Intracellular ATP Levels in CD4+ Lymphocytes Are a Risk Marker of Rejection and Infection in Renal Graft Recipients." *Transplantation Proceedings* 41 (6). Elsevier Inc.: 2106–8. doi:10.1016/j.transproceed.2009.06.136.

Picarda, Elodie, Séverine Bézie, Vanessa Venturi, Klara Echasserieau, Emmanuel Mérieau, Aurélie Delhumeau, Karine Renaudin, et al. 2014. “MHC-Derived Allopeptide Activates TCR-Biased CD8+ Tregs and Suppresses Organ Rejection.” *Journal of Clinical Investigation* 124 (6): 2497–2512. doi:10.1172/JCI71533.

Pirsch, J D, R J Ploeg, S Gange, A M D’Alessandro, S J Knechtle, H W Sollinger, M Kalayoglu, and F O Belzer. 1996. “Determinants of Graft Survival after Renal Transplantation.” *Transplantation* 61 (11): 1581–86. doi:10.1097/00007890-199606150-00006.

Plunkett, F. J., O. Franzese, H. M. Finney, J. M. Fletcher, L. L. Belaramani, M. Salmon, I. Dokal, D. Webster, a. D. G. Lawson, and a. N. Akbar. 2007. “The Loss of Telomerase Activity in Highly Differentiated CD8+CD28-CD27- T Cells Is Associated with Decreased Akt (Ser473) Phosphorylation.” *The Journal of Immunology* 178 (12): 7710–19. doi:10.4049/jimmunol.178.12.7710.

Pochini, Lorena, Mariafrancesca Scalise, Michele Galluccio, and Cesare Indiveri. 2014. “Membrane Transporters for the Special Amino Acid Glutamine: Structure/function Relationships and Relevance to Human Health.” *Frontiers in Chemistry* 2 (August): 1–23. doi:10.3389/fchem.2014.00061.

Poirier, N., N. Dilek, C. Mary, S. Ville, F. Coulon, J. Branchereau, X. Tillou, et al. 2015. “FR104, an Antagonist Anti-CD28 Monovalent Fab’ Antibody, Prevents Alloimmunization and Allows Calcineurin Inhibitor Minimization in Nonhuman Primate Renal Allograft.” *American Journal of Transplantation* 15 (1): 88–100. doi:10.1111/ajt.12964.

Poirier, N., C. Mary, N. Dilek, J. Hervouet, D. Minault, G. Blancho, and B. Vanhove. 2012. “Preclinical Efficacy and Immunological Safety of FR104, an Antagonist Anti-CD28 Monovalent Fab’ Antibody.” *American Journal of Transplantation* 12 (10): 2630–40. doi:10.1111/j.1600-6143.2012.04164.x.

Pollizzi, Kristen N., and Jonathan D. Powell. 2014. “Integrating Canonical and Metabolic Signalling Programmes in the Regulation of T Cell Responses.” *Nature Reviews. Immunology* 14 (7). Nature Publishing Group: 435–46. doi:10.1038/nri3701.

Porstmann, Thomas, Claudio R. Santos, Beatrice Griffiths, Megan Cully, Mary Wu, Sally Leevers, John R. Griffiths, Yuen Li Chung, and Almut Schulze. 2008. “SREBP Activity Is Regulated by mTORC1 and Contributes to Akt-Dependent Cell Growth.” *Cell Metabolism* 8 (3). Elsevier Inc.: 224–36. doi:10.1016/j.cmet.2008.07.007.

Quaglia, M., T. Cena, R. Fenoglio, C. Musetti, D. Cagna, E. Radin, S. Roggero, a. Amoroso, C. Magnani, and P. Stratta. 2014. “Immune Function Assay (Immunknow) Drop Over First 6 Months After Renal Transplant: A Predictor of Opportunistic Viral Infections?” *Transplantation Proceedings* 46 (7). Elsevier Inc.: 2220–23. doi:10.1016/j.transproceed.2014.07.050.

Razonable, R R, a Humar, and the a S T Infectious Diseases Community of Practice. 2013. “Cytomegalovirus in Solid Organ Transplantation.” *American Journal of Transplantation* 13 (s4): 93–106. doi:10.1111/ajt.12103.

Reinke, Simon, Sven Geissler, William R Taylor, Katharina Schmidt-Bleek, Kerstin Juelke, Verena Schwachmeyer, Michael Dahne, et al. 2013. "Terminally Differentiated CD8^{+} T Cells Negatively Affect Bone Regeneration in Humans." *Science Translational Medicine* 5 (177): 177ra36. doi:10.1126/scitranslmed.3004754.

Ringsrud, Karen M. 2001. "Cells in the Urine." *Laboratory Medicine* 32 (3): 153–55. doi:10.1309/60JQ-6F5F-1V9E-AGGB.

Roberti, I, L Reisman, L Burrows, and K V Lieberman. 1995. *Urine Cytology and Urine Flow Cytometry in Renal Transplantation--A Prospective Double Blind Study. Transplantation*. Vol. 59. doi:10.1097/00007890-199559040-00009.

Robertson, Helen, Simi Ali, Benjamin J. Mcdonnell, Alastair D. Burt, and John A. Kirby. 2004. "Chronic Renal Allograft Dysfunction: The Role of T Cell-Mediated Tubular Epithelial to Mesenchymal Cell Transition." *Journal of the American Society of Nephrology* 15 (2): 390–97. doi:10.1097/01.ASN.0000108521.39082.E1.

Robertson, Helen, Janice Wheeler, John A Kirby, and Adrian R Morley. 1996. "Renal Allograft Rejection--in Situ Demonstration of Cytotoxic Intratubular Cells." *Transplantation* 61 (10): 1546–49. doi:10.1097/00007890-199605270-00023.

Russell, J D, M L Beecroft, D Ludwin, and D N Churchill. 1992. "The Quality of Life in Renal Transplantation--A Prospective Study." *Transplantation* 54 (4): 656–60. doi:10.1097/00007890-199210000-00018.

Sagoo, Pervinder, Esperanza Perucha, Birgit Sawitzki, Stefan Tomiuk, David A Stephens, Patrick Miqueu, Stephanie Chapman, et al. 2010. "Development of a Cross-Platform Biomarker Signature to Detect Renal Transplant Tolerance in Humans." *The Journal of Clinical Investigation* 120 (6): 1848–61. doi:10.1172/JCI39922.

Sallusto, F, D Lenig, R Förster, M Lipp, and a Lanzavecchia. 1999. "Two Subsets of Memory T Lymphocytes with Distinct Homing Potentials and Effector Functions." *Nature* 401 (October): 708–12. doi:10.1038/44385.

Salou, Marion, Alexandra Garcia, Laure Michel, Anne Gainche-Salmon, Delphine Loussouarn, Bryan Nicol, Flora Guillot, et al. 2015. "Expanded CD8 T-Cell Sharing between Periphery and CNS in Multiple Sclerosis." *Annals of Clinical and Translational Neurology* 2 (6): 609–22. doi:10.1002/acn3.199.

San Segundo, D., G. Fernández-Fresnedo, E. Rodrigo, J. C. Ruiz, M. González, C. Gómez-Alamillo, M. Arias, and M. López-Hoyos. 2012. "High Regulatory T-Cell Levels at 1 Year Posttransplantation Predict Long-Term Graft Survival among Kidney Transplant Recipients." *Transplantation Proceedings* 44 (9). Elsevier Inc.: 2538–41. doi:10.1016/j.transproceed.2012.09.083.

San Segundo, David, María Ángeles Ballesteros, Sara Naranjo, Felipe Zurbano, Eduardo Miñambres, and Marcos López-Hoyos. 2013. "Increased Numbers of Circulating CD8 Effector Memory T Cells before Transplantation Enhance the Risk of Acute Rejection in Lung Transplant Recipients." *PLoS ONE* 8 (11): 1–9. doi:10.1371/journal.pone.0080601.

San Segundo, David, Olga Millán, Pedro Muñoz-Cacho, Francisco Boix, Estela Paz-Artal, Paloma Talayero, José María Morales, et al. 2014. "High Proportion of Pretransplantation Activated Regulatory T Cells (CD4+CD25highCD62L+CD45RO+) Predicts Acute Rejection in Kidney Transplantation." *Transplantation* 98 (11): 1213–18. doi:10.1097/TP.0000000000000202.

Schell, John C, and Jared Rutter. 2013. "The Long and Winding Road to the Mitochondrial Pyruvate Carrier." *Cancer & Metabolism* 1 (1): 6. doi:10.1186/2049-3002-1-6.

Schnitzler, M A, R S Woodward, D C Brennan, D L Phelan, E L Spitznagel, S B Boxerman, W C Dunagan, and T C Bailey. 1997. "Cytomegalovirus and HLA-A, B, and DR Locus Interactions: Impact on Renal Transplant Graft Survival." *American Journal of Kidney Diseases : The Official Journal of the National Kidney Foundation* 30 (6): 766–71.

Sellarés, J., J. Reeve, a. Loupy, M. Mengel, B. Sis, a. Skene, D. G. De Freitas, et al. 2013. "Molecular Diagnosis of Antibody-Mediated Rejection in Human Kidney Transplants." *American Journal of Transplantation* 13 (4): 971–83. doi:10.1111/ajt.12150.

Shen, Tao, Jiajia Zheng, Chunhui Xu, Jia Liu, Weidong Zhang, Fengmin Lu, and Hui Zhuang. 2010. "PD-1 Expression on Peripheral CD8+ TEM/TEMRA Subsets Closely Correlated with HCV Viral Load in Chronic Hepatitis C Patients." *Virology Journal* 7: 310. doi:10.1186/1743-422X-7-310.

Sigdel, Tara K., Oriol Bestard, Tim Q. Tran, Szu-Chuan Hsieh, Silke Roedder, Izabella Damm, Flavio Vincenti, and Minnie M. Sarwal. 2015. "A Computational Gene Expression Score for Predicting Immune Injury in Renal Allografts." *Plos One* 10 (9): e0138133. doi:10.1371/journal.pone.0138133.

Simon, Tania, Gerhard Opelz, Manfred Wiesel, Ralf C Ott, and Caner Süsal. 2003. "Serial Peripheral Blood Perforin and Granzyme B Gene Expression Measurements for Prediction of Acute Rejection in Kidney Graft Recipients." *American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 3 (9): 1121–27. doi:10.1034/j.1600-6143.2003.00187.x.

Sinclair, Linda V, Julia Rolf, Elizabeth Emslie, Yun-Bo Shi, Peter M Taylor, and Doreen a Cantrell. 2013. "Control of Amino-Acid Transport by Antigen Receptors Coordinates the Metabolic Reprogramming Essential for T Cell Differentiation." *Nature Immunology* 14 (5): 500–508. doi:10.1038/ni.2556.

Singer, Katrin, Michael Kastenberger, Eva Gottfried, Christine G. Hammerschmied, Maike Büttner, Michael Aigner, Barbara Seliger, et al. 2011. "Warburg Phenotype in Renal Cell Carcinoma: High Expression of Glucose-Transporter 1 (GLUT-1) Correlates with Low CD8+ T-Cell Infiltration in the Tumor." *International Journal of Cancer* 128 (9): 2085–95. doi:10.1002/ijc.25543.

Sipula, Ian J., Nicholas F. Brown, and German Perdomo. 2006. "Rapamycin-Mediated Inhibition of Mammalian Target of Rapamycin in Skeletal Muscle Cells Reduces Glucose Utilization and Increases Fatty Acid Oxidation." *Metabolism: Clinical and Experimental* 55 (12): 1637–44. doi:10.1016/j.metabol.2006.08.002.

Sis, B., P. M. Campbell, T. Mueller, C. Hunter, S. M. Cockfield, J. Cruz, C. Meng, D. Wishart, K. Solez, and P. F. Halloran. 2007. "Transplant Glomerulopathy, Late Antibody-Mediated Rejection and the ABCD Tetrad in Kidney Allograft Biopsies for Cause." *American Journal of Transplantation* 7 (7): 1743–52. doi:10.1111/j.1600-6143.2007.01836.x.

Smith, R. Neal, and Robert B. Colvin. 2012. "Chronic Alloantibody Mediated Rejection." *Seminars in Immunology* 24 (2). Elsevier Ltd: 115–21. doi:10.1016/j.smim.2011.09.002.

Sobao, Y., H. Tomiyama, S. Nakamura, H. Sekihara, K. Tanaka, and M. Takiguchi. 2001. "Visual Demonstration of Hepatitis C Virus-Specific Memory CD8+ T-Cell Expansion in Patients with Acute Hepatitis C." *Hepatology* 33: 287–94. doi:10.1053/jhep.2001.21164.

Solez, K, R A Axelsen, H Benediktsson, J F Burdick, A H Cohen, R B Colvin, B P Croker, D Droz, M S Dunnill, and P F Halloran. 1993. "International Standardization of Criteria for the Histologic Diagnosis of Renal Allograft Rejection: The Banff Working Classification of Kidney Transplant Pathology." *Kidney International* 44 (2): 411–22. doi:10.1038/ki.1993.259.

Solez, K, and L C Racusen. 2013. "The Banff Classification Revisited." *Kidney Int* 83 (2). Nature Publishing Group: 201–6. doi:10.1038/ki.2012.395.

Solez, K., R. B. Colvin, L. C. Racusen, B. Sis, P. F. Halloran, P. E. Birk, P. M. Campbell, et al. 2007. "Banff '05 Meeting Report: Differential Diagnosis of Chronic Allograft Injury and Elimination of Chronic Allograft Nephropathy ('CAN')." *American Journal of Transplantation* 7 (3): 518–26. doi:10.1111/j.1600-6143.2006.01688.x.

Squibb, Briston-Myers. 2008. "Glucophage ® XR Label," 3–32.

Starzl, T. E. 1995. "Peter Brian Medawar: Father of Transplantation." *Journal of the American College of Surgeons* 180 (3): 332–36.

Stemberger, Christian, Katharina M. Huster, Martina Koffler, Florian Anderl, Matthias Schiemann, Hermann Wagner, and Dirk H. Busch. 2007. "A Single Naive CD8+ T Cell Precursor Can Develop into Diverse Effector and Memory Subsets." *Immunity* 27 (6): 985–97. doi:10.1016/j.immuni.2007.10.012.

Stemberger, Christian, Michael Neuenhahn, Veit R Buchholz, and Dirk H Busch. 2007. "Origin of CD8+ Effector and Memory T Cell Subsets." *Cellular & Molecular Immunology* 4 (December): 399–405.

Strioga, Marius, Vita Pasukoniene, and Dainius Characiejus. 2011. "CD8+CD28- and CD8+CD57+ T Cells and Their Role in Health and Disease." *Immunology* 134 (1): 17–32. doi:10.1111/j.1365-2567.2011.03470.x.

Suciu-Foca, Nicole, John S. Manavalan, Luigi Scotto, Seunghee Kim-Schulze, Sara Galluzzo, Afzal J. Naiyer, Jianshe Fan, George Vlad, and Raffaello Cortesini. 2005. "Molecular Characterization of Allospecific T Suppressor and Tolerogenic Dendritic Cells: Review." *International Immunopharmacology* 5 (1): 7–11. doi:10.1016/j.intimp.2004.09.003.

Sukumar, Madhusudhanan, Jie Liu, Yun Ji, Murugan Subramanian, Joseph G. Crompton, Zhiya Yu, Rahul Roychoudhuri, et al. 2013. "Inhibiting Glycolytic Metabolism Enhances CD8+ T Cell Memory and Antitumor Function." *Journal of Clinical Investigation* 123 (10): 4479–88. doi:10.1172/JCI69589.

Suzuki, M, a L Jagger, C Konya, Y Shimojima, S Pryshchep, J J Goronzy, and C M Weyand. 2012. "CD8+CD45RA+CCR7+FOXP3+ T Cells with Immunosuppressive Properties: A Novel Subset of Inducible Human Regulatory T Cells." *J Immunol* 189 (5): 2118–30. doi:10.4049/jimmunol.1200122.

Takemoto, Steve, Friedrich K. Port, Frans H J Claas, and Rene J. Duquesnoy. 2004. "HLA Matching for Kidney Transplantation." *Human Immunology* 65 (12): 1489–1505. doi:10.1016/j.humimm.2004.06.008.

Takemoto, Steven K., Paul I. Terasaki, David W. Gjertson, and J. Michael Cecka. 2000. "Twelve Years' Experience with National Sharing of HLA-Matched Cadaveric Kidneys for Transplantation." *The New England Journal of Medicine* 343 (15).

Tan, P, C Anasetti, J a Hansen, J Melrose, M Brunvand, J Bradshaw, J a Ledbetter, and P S Linsley. 1993. "Induction of Alloantigen-Specific Hyporesponsiveness in Human T Lymphocytes by Blocking Interaction of CD28 with Its Natural Ligand B7/BB1." *The Journal of Experimental Medicine* 177 (1): 165–73. doi:10.1084/jem.177.1.165.

Taylor, C J, K I Welsh, C M Gray, M Bunce, A M Bayne, P M Sutton, D W Gray, A Ting, and P J Morris. 1993. "Clinical and Socioeconomic Benefits of Serological HLA-DR Matching for Renal Transplantation over Three Eras of Immunosuppression Regimens at a Single Unit." *Clinical Transplants*, 233–41.

Tomiyama, Hiroko, Tomoko Matsuda, and Masafumi Takiguchi. 2002. "Differentiation of Human CD8+ T Cells from a Memory to Memory/Effector Phenotype." *The Journal of Immunology* 168: 5538–50. doi:10.4049/jimmunol.168.11.5538.

Tomiyama, Hiroko, Hiroshi Takata, Tomoko Matsuda, and Masafumi Takiguchi. 2004. "Phenotypic Classification of Human CD8+ T Cells Reflecting Their Function: Inverse Correlation between Quantitative Expression of CD27 and Cytotoxic Effector Function." *European Journal of Immunology* 34: 999–1010. doi:10.1002/eji.200324478.

Tonelli, M., N. Wiebe, G. Knoll, a. Bello, S. Browne, D. Jadhav, S. Klarenbach, and J. Gill. 2011. "Systematic Review: Kidney Transplantation Compared with Dialysis in Clinically Relevant Outcomes." *American Journal of Transplantation* 11 (10): 2093–2109. doi:10.1111/j.1600-6143.2011.03686.x.

Trivedi, H. L. 2007. "Immunobiology of Rejection and Adaptation." *Transplantation Proceedings* 39 (3): 647–52. doi:10.1016/j.transproceed.2007.01.047.

Urbschat, Anja, Nicholas Obermüller, and Axel Haferkamp. 2011. "Biomarkers of Kidney Injury." *Biomarkers : Biochemical Indicators of Exposure, Response, and Susceptibility to Chemicals* 16 Suppl 1: S22–30. doi:10.3109/1354750X.2011.587129.

Van der Bij, W, and R Speich. 2001. “Management of Cytomegalovirus Infection and Disease after Solid-Organ Transplantation.” *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America* 33 Suppl 1 (Suppl 1): S32–37. doi:10.1086/320902.

Van der Windt, Gerritje J W, David O’Sullivan, Bart Everts, Stanley Ching-Cheng Huang, Michael D Buck, Jonathan D Curtis, Chih-Hao Chang, et al. 2013. “CD8 Memory T Cells Have a Bioenergetic Advantage That Underlies Their Rapid Recall Ability.” *Proceedings of the National Academy of Sciences of the United States of America* 110 (35): 14336–41. doi:10.1073/pnas.1221740110.

Van der Windt, Gerritje J W, and Erika L. Pearce. 2012. “Metabolic Switching and Fuel Choice during T-Cell Differentiation and Memory Development.” *Immunological Reviews* 249 (1): 27–42. doi:10.1111/j.1600-065X.2012.01150.x.

Van Doesum, W. B., W. H. Abdulahad, M. C R F van Dijk, S. Dolff, W. J. van Son, C. a. Stegeman, and J. S F Sanders. 2014. “Characterization of Urinary CD4+ and CD8+ T Cells in Kidney Transplantation Patients with Polyomavirus BK Infection and Allograft Rejection.” *Transplant Infectious Disease*, 733–43. doi:10.1111/tid.12273.

Vander Heiden, M. G., Cantley, L.C., Thompson, C.B. 2009. “Understanding the Warburg Effect: The Metabolic Requirements of Cells Proliferation.” *Science* 324 (5930): 1029–33. doi:10.1126/science.1160809.Understanding.

Villanova, Federica, Paola Di Meglio, Margaret Inokuma, Nima Aghaeepour, Esperanza Perucha, Jennifer Mollon, Laurel Nomura, et al. 2013. “Integration of Lyoplate Based Flow Cytometry and Computational Analysis for Standardized Immunological Biomarker Discovery.” *PLoS ONE* 8 (7). doi:10.1371/journal.pone.0065485.

Viswanathan, Gautham, and Ashish Upadhyay. 2011. “Assessment of Proteinuria.” *Advances in Chronic Kidney Disease* 18 (4). Elsevier Ltd: 243–48. doi:10.1053/j.ackd.2011.03.002.

Voskoboinik, Ilia, James C. Whisstock, and Joseph a. Trapani. 2015. “Perforin and Granzymes: Function, Dysfunction and Human Pathology.” *Nature Reviews Immunology* 15 (6). Nature Publishing Group: 388–400. doi:10.1038/nri3839.

Waikar, Sushrut S., Venkata S. Sabbisetti, and Joseph V. Bonventre. 2010. “Normalization of Urinary Biomarkers to Creatinine during Changes in Glomerular Filtration Rate.” *Kidney International* 78 (5): 486–94. doi:10.1016/j.biotechadv.2011.08.021.Secreted.

Wang, George C, and Vincenzo Casolaro. 2014. “Immunologic Changes in Frail Older Adults.” *Translational Medicine @ UniSa* 9 (1): 1–6.

Wang, Ruoning, Christopher P. Dillon, Lewis Zhichang Shi, Sandra Milasta, Robert Carter, David Finkelstein, Laura L. McCormick, et al. 2011. “The Transcription Factor Myc Controls Metabolic Reprogramming upon T Lymphocyte Activation.” *Immunity* 35 (6). Elsevier Inc.: 871–82. doi:10.1016/j.immuni.2011.09.021.

Warburg, Otto Heinrich. 1928. “The Chemical Constitution of Respiration Ferment.” *Science* 68: 437–43. doi:10.1007/s11999-010-1534-y.

Wasinski, Frederick, Marcos F. Gregnani, Fábio H. Ornellas, Aline V. N. Bacurau, Niels O. Câmara, Ronaldo C. Araujo, and Reury F. Bacurau. 2014. “Lymphocyte Glucose and Glutamine Metabolism as Targets of the Anti-Inflammatory and Immunomodulatory Effects of Exercise.” *Mediators of Inflammation* 2014: 1–10. doi:10.1155/2014/326803.

Wasung, Michael E, Lakhmir S Chawla, and Magdalena Madero. 2015. “Biomarkers of Renal Function , Which and When ?” *Clinica Chimica Acta* 438 (1). Elsevier B.V.: 350–57. doi:10.1016/j.cca.2014.08.039.

Watson, C. J E, and J. H. Dark. 2012. “Organ Transplantation: Historical Perspective and Current Practice.” *British Journal of Anaesthesia* 108 (SUPPL. 1): 29–42. doi:10.1093/bja/aer384.

Willis, Joanna C D, and Graham M Lord. 2015. “Immune Biomarkers: The Promises and Pitfalls of Personalized Medicine.” *Nature Reviews Immunology* 15 (March). Nature Publishing Group: 1–7. doi:10.1038/nri3820.

Wofford, Jessica A., Heather L. Wieman, Sarah R. Jacobs, Yuxing Zhao, and Jeffrey C. Rathmell. 2008. “IL-7 Promotes Glut1 Trafficking and Glucose Uptake via STAT5-Mediated Activation of Akt to Support T-Cell Survival.” *Blood* 111 (4): 2101–11. doi:10.1182/blood-2007-06-096297.

Wongsaroj, Patarapha, Joseph Kahwaji, Ashley Vo, and Stanley C Jordan. 2015. “Modern Approaches to Incompatible Kidney Transplantation.” *World Journal of Nephrology* 4 (3): 354–62. doi:10.5527/wjn.v4.i3.354.

Wood, K.J., and R. Goto. 2012. “Mechanisms of Rejection: Current Perspectives.” *Transplantation* 93 (1): 1. doi:10.1097/TP.0b013e31823cab44.

Xu, Xiaojin, Lilin Ye, Koichi Araki, and Rafi Ahmed. 2012. “MTOR, Linking Metabolism and Immunity.” *Seminars in Immunology* 24 (6). Elsevier Ltd: 429–35. doi:10.1016/j.smim.2012.12.005.

Xystrakis, Emmanuel, Anne S Dejean, Isabelle Bernard, Philippe Druet, Roland Liblau, Daniel Gonzalez-dunia, and Abdelhadi Saoudi. 2009. “Identification of a Novel Natural Regulatory CD8 T-Cell Subset and Analysis of Its Mechanism of Regulation.” *Blood* 104 (10): 3294–3301. doi:10.1182/blood-2004-03-1214.

Yamada, Y., S. Boskovic, a. Aoyama, T. Murakami, P. Putheti, R. N. Smith, T. Ochiai, et al. 2012. “Overcoming Memory T-Cell Responses for Induction of Delayed Tolerance in Nonhuman Primates.” *American Journal of Transplantation* 12 (2): 330–40. doi:10.1111/j.1600-6143.2011.03795.x.

Yap, Michelle, Françoise Boeffard, Emmanuel Clave, Annaick Pallier, Richard Danger, Magali Giral, Jacques Dantal, et al. 2014. “Expansion of Highly Differentiated Cytotoxic Terminally Differentiated Effector Memory CD8+ T Cells in a Subset of Clinically Stable Kidney Transplant Recipients: A Potential Marker for Late Graft Dysfunction.”

Journal of the American Society of Nephrology : JASN 25 (8): 1856–68. doi:10.1681/ASN.2013080848.

Yin, Yiming, Seung-chul Choi, Zhiwei Xu, Daniel J Perry, Howard Seay, Byron P Croker, Eric S Sobel, Todd M Brusko, and Laurence Morel. 2015. “Normalization of CD4 + T Cell Metabolism Reverses Lupus.” *Science Translational Medicine* 7 (274): 1–13.

6 Appendix

6.1 Summary of Thesis in French

CARACTERISATION DE LA DIFFERENCIATION DES LYMPHOCYTES T CD8$^+$ ET DE LEUR PROFIL IMMUNOMETABOLIQUE EN TRANSPLANTATION RENALE

La transplantation rénale est le meilleur traitement pour les maladies rénales en phase terminale, allongeant l'espérance de vie et améliorant la qualité de vie des patients (Tonelli et al.2011). Cependant, cette solution thérapeutique est limitée par des facteurs immunologiques, mais aussi logistiques. Ce dernier concerne principalement la pénurie d'organes. En effet, en 2012, sur 57 903 patients recensés aux Etats-Unis en attente de transplantation rénale, seulement 17 282 en ont bénéficié, soulignant un besoin largement supérieur à la disponibilité des organes (Department of Health and Human Services 2014). Par ailleurs, les patients transplantés doivent malheureusement se plier à un traitement immunosuppresseur à vie pour maitriser le risque de rejet d'allogreffe. Si ces traitements sont efficaces pour prévenir du rejet aigu, le rejet chronique, dont les mécanismes restent mal compris, met en danger la survie à long terme du greffon. Les biomarqueurs utilisés actuellement en clinique ne permettent de détecter les épisodes de rejet qu'après l'apparition de lésions dans le greffon. C'est pourquoi il est important dans le domaine de la transplantation de mieux comprendre les mécanismes du rejet d'allogreffe et de trouver de nouveaux biomarqueurs plus fiables et permettant prédire plus tôtle rejet.

Miqueu et al. ont montré que le rejet d'allogreffe rénale chronique était associé à un répertoire TCR Vβ altéré en périphérie et à une augmentation du ratio T CD8$^+$/CD4$^+$, alors que ce répertoire ne présente pas d'accumulation de clones T sélectionnés chez les patients tolérants. Chez les patients stables, l'utilisation du répertoire est hétérogène allant d'une répartition gaussienne à des accumulations de clones T. De plus, Brouard et al. ont montré que l'altération du répertoire TCR Vβ chez les patients transplantés concerne principalement le compartiment CD8 (Brouard et al. 2005). Par ailleurs, Baeten et al. ont démontré que les

cellules T $CD8^+$ cytotoxiques étaient impliquées dans le rejet chronique du greffon (Baeten et al. 2006).

Les cellules T $CD8^+$ ont été montrées comme hautement impliquées dans les lésions d'allogreffe rénale à caractère cellulaire, induisant la dysfonction chronique du greffon malgré l'utilisation de drogues immunosuppressives de type calcineurine pour inhiber leur réponse allogénique. Le marqueur CD45RA combiné au marqueur de migration vers les ganglions CCR7 (aussi appelé CD197) peuvent être utilisés pour identifier les cellules naïves, effectrices mémoires (EM), centrales mémoires (CM) et mémoire au stade de différentiation terminale (TEMRA) parmi les cellules CD8 (Sallusto et al. 1999). L'activation des cellules naïves par un antigène induit leur différenciation en cellules T effectrices ayant un haut pouvoir proliférant. Après la disparition de l'antigène, seule une fraction de la population CD8 effectrice perdure sous la forme de cellules $CD8^+$ EM et CM.

Ces dernières années, le rôle du métabolisme suscite de nouveau l'intérêt de la recherche. En effet, l'immunométabolisme des lymphocytes ne permettrait pas seulement la production d'énergie mais serait aussi lié à leur différenciation et leur prolifération.

A l'état basal, les cellules T naïves utilisent la phosphorylation oxydative (OXPHOS) pour produire l'ATP (Guppy, Greiner, and Brand 1993). Le catabolisme de dérivés glucidiques, lipidiques et protéiques est en effet la méthode la plus efficace pour générer de l'ATP. A l'oxydation mitochondriale du glucose s'ajoute la β-oxydation des acides gras, particulièrement utilisée par les cellules mémoires quiescentes. L'oxydation des acides gras (FAO) est nécessaire pour le développement des cellules T mémoires, un défaut de FAO affectant lourdement la population de cellules T CD8 mémoires (Lochner, Berod, and Sparwasser 2015; O'Sullivan et al. 2014). Cependant, lorsque les cellules T sont activées, elles subissent l'effet Warburg, reprogrammant leur métabolisme d'un système OXPHOS à

un système de glycolyse aérobique et de glutaminolyse (MacIver, Michalek, and Rathmell 2013). Ce changement glycolytique aide à la prolifération rapide des lymphocytes activés en terme de besoins énergétiques mais aussi en terme d'intermédiaires métaboliques. Ces derniers servent à la biosynthèse de macromolécules essentielles pour la croissance et la prolifération massive des cellules, ainsi que pour la génération de cytokines et de molécules cytotoxiques, pour l'expansion des cellules CD8 effectrices pendant la réponse immune (Lunt and Vander Heiden 2011).

Cette thèse se compose de deux parties. La première partie est centrée sur la caractérisation de la distribution des populations de cellules T $CD8^+$ en périphérie chez les patients transplantés rénaux stables à long terme, et évalue l'intérêt de l'utilisation des cellules T $CD8^+$ TEMRA comme marqueurs de prédiction du rejet d'allogreffe. Nous avons montré que les patients transplantés rénaux, dont la fonction du greffon est stable un an après la greffe et présentant un répertoire TCR Vβ altéré, ont un risque 1,96 fois plus élevé de développer un rejet chronique comparés aux patients dont le répertoire TCR Vβ n'est pas altéré (Publication #1). De plus, chez ces patients, une fréquence plus élevée de cellules $CD8^+$ TEMRA corrèle avec l'altération du répertoire TCR Vβ. Cette étude a aboutit à tester si l'inclusion de paramètres immunologiques (en l'occurrence la fréquence des sous-populations T CD8) était à même d'améliorer les capacités pronostiques du KTFS (Kidney Transplant Failure Score), score de prédiction du retour en dialyse dans les 8 années post-transplantation basé sur des variables cliniques (Publication #2) Nous avons montré que la mesure de la fréquence des cellules $CD8^+$TEMRA ou de celles des cellules $CD27^+CD28^-$, $GZMB^+PERF^+$ et $GZMB^-PERF^+$ dans le calcul du KTFS permettait d'améliorer l'aire sous la courbe du KTFS de 0,64 à 0,76. Cette augmentation se traduit par une meilleure classification de 26,1% des patients. Enfin, nous avons comparé trois nomenclatures différentes pour identifier les populations de cellules T $CD8^+$ dans le but d'identifier la plus fiable pour surveiller le compartiment CD8

chez les patients transplantés rénaux (Publication #3). Nous avons ainsi montré que l'utilisation de la nomenclature CD45RA/CD28 permet non seulement d'identifier les sous-populations naives et TEMRA mais permet également d'étudier les cellules $CD8^+$ EM aux stades précoces et tardifs de différenciation. L'utilisation de cette nomenclature pourrait avoir notamment un grand intérêt pour le suivi des patients transplantés traités avec la nouvelle génération d'anti-CD28.

La deuxième partie de cette thèse est centrée sur la caractérisation du programme immunométabolique impliqué dans la fonction des cellules T $CD8^+$ chez les volontaires sains, les patients transplantés rénaux, et les patients atteints de sclérose en plaques. Nous avons utilisé un analyseur de flux extracellulaire (Seahorse) pour évaluer les changements du programme immunométabolique de différentes populations de cellules T $CD8^+$. Nous avons notamment étudié l'importance des différentes voies métaboliques pour la fonction effectrice des cellules T CD8. Nous avons montré que chez les volontaires sains, les cellules EM et TEMRA ont un niveau basal d'activité glycolytique supérieur aux cellules naïves, et qu'elles peuvent maintenir un niveau élevé de glycolyse et de respiration mitochondriale après stimulation contrairement aux cellules naïves. De plus, les cellules TEMRA et EM présentent une concentration plus élevée en ATP stockée en condition basale comparées aux cellules naïves. Lors d'une activation, les cellules CD8 naïves commencent à produire de l'ATP alors que les cellules CD8 EM et TEMRA vident rapidement leur stock d'ATP avant d'en générer d'autre. Par ailleurs, en utilisant le 2-DG pour bloquer la voie glycolytique, nous avons montré que la glycolyse est essentielle aux CD8 pour la production de cytokines proinflammatoires.

La partie annexe de la cette thèse comprend un résumé de cette thèse en français, ainsi que deux articles actuellement soumis. Le premier article intitulé "Targeting CD8 T Cell Metabolism in Transplantation" est une revue d'articles portant sur l'immunométabolisme des

cellules T CD8 en transplantation. Cette revue reprend dans les grandes lignes l'introduction de cette thèse, elle a donc été placée en annexe par soucis de redondance. Le deuxième article intitulé « B cell depletion therapy impact CD8 T cells in ANCA-associated vasculitis" résulte d'un projet pour lequel j'ai participé à l'immunophénotypage, mais étant éloigné du sujet principal de ma thèse, je l'ai placé en annexe.

6.2 Supplemental Review Article

Targeting CD8 T Cell Metabolism in Transplantation

Published in Frontiers of Immunology in 2015.

frontiers in Immunology

MINI REVIEW
published: 23 October 2015
doi: 10.3389/fimmu.2015.00547

Targeting CD8 T-cell metabolism in transplantation

Michelle Yap[1,2], Sophie Brouard[1,3,4,5], Claire Pecqueur[2,6] and Nicolas Degauque[1,3]*

[1] UMR 1064, INSERM, Nantes, France, [2] Faculté de Médecine, Université de Nantes, Nantes, France, [3] CHU de Nantes, ITUN, Nantes, France, [4] CIC Biothérapie, Nantes, France, [5] CHU Nantes, CRB, Nantes, France, [6] UMR 892, INSERM, Nantes, France

Infiltration of effector CD8 T cells plays a major role in allograft rejection, and increases in memory and terminally differentiated effector memory CD8 T cells are associated with long-term allograft dysfunction. Alternatively, CD8 regulatory T cells suppress the inflammatory responses of effector lymphocytes and induce allograft tolerance in animal models. Recently, there has been a renewed interest in the field of immunometabolics and its important role in CD8 function and differentiation. The purpose of this review is to highlight the key metabolic pathways involved in CD8 T cells and to discuss how manipulating these metabolic pathways could lead to new immunosuppressive strategies for the transplantation field.

Keywords: CD8 lymphocytes, transplantation, metabolism, glycolysis, mitochondria, regulation, therapeutics

OPEN ACCESS

Edited by:
Chiara Bonini,
San Raffaele Scientific Institute, Italy

Reviewed by:
Luca Gattinoni,
National Cancer Institute, USA
Nicoletta Cieri,
University of Milan, Italy

****Correspondence:***
Nicolas Degauque
nicolas.degauque@univ-nantes.fr

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *30 July 2015*
Accepted: *12 October 2015*
Published: *23 October 2015*

Citation:
Yap M, Brouard S, Pecqueur C and Degauque N (2015) Targeting CD8 T-cell metabolism in transplantation. Front. Immunol. 6:547. doi: 10.3389/fimmu.2015.00547

INTRODUCTION

To efficiently protect mammalian against pathogen infection, the response of the adaptive T-cell immune system follows a three-step process: an initial phase of T-cell priming followed by a massive clonal expansion and differentiation, a contraction phase leading to the clearance of most effector cells, and the generation of memory immune T cells. Numerous subsets of memory CD8 T cells have been described with different functions and localization within the body. Seminal study by Sallusto et al. identified that isoform CD45RA and lymph node homing marker CCR7 allow the discrimination of naive and subsets of memory CD8 cells (central memory, CM; effector memory, EM; terminally differentiated effector memory cells, TEMRA) (1). The inclusion of additional markers, such as CD27, CD28, CD57, or TBET, can narrow down the identification of specific memory subsets to decipher their relative function during the recall response and to identify checkpoint regulating their differentiation and their survival (2). T-cell response to antigen stimulation and activating their effector functions are energetically demanding processes. Over the last decade, numerous reports have highlighted that the generation of effector and memory cells is not only regulated by the inflammatory milieu but also by metabolic processes.

In the 1920s, Otto Warburg observed that cancer cells consumed much higher amounts of glucose compared with normal cells and that cancer cells mainly utilize glycolysis over mitochondrial respiration, even in the presence of oxygen (3, 4). This metabolic strategy, termed the Warburg effect, has also been observed in activated lymphocytes (5–7). Despite being less efficient albeit rapid at producing ATP, the aerobic glycolysis enables cells to generate the biosynthetic precursors necessary to support rapid growth and proliferation as well as to maintain the redox balance (8). The renewed interest in the metabolic processes involved in T-cell biology has also revealed that evolutionary conserved pathways, such as the mammalian target of rapamycin (mTOR) pathway, are common to both immune responses and metabolism regulation (9). The goal of this review is to summarize the link between

CD8 T-cell subsets and their metabolic adaptations and to evaluate how targeting this metabolic programming could be a new possible therapeutic treatment, with a special focus on transplantation.

T CELL AND TRANSPLANT OUTCOME: KNOWN TARGETS THAT REQUIRED NEW THERAPEUTIC TOOLS

Involvement of T lymphocytes in solid organ transplant rejection is a well-researched topic in the transplantation field; however, most of the research focuses on the various roles of the different CD4 lymphocyte subpopulations. Few investigate the role of CD8 cells in transplantation with a main focus on their cytotoxic and proinflammatory functions and how deleterious they are to long-term allograft health (10–13). In acute allograft rejection, infiltrating CD8 T cells play a major role in tubulitis and tissue necrosis. Cytotoxic molecules, such as perforin and granzyme-B, as well as proinflammatory cytokines, such as IFN-γ, secreted by CD8 T cells have been shown to contribute to the rejection response (14, 15). Furthermore, memory CD8 T cells have been shown to act as major barriers for transplant tolerance induction protocols (16, 17). Additionally, recent immunomonitoring research has shown that an increase in certain CD8 populations in transplant patients is associated with allograft rejection and loss. Elevated effector, memory, and TEMRA CD8 subsets in the peripheral circulation are associated with increased occurrence of acute and chronic allograft dysfunction (18–22). As a result, immunomodulation of the CD8 population is an important component for long-term allograft survival and targeting their specific metabolic processes could represent a new mean to foster graft survival.

There is budding interest into a small subpopulation of CD8 lymphocytes with regulatory and suppressive functions. While most of the research has been focused on $CD4^+FOXP3^+$Tregs, there is growing interest in the other types of regulatory T cells, which include the CD8 Tregs (23–25). There have been several different nomenclatures proposed in the literature, which can be used to identify this subset of cells, a few examples being $CD8^+CD28^-$, $CD8^+CD45RC^{low}$, and $CD8^+FOXP3^+$ (26–28). CD8 Treg populations have potent suppressive ability against effector lymphocytes and promote tolerant induction in rodent transplant models, thus making them an attractive target for promoting allograft survival (23, 24, 28–30). Immunometabolic regulation of CD8 T cells could be used as a means to manipulate the CD8 T-cell immune function for effective immunosuppression. However, little is known regarding their metabolic adaptation during the induction and the expansion phases.

CD8 T-CELL METABOLIC PROGRAMMING CHANGES ARE SPECIFIC TO CD8 SUBSETS AND FUNCTION

Metabolic Demands in Quiescent T Cells

In resting state, naive T cells rely on oxidative phosphorylation (OXPHOS) to produce adenosine 5′-triphosphate (ATP) (31). This metabolic pathway is the most effective method to generate ATP by catabolizing metabolic intermediates derived from glucose, fatty acids, and amino acids. The low rate of energy metabolism needed by quiescent cells to maintain their housekeeping functions is instructed by cytokine-dependent signals and preferentially IL-7-mediated signals. Indeed, IL-7 is a crucial cytokine to sustain the homeostasis of naive cells and their survival and is also an important regulator of the glucose transporter GLUT-1 (32). The IL-7 receptor (IL-7R) is largely expressed in naive lymphocytes and downregulated when naive CD8 T cells differentiate into the effector phenotype. IL-7 causes activation of JAK1 and JAK3, which in turn activates STAT5 and the PI3K/AKT pathways (32–34). IL-7 allows a basal glucose metabolism, which has been shown to be essential for quiescent T-cell survival (32).

In addition to mitochondrial glucose oxidation, resting T cells generate energy through the β-oxidation of fatty acids and especially quiescent memory subsets (**Figure 1A**). Fatty acid oxidation (FAO) is integral to the development of memory T cells, and when FAO is impeded, the memory CD8 T-cell population is heavily affected (35, 36). Pearce et al. showed that mice which lack tumor necrosis receptor-associated factor 6 (TRAF6) cannot upregulate FAO, and while these mice were able to mount a normal effector CD8 response, they were unable to form a CD8 memory population after bacterial infection (37). Additionally, TRAF6-deficient CD8 cells could not properly activate AMP-activated kinase (AMPK); however, metformin, an AMPK activator, was able to rescue FAO in the TRAF6-deficient CD8 cells and promote the development of the memory subset (37). Interestingly, the fatty acids used are not acquired from their external environment. Instead, FAO is supported in memory T cells by the synthesis of triacylglycerols (TAGs) from externally acquired glucose. IL-7 has been shown to upregulate the expression of glycerol channel aquaporin 9 (AQP9) and increased TAG synthesis in antigen-experienced CD8 T cells. The resulting TAGs are hydrolyzed by lysosomal acid lipase into free fatty acids for FAO (36, 38). The generation of potent CD8 memory T cells is also dependent on mTOR-related signaling since rapamycin, a mTOR inhibitor, promotes memory T-cell differentiation (39, 40). Rapamycin treatment during the CD8 expansion phase has been shown to increase the resulting quantity of CD8 memory cells; furthermore, rapamycin given during the contraction phase results in memory CD8 cells that are highly functional and can mount a better memory response (39, 41, 42).

Activation of CD8 T Cells Leads to Metabolic Reprogramming

Proliferation and potent effector functions are an energetically demanding process that requires a metabolic adaptation in order to fulfill the needs of T cells. Upon activation, CD8 T cells reprogram their metabolism from OXPHOS to aerobic glycolysis and glutaminolysis (**Figure 1B**) (5). This glycolytic shift supports the rapid proliferation of activated lymphocytes not only in terms of energy requirement but also in terms of metabolic intermediates (43). These metabolic intermediates allow the biosynthesis of macromolecules essential for the massive cell growth and proliferation required as effector CD8 cells expand during the immune response. For example, these intermediates generated from the glycolytic pathway are used to generate nucleotides, amino acids, and lipids, which in turn will be used to synthesize nucleic acids, lipids, and proteins needed in order to duplicate the cellular biomass (8, 43).

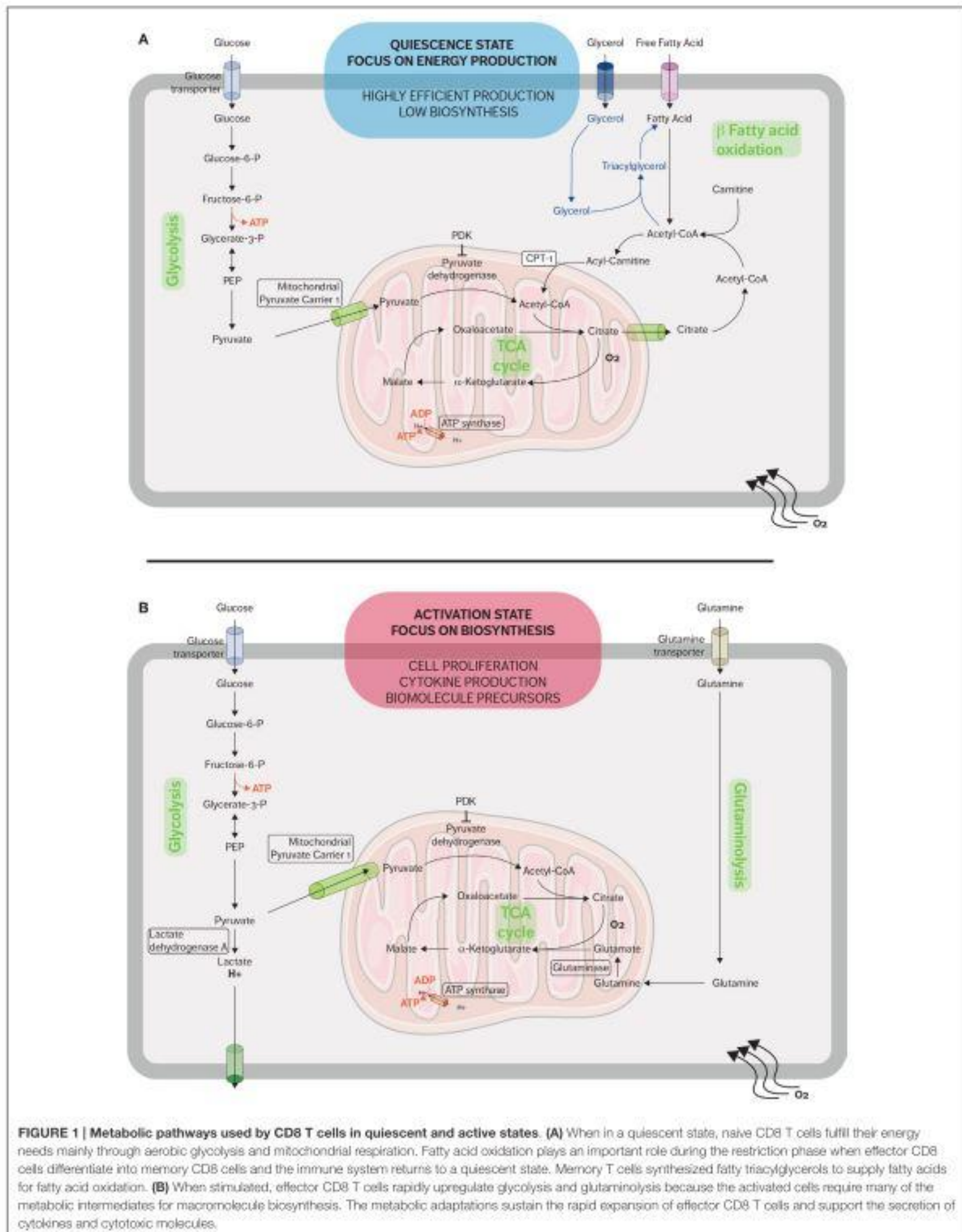


FIGURE 1 | Metabolic pathways used by CD8 T cells in quiescent and active states. **(A)** When in a quiescent state, naive CD8 T cells fulfill their energy needs mainly through aerobic glycolysis and mitochondrial respiration. Fatty acid oxidation plays an important role during the restriction phase when effector CD8 cells differentiate into memory CD8 cells and the immune system returns to a quiescent state. Memory T cells synthesized fatty triacylglycerols to supply fatty acids for fatty acid oxidation. **(B)** When stimulated, effector CD8 T cells rapidly upregulate glycolysis and glutaminolysis because the activated cells require many of the metabolic intermediates for macromolecule biosynthesis. The metabolic adaptations sustain the rapid expansion of effector CD8 T cells and support the secretion of cytokines and cytotoxic molecules.

T-cell activation requires two signals: one from the T-cell receptor (TCR) and the other from costimulatory receptors. TCR signaling alone is not sufficient for inducing major changes to the metabolic programming (44). The increased glucose metabolism seen in activated lymphocytes is due to CD28 costimulation signaling, which in turn activates the PI3K/AKT pathway (**Figure 2**) (44–46), leading to an increase of nutriment uptake, cell-surface expression, and function of glucose transporter. However, it is becoming apparent that there are specific metabolic adaptations dependent on the T-cell subset. Gubser et al. showed that the effector memory CD8 T cells are able to switch to glycolysis more rapidly as compared to naive CD8 T cells (47). Furthermore, effector memory CD8 T cells are able to sustain this higher glycolytic rate. Glycolysis has also been implicated in posttranscriptional control of cytokine secretion in activated lymphocytes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an enzyme involved in the glycolytic process and has been shown to act as an mRNA-binding protein. Chang et al. demonstrated that interferon-γ (IFNγ) secretion was suppressed in activated lymphocytes which were cultured in galactose and thus could not utilize the glycolytic pathway. The depression in the IFNγ secretion is due to the binding of GAPDH to IFNγ transcripts. Therefore, glycolysis in lymphocytes has a secondary role as a regulator of GAPDH binding of IFNγ (48). The drastic diversion of energy generation from OXPHOS to aerobic glycolysis induced by T-cell activation may be oversimplified as a higher mitochondrial mass of memory CD8 T cells compared to naive CD8 T cells has been reported and linked to a higher enhancement of both OXPHOS and glycolysis in memory CD8 T cells (49).

In addition to increased glycolytic activity, activated T cells also upregulate glutamine metabolism (glutaminolysis). Glutamine, a common amino acid found in human plasma, can serve as an alternative source of energy. Activated lymphocytes require glutamine for cell proliferation and cytokine secretion, and CD28 costimulation enhances glutamine uptake and increases glutamine transporter expression (50, 51). Several metabolic TCA cycle intermediates, such as citrate, α-ketoglutarate, and oxaloacetate, are crucial for proliferation as precursor for lipid and amino acids synthesis. Glutamine undergoes anaplerotic reaction to produce oxaloacetate and α-ketoglutarate metabolism through glutamate. Glutaminolysis also replenishes NADPH pool, which is also needed by proliferating lymphocytes to support lipid and nucleotide biosynthesis as well as maintaining the redox balance (51, 52).

METABOLIC TARGETS FOR IMMUNOMODULATING CD8 T CELLS

There are two possible approaches to manipulating the metabolic programming of CD8 cells as a means of immunosuppression for transplantation. The first would be to target metabolic pathways that are involved in cytokine production and cytotoxic activity of effector and effector memory cells in order to shut down CD8 T cells' ability to cause damage to allografts (**Figure 2**). The other method would be to target metabolic pathways to enhance and promote the proliferation of CD8 Tregs, which will in turn cause a suppressive effect on the CD8 effector population, thus prolonging allograft half-life and possibly favoring tolerance induction.

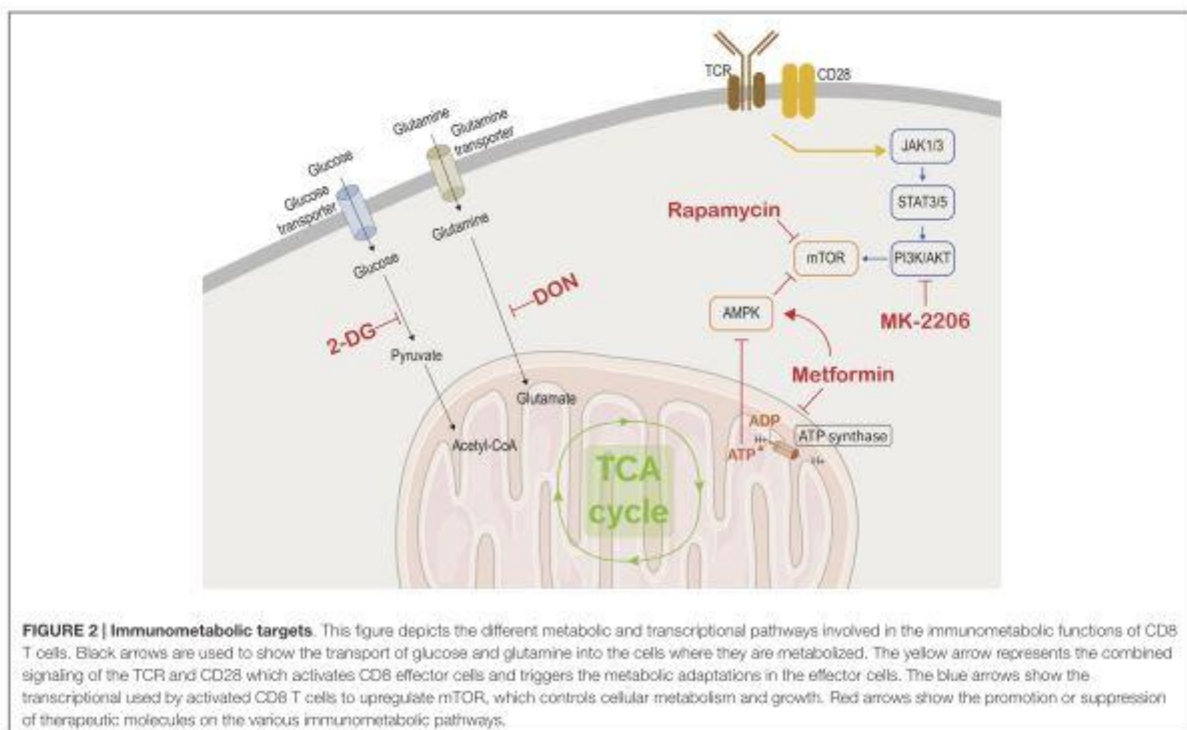


FIGURE 2 | Immunometabolic targets. This figure depicts the different metabolic and transcriptional pathways involved in the immunometabolic functions of CD8 T cells. Black arrows are used to show the transport of glucose and glutamine into the cells where they are metabolized. The yellow arrow represents the combined signaling of the TCR and CD28 which activates CD8 effector cells and triggers the metabolic adaptations in the effector cells. The blue arrows show the transcriptional used by activated CD8 T cells to upregulate mTOR, which controls cellular metabolism and growth. Red arrows show the promotion or suppression of therapeutic molecules on the various immunometabolic pathways.

Shutting Down the Warburg Effect in Effector CD8 T Cells

As previously discussed, glycolysis and glutaminolysis are two key metabolic pathways that are imperative for proper CD8 effector function. Finding methods to interrupt these pathways in allograft infiltrating effector cells would be a mean stopping allograft rejection. 2-Deoxy-D-glucose (2-DG) is a glucose analog that inhibits glycolysis by blocking hexokinase function. Many studies showed the potent effect of 2-DG in inhibiting the cytotoxic function of effector CD8 cells (47, 53–55). In a similar fashion, blocking glutaminolysis with a glutamine antagonist such as 6-diazo-5-oxo-L-norleucine (DON) inhibits lymphocyte proliferation (56). Additionally, blocking glucose and glutamine transporters disrupt lymphocyte activation and affect memory differentiation (57–60).

Targeting major bioenergetic pathways seems at first glance a rather dangerous means to control an immune response and major side effects such as a massive toxicity to normal tissue is expected. Animal models and ongoing therapeutic use of metabolic interferences therapies prove that such strategy is safe and feasible. For instance, leflunomide and its active metabolite teriflunomide prevent *de novo* biosynthesis of pyrimidine and efficiently inhibit the development of EAE (61). In multiple sclerosis patients, teriflunomide, compared to placebo, significantly reduces relapse rates, disability progression (at the higher dose), and MRI evidence of disease activity (62). Interestingly, whereas a link with metabolism has not been established, leflunomide or analogs have also been shown to be effective in prolonging graft survival and even to induce tolerance in a model of heart allograft transplantation (63). Metabolic interferences have been mainly studied in the cancer field. Given the similarity of metabolic adaptation between cancer cells and activated T cells, immunometabolic regulation of CD8 T cells could be used as a means to manipulate the CD8 T-cell immune function for effective immunosuppression. However, the doses required to eradicate all malignant cells and those required to control auto-or alloreactive T cells might be radically different. Cancer therapy aims to eradicate all cancer cells, whereas transplant therapy aims to control alloreactive T cells. Given their effector nature, alloreactive T cells are likely to be characterized by a higher use of glycolysis as compared to quiescent T cells. The selective targeting of glycolysis processes will thus focus preferentially alloreactive and not quiescent (naive or memory) T cells.

Targeting Transcriptional Regulators of Immunometabolism

Since metabolic adaptation is required to support T-cell activation and function, nutrient availability or limitation will affect these processes. A recent publication has shown that adenosine monophosphate-activated protein kinase (AMPK) couples T-cell function to nutrient availability (64). AMPK is a serine-threonine kinase that is sensitive to energy levels and is activated during cellular stress. By sensing the AMP/ATP ratio, AMPK senses energy deficiency and favors pathways leading to ATP production while inhibiting ATP-consuming pathway. It increases catabolic processes and inhibits anabolic processes to increase ATP production when activated. AMPK upregulates fatty acid β-oxidation by promoting the transfer of long-chain fatty acids into the mitochondria via carnitine palmitoyltransferase 1 (CPT1) (5). Metformin, a drug commonly used in diabetes treatment, blocks mitochondrial complex I, which has the downstream effect of promoting AMPK activity. Interestingly, metformin fosters memory CD8 T-cell differentiation in mice (37). In agreement with these results, it has been shown that autoreactive T cells can be efficiently controlled by the coadministration of 2-DG and metformin in a mouse model of systemic lupus erythematosus (SLE) (65). However, as meformin also inhibits OXPHOS, *in vitro* or *in vivo* administration is likely to have a broader target than solely the memory compartment.

The PI3K/AKT pathway is another key pathway that integrates immune stimulation and nutrient uptake (9). Blocking PI3K/AKT pathway would therefore be another way to suppress the effector function of CD8 cells. The core kinases of this pathway are AKT, AMPK, and mTOR. An intimate positive and negative cross-regulation of these protein kinases has been shown and this topic has been covered in-depth in several publications (5, 6, 9). AKT pathway is optimally activated by the coligation of TCR and CD28 and leads to the increase of glycolysis, via an increase of glucose uptake and the enhancement of rate-limiting glycolytic enzymes hexokinase and phosphofructokinase (66). Preventing activation of this pathway could be accomplished by inhibiting the costimulation signaling provided by CD28 or by directly inhibiting AKT activity. FR104, an anti-CD28 antagonist antibody, has been shown to prevent lymphocyte activation and proliferation in a murine model (67). Furthermore, this drug has been shown to be effective in reducing allograft rejection in both murine and non-human primate models of transplantation (68, 69). Another possible strategy would be to target AKT directly through the use of one of the AKT inhibitors currently in development (70). For example, AKT inhibitor MK-2206 treatment in a murine model increased the differentiation of naive CD8 cells into central memory CD8 cells and diminished terminal differentiation in the CD8 population (71). Additionally, AKT can be modulated upstream by inhibiting focal adhesion kinase (FAK), a well-established regulator of the PI3K/AKT pathway. Inhibiting FAK in Ewing sarcoma cells results in downregulation of both AKT and mTOR and impaired cell growth and colony formation (72, 73). Donor lymphocyte infusions following allogeneic stem cell transplantation are performed to enhance the graft-versus-tumor (GVT) effect, and minor histocompatibility antigen (MiHA)-specific CD8 T cells play an important role in this GVT response. It has been hypothesized that adoptive MiHA-specific CD8 T-cell transfer would lead to a more efficacious GVT response while also minimized graft-versus-host disease (GVHD), a harmful effect which is also observed in allogeneic stem cell transplant patients. AKT signal inhibition during *ex vivo* priming of naive precursor cells resulted in the generation of stem cell-like MiHA-specific CD8 T cells. Additionally, these cells have a superior proliferation capacity and antitumor effects in a murine model (74). While clinical research on these developmental AKT inhibitors has been mainly focused on the field of oncology and allogeneic stem cell transplantation, there could be possible use for them in the solid organ transplantation field.

Boosting Treg Populations with Metabolic Programming

In the field of CD4 Tregs, there has been a lot of progress in finding viable ways to use them in clinical practices. Several clinical trials are currently in progress to evaluate the safety and efficacy of adoptive transfer of CD4 Tregs in allograft transplant recipients (75). However, studies concerning CD8 Tregs are at more exploratory stages.

Rapamycin is an antifungal macrolide that is produced by bacteria discovered on Easter Island which targets and inhibits mTOR and is already used as a part of immunosuppressive regimens in transplantation. mTOR is a serine/threonine kinase that regulates cell survival, growth, and energy metabolism. mTOR can form two distinct complexes, mTOR complex 1 (mTORC1), which is rapamycin sensitive, and mTOR complex 2 (mTORC2), which is rapamycin insensitive and much less studied compared to mTORC1 (41, 76–79). mTORC1 is the master regulator of cell growth and metabolism and can be activated through either PI3K or AKT signaling. Upon activation, mTORC1 promotes ribosome biogenesis and increases protein translation and synthesis (6, 78, 79). mTORC1 also promotes lipid biosynthesis and regulates mitochondrial metabolism and biogenesis through sterol regulatory element-binding proteins (SREBP) which are involved in positively regulating lipid homeostasis (80, 81). Rapamycin targets and inhibits mTOR activity, thus interfering with G1 phase cell cycle activity and inhibits interleukin-2 (IL-2)-driven proliferation in T lymphocytes. This leads to a potent immunosuppressive effect. Consequently, mTOR inhibitors have been adopted into use as a part of immunosuppressive regimens in solid organ transplantation (82, 83). In addition to the immunosuppressive effects of rapamycin, it has also been shown to induce the preferential growth of $CD4^+CD25^+FOXP3^+$ Tregs *in vitro* (84). Evidence is emerging that rapamycin treatment *in vitro* can also induce $CD8^+CD28^-$ Tregs in murine models (85), indicating that adoptive transfer strategies to induce allograft tolerance that are currently under investigation for CD4 Tregs could potentially be adjusted for CD8 Tregs.

Another immunosuppressive drug that has been shown to be able to induce preferential expansion of CD8 Tregs *in vitro* is CTLA-4-Ig belatacept, a recently approved drug for transplant immunosuppression treatment (86). A CTLA-4 antagonist would affect the metabolic function of effector CD8 cells by blocking CD28 costimulation signaling, which has the downstream effects of impeding AKT activation and downregulating glucose and glutamine uptake. Additionally, Barbon et al. showed that by first alloanergizing human PBMCs with belatacept and then allostimulating the cells caused an increase in the frequency of $CD8^+CD28^-$ T cells. Furthermore, repeated rounds of allorestimulation after alloanergization would continue to expand the $CD8^+CD28^-$ Tregs, which were able to suppress the proliferation of CD4 T cells in a dose-dependent manner (86). Additionally, Barbon et al. reported on three hematopoietic stem cell transplant recipients with a marked increase in blood circulating $CD8^+CD28^-$ T cells between days 20 and 40 post-transplantation (86). While these findings are still preliminary, it is promising evidence that CD8 Tregs could play a future role in controlling allograft rejection.

CONCLUSION

When CD8 T cells go from a quiescent to an activated state, there are also major alterations to their metabolic programming. Upregulation of glycolysis and glutaminolysis has been shown to be important not only to fulfill the energy requirements of the activated effector CD8 cells but also to provide the biosynthetic materials needed for this rapidly expanding population. Research into the immunometabolics of CD8 cells not only expands our understanding about CD8 T cells but also provides new pharmaceutical targets that could help to reduce the negative effect of these cells in an allograft immune response.

ACKNOWLEDGMENTS

The International Fellowship Grant from ProGreffe Fondation supports MY. This work was realized in the context of the IHU-Cesti project which received French government financial support managed by the National Research Agency via the "Investment into the Future" program ANR-10-IBHU-005. The IHU-Cesti project is also supported by Nantes Metropole and the Pays de la Loire Region. This work was realized in the context of the LabEX IGO program supported by the National Research Agency via the investment of the future program ANR-11-LABX-0016-01.

REFERENCES

1. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* (1999) **401**:708–12. doi:10.1038/44385
2. Appay V, van Lier RA, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A* (2008) **73**:975–83. doi:10.1002/cyto.a.20643
3. Chen X, Yanrong Q, Wu S. The Warburg effect: evolving interpretations of an established concept. *Free Radic Biol Med* (2015) **79**:253–63. doi:10.1016/j.freeradbiomed.2014.08.027
4. Warburg OH. The chemical constitution of respiration ferment. *Science* (1928) **68**:437–43. doi:10.1007/s11999-010-1534-y
5. MacIver NJ, Michalek RD, Rathmell JC. Metabolic regulation of T lymphocytes. *Annu Rev Immunol* (2013) **31**:259–83. doi:10.1146/annurev-immunol-032712-095956
6. Gaber T, Strehl C, Sawitzki B, Hoff P, Buttgereit F. Cellular energy metabolism in T-lymphocytes. *Int Rev Immunol* (2015) **34**(1):34–49. doi:10.3109/08830185.2014.956358
7. Bental M, Deutsch C. Metabolic changes in activated T cells: an NMR study of human peripheral blood lymphocytes. *Magn Reson Med* (1993) **29**(3):317–26. doi:10.1002/mrm.1910290307
8. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cells proliferation. *Science* (2009) **324**(5930):1029–33. doi:10.1126/science.1160809
9. Pollizzi KN, Powell JD. Integrating canonical and metabolic signalling programmes in the regulation of T cell responses. *Nat Rev Immunol* (2014) **14**(7):435–46. doi:10.1038/nri3701
10. Cornell LD, Neal Smith R, Colvin RB. Kidney transplantation: mechanisms of rejection and acceptance. *Annu Rev Pathol* (2008) **3**:189–220. doi:10.1146/annurev.pathol.3.121806.151508
11. Issa F, Schiopu A, Wood KJ. Role of T cells in graft rejection and transplantation tolerance. *Exp Rev Clin Immunol* (2010) **6**(1):155–69. doi:10.1586/eci.09.64

12. Bueno V, De Nefrologia D, De Medicina EP. The role of CD8 + T cells during allograft rejection. *Braz J Med Biol Res* (2002) **35**:1247–58. doi:10.1590/S0100-879X2002001100001
13. Wood KJ, Goto R. Mechanisms of rejection: current perspectives. *Transplantation* (2012) **93**(1):1. doi:10.1097/TP.0b013e31823cab44
14. Robertson H, Wheeler J, Kirby JA, Morley AR. Renal allograft rejection – in situ demonstration of cytotoxic intratubular cells. *Transplantation* (1996) **61**(10):1546–9. doi:10.1097/00007890-199605270-00023
15. Diamond AS, Gill RG. An essential contribution by IFN-gamma to CD8+ T cell-mediated rejection of pancreatic islet allografts. *J Immunol* (2000) **165**(1):247–55. doi:10.4049/jimmunol.165.1.247
16. Yamada Y, Boskovic S, Aoyama A, Murakami T, Putheti P, Smith RN, et al. Overcoming memory T-cell responses for induction of delayed tolerance in nonhuman primates. *Am J Transplant* (2012) **12**(2):330–40. doi:10.1111/j.1600-6143.2011.03795.x
17. Koyama I, Nadazdin O, Boskovic S, Ochiai T, Smith RN, Sykes M, et al. Depletion of CD8 memory T cells for induction of tolerance of a previously transplanted kidney allograft. *Am J Transplant* (2007) **7**:1055–61. doi:10.1111/j.1600-6143.2006.01703.x
18. Betjes MG, Meijers RW, de Wit EA, Weimar W, Litjens NH. Terminally differentiated CD8+ Temra cells are associated with the risk for acute kidney allograft rejection. *Transplantation* (2012) **94**(1):63–9. doi:10.1097/TP.0b013e31825306ff
19. Baeten D, Louis S, Braud C, Braudeau C, Ballet C, Moizant F, et al. Phenotypically and functionally distinct CD8+ lymphocyte populations in long-term drug-free tolerance and chronic rejection in human kidney graft recipients. *J Am Soc Nephrol* (2006) **17**:294–304. doi:10.1681/ASN.2005020178
20. San Segundo D, Ballesteros MÁ, Naranjo S, Zurbano F, Miñambres E, López-Hoyos M. Increased numbers of circulating CD8 effector memory T cells before transplantation enhance the risk of acute rejection in lung transplant recipients. *PLoS One* (2013) **8**(11):e80601. doi:10.1371/journal.pone.0080601
21. Ordonez L, Bernard I, Chabod M, Augusto JF, Lauwers-Cances V, Cristini C, et al. A higher risk of acute rejection of human kidney allografts can be predicted from the level of CD45RC expressed by the recipients' CD8 T cells. *PLoS One* (2013) **8**(7):e69791. doi:10.1371/journal.pone.0069791
22. Yap M, Boeffard F, Clave E, Pallier A, Danger R, Giral M, et al. Expansion of highly differentiated cytotoxic terminally differentiated effector memory CD8+ T cells in a subset of clinically stable kidney transplant recipients: a potential marker for late graft dysfunction. *J Am Soc Nephrol* (2014) **25**(8):1856–68. doi:10.1681/ASN.2013080848
23. Ligocki AJ, Niederkorn JY. Advances on non-CD4 + Foxp3+ T regulatory cells. *Transplantation* (2015) **99**(8):1. doi:10.1097/TP.0000000000000813
24. Ciubotariu R, Vasilescu R, Ho E, Cinti P, Cancedda C, Poli L, et al. Detection of T suppressor cells in patients with organ allografts. *Hum Immunol* (2001) **62**(00):15–20. doi:10.1016/S0198-8859(00)00226-3
25. Picarda E, Bézie S, Venturi V, Echasserieau K, Mérieau E, Delhumeau A, et al. MHC-derived allopeptide activates TCR-biased CD8+ tregs and suppresses organ rejection. *J Clin Invest* (2014) **124**(6):2497–512. doi:10.1172/JCI71533
26. Xystrakis E, Dejean AS, Bernard I, Druet P, Liblau R, Gonzalez-Dunia D, et al. Identification of a novel natural regulatory CD8 T-cell subset and analysis of its mechanism of regulation. *Blood* (2009) **104**(10):3294–301. doi:10.1182/blood-2004-03-1214
27. Suzuki M, Jagger AL, Konya C, Shimojima Y, Pryshchep S, Goronzy JJ, et al. CD8+CD45RA+CCR7+FOXP3+ T cells with immunosuppressive properties: a novel subset of inducible human regulatory T cells. *J Immunol* (2012) **189**(5):2118–30. doi:10.4049/jimmunol.1200122
28. Liu Y, Chen N, Chen G, You P. The protective effect of CD8+CD28- T suppressor cells on the acute rejection responses in rat liver transplantation. *Transplant Proc* (2007) **39**(10):3396–403. doi:10.1016/j.transproceed.2007.06.089
29. Lin YX, Yan LN, Li B, Wang LL, Wen TF, Zeng Y, et al. A significant expansion of CD8+ CD28- T-suppressor cells in adult-to-adult living donor liver transplant recipients. *Transplant Proc* (2009) **41**(10):4229–31. doi:10.1016/j.transproceed.2009.09.072
30. Li XL, Ménoret S, Bezie S, Caron L, Chabannes D, Hill M, et al. Mechanism and localization of CD8 regulatory T cells in a heart transplant model of tolerance. *J Immunol* (2010) **185**(2):823–33. doi:10.4049/jimmunol.1000120
31. Guppy M, Greiner E, Brand K. The role of the crabtree effect and an endogenous fuel in the energy metabolism of resting and proliferating thymocytes. *Eur J Biochem* (1993) **212**(1):95–9. doi:10.1111/j.1432-1033.1993.tb17637.x
32. Wofford JA, Wieman HL, Jacobs SR, Zhao Y, Rathmell JC. IL-7 promotes Glut1 trafficking and glucose uptake via STAT5-mediated activation of Akt to support T-cell survival. *Blood* (2008) **111**(4):2101–11. doi:10.1182/blood-2007-06-096297
33. Pallard C, Stegmann AP, van Kleffens T, Smart F, Venkitaraman A, Spits H. Distinct roles of the phosphatidylinositol 3-kinase and STAT5 pathways in IL-7-mediated development of human thymocyte precursors. *Immunity* (1999) **10**(5):525–35. doi:10.1016/S1074-7613(00)80052-7
34. Crawley AM, Vranjkovic A, Faller E, McGuinty M, Busca A, Burke SC, et al. Jak/STAT and PI3K signaling pathways have both common and distinct roles in IL-7-mediated activities in human CD8+ T cells. *J Leukoc Biol* (2014) **95**(1):117–27. doi:10.1189/jlb.0313122
35. Lochner M, Berod L, Sparwasser T. Fatty acid metabolism in the regulation of T cell function. *Trends Immunol* (2015) **36**(2):81–91. doi:10.1016/j.it.2014.12.005
36. O'Sullivan D, van der Windt GJ, Huang SC, Curtis JD, Chang CH, Buck MD, et al. Memory CD8(+) T cells use cell-intrinsic lipolysis to support the metabolic programming necessary for development. *Immunity* (2014) **41**(1):75–88. doi:10.1016/j.immuni.2014.06.005
37. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang LS, et al. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* (2009) **460**(7251):103–7. doi:10.1038/nature08097
38. Cui G, Staron MM, Gray SM, Ho P-C, Amezquita RA, Wu J, et al. IL-7-induced glycerol transport and TAG synthesis promotes memory CD8(+) T cell longevity. *Cell* (2015) **161**(4):750–61. doi:10.1016/j.cell.2015.03.021
39. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF. mTOR regulates memory CD8 T-cell differentiation. *Nature* (2009) **460**(7251):108–12. doi:10.1038/nature08155
40. Sipula IJ, Brown NF, Perdomo G. Rapamycin-mediated inhibition of mammalian target of rapamycin in skeletal muscle cells reduces glucose utilization and increases fatty acid oxidation. *Metabolism* (2006) **55**(12):1637–44. doi:10.1016/j.metabol.2006.08.002
41. Xu X, Ye L, Araki K, Ahmed R. MTOR, linking metabolism and immunity. *Semin Immunol* (2012) **24**(6):429–35. doi:10.1016/j.smim.2012.12.005
42. Araki K, Youngblood B, Ahmed R. The role of mTOR in memory CD8+ T-cell differentiation. *Immunol Rev* (2010) **235**(1):234–43. doi:10.1111/j.0105-2896.2010.00898.x
43. Lunt SY, Vander Heiden MG. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol* (2011) **27**(1):441–64. doi:10.1146/annurev-cellbio-092910-154237
44. Frauwirth KA, Riley JL, Harris MH, Parry RV, Rathmell JC, Plas DR, et al. The CD28 signaling pathway regulates glucose metabolism. *Immunity* (2002) **16**(6):769–77. doi:10.1016/S1074-7613(02)00323-0
45. Jacobs SR, Herman CE, Maciver NJ, Wofford JA, Wieman HL, Hammen JJ, et al. Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways. *J Immunol* (2008) **180**(7):4476–86. doi:10.4049/jimmunol.180.7.4476
46. Parry RV, Reif K, Smith G, Sansom DM, Hemmings BA, Ward SG. Ligation of the T cell co-stimulatory receptor CD28 activates the serine-threonine protein kinase protein kinase B. *Eur J Immunol* (1997) **27**(10):2495–501. doi:10.1002/eji.1830271006
47. Gubser PM, Bantug GR, Razik L, Fischer M, Dimeloe S, Hoenger G, et al. Rapid effector function of memory CD8+ T cells requires an immediate-early glycolytic switch. *Nat Immunol* (2013) **14**(10):1064–72. doi:10.1038/ni.2687
48. Chang CH, Curtis JD, Maggi LB Jr, Faubert B, Villarino AV, O'Sullivan D, et al. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* (2013) **153**(6):1239–51. doi:10.1016/j.cell.2013.05.016
49. van der Windt GJ, O'Sullivan D, Everts B, Huang SC, Buck MD, Curtis JD, et al. CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. *Proc Natl Acad Sci U S A* (2013) **110**(35):14336–41. doi:10.1073/pnas.1221740110
50. Carr EL, Kelman A, Wu GS, Gopaul R, Senkevitch E, Aghvanyan A, et al. Glutamine uptake and metabolism are coordinately regulated by ERK/MAPK during T lymphocyte activation. *J Immunol* (2010) **185**(2):1037–44. doi:10.4049/jimmunol.0903586
51. van der Windt GJW, Pearce EL. Metabolic switching and fuel choice during T-cell differentiation and memory development. *Immunol Rev* (2012) **249**(1):27–42. doi:10.1111/j.1600-065X.2012.01150.x

52. Wasinski F, Gregnani MF, Ornellas FH, Bacurau AV, Câmara NO, Araujo RC. Lymphocyte glucose and glutamine metabolism as targets of the anti-inflammatory and immunomodulatory effects of exercise. *Mediators Inflamm* (2014) **2014**:1–10. doi:10.1155/2014/326803
53. O'Sullivan D, Pearce EL. Targeting T cell metabolism for therapy. *Trends Immunol* (2015) **36**(2):1–10. doi:10.1016/j.it.2014.12.004
54. Cham CM, Gajewski TF. Glucose availability regulates IFN-gamma production and p70S6 kinase activation in CD8+ effector T cells. *J Immunol* (2005) **174**(8):4670–7. doi:10.4049/jimmunol.174.8.4670
55. Sukumar M, Liu J, Ji Y, Subramanian M, Crompton JG, Yu Z, et al. Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function. *J Clin Invest* (2013) **123**(10):4479–88. doi:10.1172/JCI69589
56. Wang R, Dillon CP, Shi LZ, Milasta S, Carter R, Finkelstein D, et al. The transcription factor myc controls metabolic reprogramming upon T lymphocyte activation. *Immunity* (2011) **35**(6):871–82. doi:10.1016/j.immuni.2011.09.021
57. Sinclair LV, Rolf J, Emslie E, Shi YB, Taylor PM, Cantrell DA. Control of amino-acid transport by antigen receptors coordinates the metabolic reprogramming essential for T cell differentiation. *Nat Immunol* (2013) **14**(5):500–8. doi:10.1038/ni.2556
58. Singer K, Kastenberger M, Gottfried E, Hammerschmied CG, Büttner M, Aigner M, et al. Warburg phenotype in renal cell carcinoma: high expression of glucose-transporter 1 (GLUT-1) correlates with low CD8+ T-cell infiltration in the tumor. *Int J Cancer* (2011) **128**(9):2085–95. doi:10.1002/ijc.25543
59. Anastasiou D, Poulogiannis G, Asara JM, Boxer MB, Jiang JK, Shen M, et al. Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. *Science* (2011) **334**(6060):1278–83. doi:10.1126/science.1211485.Inhibition
60. Macintyre AN, Gerriets VA, Nichols AG, Michalek RD, Rudolph MC, Deoliveira D, et al. The glucose transporter Glut1 is selectively essential for CD4 T cell activation and effector function. *Cell Metab* (2014) **20**(1):61–72. doi:10.1016/j.cmet.2014.05.004
61. Korn T, Magnus T, Toyka K, Jung S. Modulation of effector cell functions in experimental autoimmune encephalomyelitis by leflunomide – mechanisms independent of pyrimidine depletion. *J Leukoc Biol* (2004) **76**(5):950–60. doi:10.1189/jlb.0504308
62. O'Connor P, Wolinsky JS, Confavreux C, Comi G, Kappos L, Olsson TP, et al. Randomized trial of oral teriflunomide for relapsing multiple sclerosis. *N Engl J Med* (2011) **365**(14):1293–303. doi:10.1056/NEJMoa1014656
63. Le Texier L, Thebault P, Lavault A, Usal C, Merieau E, Quillard T, et al. Long-term allograft tolerance is characterized by the accumulation of B cells exhibiting an inhibited profile. *Am J Transplant* (2011) **11**(3):429–38. doi:10.1111/j.1600-6143.2010.03336.x
64. Blagih J, Coulombe F, Vincent EE, Dupuy F, Galicia-Vázquez G, Yurchenko E, et al. The energy sensor AMPK regulates T cell metabolic adaptation and effector responses in vivo. *Immunity* (2015) **42**(1):41–54. doi:10.1016/j.immuni.2014.12.030
65. Yin Y, Choi SC, Xu Z, Perry DJ, Seay H, Croker BP, et al. Normalization of CD4 + T cell metabolism reverses lupus. *Sci Transl Med* (2015) **7**(274):1–13. doi:10.1126/scitranslmed.aaa0835
66. Frauwirth KA, Thompson CB. Regulation of T lymphocyte metabolism. *J Immunol* (2004) **172**(8):4661–5. doi:10.4049/jimmunol.172.8.4661
67. Mary C, Coulon F, Poirier N, Dilek N, Martinet B, Blancho G, et al. Antagonist properties of monoclonal antibodies targeting human CD28. *MAbs* (2013) **5**(1):47–55. doi:10.4161/mabs.22697
68. Poirier N, Mary C, Dilek N, Hervouet J, Minault D, Blancho G, et al. Preclinical efficacy and immunological safety of FR104, an antagonist anti-CD28 monovalent fab′ antibody. *Am J Transplant* (2012) **12**(10):2630–40. doi:10.1111/j.1600-6143.2012.04164.x
69. Poirier N, Dilek N, Mary C, Ville S, Coulon F, Branchereau J, et al. FR104, an antagonist anti-CD28 monovalent fab′ antibody, prevents alloimmunization and allows calcineurin inhibitor minimization in nonhuman primate renal allograft. *Am J Transplant* (2015) **15**(1):88–100. doi:10.1111/ajt.12964
70. Alexander W. Inhibiting the Akt pathway in cancer treatment: three leading candidates. *P T* (2011) **36**(4):225–7.
71. Abu Eid R, Friedman KM, Mkrtichyan M, Walens A, King W, Janik J, et al. Akt1 and -2 inhibition diminishes terminal differentiation and enhances central memory CD8 + T-cell proliferation and survival. *OncoImmunology* (2015) **4**(5):e1005448. doi:10.1080/2162402X.2015.1005448
72. Zhao X, Guan J-L. Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. *Adv Drug Deliv Rev* (2011) **63**(8):610–5. doi:10.1016/j.addr.2010.11.001
73. Crompton BD, Carlton AL, Thorner AR, Christie AL, Du J, Calicchio ML, et al. High-throughput tyrosine kinase activity profiling identifies FAK as a candidate therapeutic target in Ewing sarcoma. *Cancer Res* (2013) **73**(9):2873–83. doi:10.1158/0008-5472.CAN-12-1944
74. van der Waart AB, van de Weem NM, Maas F, Kramer CS, Kester MG, Falkenburg JH, et al. Inhibition of Akt signaling promotes the generation of superior tumor-reactive T cells for adoptive immunotherapy. *Blood* (2014) **124**(23):3490–500. doi:10.1182/blood-2014-05-578583
75. Edozie FC, Nova-Lamperti EA, Povoleri GA, Scottà C, John S, Lombardi G, et al. Regulatory T-cell therapy in the induction of transplant tolerance: the issue of subpopulations. *Transplantation* (2014) **98**(4):370–9. doi:10.1097/TP.0000000000000243
76. Heitman J, Movva NR, Hall MN. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* (1991) **253**(5022):905–9. doi:10.1126/science.1715094
77. Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, et al. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* (1994) **369**(6483):756–8. doi:10.1038/369756a0
78. Laplante M, Sabatini DM. mTOR signaling at a glance. *J Cell Sci* (2009) **122**(Pt 20):3589–94. doi:10.1242/jcs.051011
79. Laplante M, Sabatini DM. Regulation of mTORC1 and its impact on gene expression at a glance. *J Cell Sci* (2013) **126**(Pt 8):1713–9. doi:10.1242/jcs.125773
80. DeBerardinis RJ, Lum JJ, Thompson CB. Phosphatidylinositol 3-kinase-dependent modulation of carnitine palmitoyltransferase 1A expression regulates lipid metabolism during hematopoietic cell growth. *J Biol Chem* (2006) **281**(49):37372–80. doi:10.1074/jbc.M608372200
81. Porstmann T, Santos CR, Griffiths B, Cully M, Wu M, Leevers S, et al. SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. *Cell Metab* (2008) **8**(3):224–36. doi:10.1016/j.cmet.2008.07.007
82. Sehgal SN. Sirolimus: its discovery, biological properties, and mechanism of action. *Transplant Proc* (2003) **35**(3):S7–14. doi:10.1016/S0041-1345(03)00211-2
83. Halloran PF. Immunosuppressive drugs for kidney transplantation. *N Engl J Med* (2004) **351**:2715–30. doi:10.1056/NEJMra033540
84. Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaupper T, Roncarolo MG. Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol* (2006) **177**(12):8338–47. doi:10.4049/jimmunol.177.12.8338
85. El Essawy B, Putheti P, Gao W, Strom TB. Rapamycin generates graft-homing murine suppressor CD8+ T cells that confer donor-specific graft protection. *Cell Transplant* (2011) **20**:1759–69. doi:10.1016/j.biotechadv.2011.08.021.Secreted
86. Barbon CM, Davies JK, Voskertchian A, Kelner RH, Brennan LL, Nadler LM, et al. Alloanergization of human T cells results in expansion of alloantigen-specific CD8(+) CD28(-) suppressor cells. *Am J Transplant* (2014) **14**:305–18. doi:10.1111/ajt.12575

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Yap, Brouard, Pecqueur and Degauque. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

6.3 Supplemental Article

B Cell Depletion Therapy Impacts CD8 T Cells in ANCA-Associated Vasculitis

Arthritis & Rheumatology

B cell depletion therapy impact CD8 T cells in ANCA-associated vasculitis.

Journal:	*Arthritis & Rheumatology*
Manuscript ID:	Draft
Wiley - Manuscript type:	Full Length
Date Submitted by the Author:	n/a
Complete List of Authors:	Néel, Antoine; CHU de Nantes, Médecine Interne; INSERM, UMR 1064 Bucchia, Marie; CHU de Nantes, Pediatrie Néel, Mélanie; INSERM, UMR 1064 Tilly, Gaelle; INSERM, UMR 1064 Yap, Michelle; INSERM, UMR 1064 Rimbert, Marie; INSERM, UMR 1064 AGARD, Christian; CHU de Nantes, Médecine Interne Hourmant, Maryvonne; CHU de Nantes, Néphrologie Perrin, Francois; CHU de Nantes, Médecine Interne Godmer, Pascal; CH Bretagne Atlantique, Médecine Interne Graveleau, Julie; CHU de Nantes, Médecine Interne Brouard, Sophie; INSERM, UMR 1064 Bressollette, Celine; CHU de Nantes, Virologie Hamidou, Mohamed; CHU de Nantes, Médecine Interne Fakhouri, Fadi; CHU de Nantes, Néphrologie Degauque, Nicolas; INSERM, UMR 1064
Keywords:	ANCA-associated Vasculitis, Cytotoxic Cells, B-Lymphocyte, Biologicals
<B>Disease Category: Please select the category from the list below that best describes the content of your manuscript.:	Vasculitis

SCHOLARONE™
Manuscripts

Running title : Rituximab and CD8 T cells in AAV.

B cell depletion therapy impact CD8 T cells in ANCA-associated vasculitis.

Antoine Néel[1,2,3], MD; Marie Bucchia[1,3]; Mélanie Néel[1,3]; Gaelle Tilly[1,4]; Michelle Yap[1,4]; Marie Rimbert[1,3], PharmD, PhD; Christian Agard[2,4], MD, PhD; Maryvonne Hourmant[1,3,4],MD, PhD; François Perrin[2], MD; Pascal Godmer[5], MD; Julie Graveleau[2], MD; Sophie Brouard[1,3,6,7], PharmD, PhD; Céline Bressollette[1,3,4], MD, PhD; Mohamed Hamidou[2,4], MD; Fadi Fakhouri[1,3,4], MD, PhD and Nicolas Degauque[1,3], PhD.

[1] INSERM, UMR 1064, Nantes, F-44093, France; [2] CHU de Nantes, Service de Médecine Interne, Nantes, F-44093, France; [3] CHU de Nantes, ITUN, Nantes, F-44093, France; [4] Université de Nantes, Faculté de Médecine, Nantes, F-44035, France; [5] Centre Hospitalier Bretagne Atlantique, Service de Médecine Interne, F-56017 Vannes, France; [6] CIC biothérapie, Nantes, F-44035, France; [7] CHU Nantes, CRB, Nantes, F-44093, France.

Corresponding author and reprint requests:

Dr Antoine Néel; Service de Médecine Interne, Centre Hospitalier Universitaire de Nantes – Hôtel Dieu, 1 Place Alexis Ricordeau, 44093 NANTES.

Tel: +33.2.40.08.33.55; Fax: +33.2.40.08.33.79; Email: antoine.neel@univ-nantes.fr

For Peer Review

Funding : La Société Nationale Française de Médecine Interne (AN), Groupama Loire Bretagne - Fondation Groupama pour la santé (AN), The International Fellowship Grant from ProGreffe Fondation (MY), the FP7 VISICORT, project that has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (grant agreement no 602470).

Conflict of interest: no conflict of interest exists.

Abstract

In anti-neutrophil cytoplasmic antibodies associated vasculitis (AAV), several clues suggest that the efficacy of B cell depletion therapy lies beyond the suppression of ANCA-producing cells and may involve B-T cell crosstalk. However, scarce data are available regarding the impact of rituximab on CD4, regulatory and CD8 T cells in this setting. Using polychromatic flow cytometry we performed a thorough immunophenotypic analysis of CD4, regulatory T and CD8 cells of 53 patients with AAV in order to compare the effects of conventional immunosuppressants (CIS) and rituximab (RTX). Cytokine/chemokine production of *in vitro* stimulated CD8 cells was assessed using a multiplex immunoassay. Among CD4 cells, we found that frequency of naïve and memory subsets and the expression of CCR5, CCR4 and CD161 were not influenced by maintenance treatment type. Similarly, Treg frequency and subsets including $CD161^{+}$, $Helios^{+}$, resting ($CD45RA^{+}$) and memory ($CD45RA^{-}$ $FoxP3^{hi}$) Tregs were comparable among RTX and CIS treated patients. By contrast, the type of maintenance treatment markedly influenced the CD8 T cell compartment. Indeed, RTX inhibited late differentiated effector memory ($CD45RA^{+}CCR7^{-}$) CD8 cell expansion whereas CIS had the opposite effect. Furthermore, we found that unlike CIS, B cell depletion therapy effectively inhibited pro-inflammatory cytokine/chemokine production by CD8 cells. In conclusion, B cell depletion therapy has a profound impact on the CD8 compartment. This observation raises the question whether the disruption of B cell help to CD8 cells could contribute to the dramatic efficacy of RTX. Whether B cell depletion therapy promotes CD8 cell exhaustion and/or inhibits immunosenescence deserves further investigations.

For Peer Review

Introduction

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) are a group of systemic auto-immune diseases characterized by necrotizing small/medium-vessel vasculitis and/or extravascular inflammation of the respiratory tract and the presence of ANCA targeting Proteinase 3 (PR3) or Myeloperoxydase (MPO). Microscopic polyangiitis (MPA) and Granulomatosis with polyangiitis (GPA) are the 2 most frequent AAV subtypes, which predominantly associates with anti-MPO and anti-PR3 ANCA, respectively (1).

These life-threatening diseases usually affect middle-aged or older adults to the elderly. Until very recently, treatment relied on the use of corticosteroids and conventional immunosuppressants (CIS) such as Cyclophosphamide (CYC), Azathioprine (AZA), Methotrexate (MTX) or Mycophenolate (MMF). These agents contributed to improve patients' survival in the past decades. Unfortunately, relapses remained a frequent issue. Furthermore, patients with AAV have an increased risk of cardiovascular diseases, infection and cancer resulting from therapeutic immunosuppression and chronic inflammation (2). In the past few years, rituximab (RTX) has emerged as a dramatically effective agent in AAV. In 2010, the RAVE trial found that RTX was as effective as CYC for remission induction (3). More recently, the MAINRITSAN trial demonstrated that long term B cell depletion therapy was even superior to CIS for remission maintenance (4).

The efficacy of RTX demonstrates that CD20 B cells play a critical role in the pathophysiology of the disease. Several clinical clues and immunological data from other autoimmune diseases suggest that the efficacy of B cell depletion therapy lies beyond the suppression of auto-antibodies producing cells. Indeed, B cells exhibit antibody-independent functions (cytokine production, antigen presentation) that can

For Peer Review

impact many effector cells, including T cells **(5)**. However, there is little data on comparing the immunological effect of RTX with that of CIS, and even less regarding the impact of RTX on T cells in AAV (6).

The primary goal of this cross-sectional study was to compare the impact of RTX and CIS on the T cell compartment in AAV. We found that patients receiving a CIS or RTX maintenance treatment exhibited similar CD4 and regulatory T cell subsets, including CD161^{+}, Helios^{+}, resting and memory Tregs. By contrast, RTX treated patients were comparable to patients in long term remission off therapy and had a decreased proportion of terminally differentiated effector memory CD8 T cells (CD45RA^{+}CCR7^{-}, i.e TEMRA cells) as compared to those receiving a CIS, which was not explained by age or CMV status. Furthermore, longitudinal analysis confirmed that the proportion of CD8 TEMRA cells increased under CIS and decreased with RTX. Finally, *in vitro* cytokines/chemokines production of CD8+ T cells from patients in remission with RTX was lower than those from patients with active disease and/or in remission under CIS.

Overall, our results demonstrate that as opposed to CIS, B cell depletion therapy inhibits CD8 TEMRA expansion and CD8 T cell cytokines/chemokines production. In view of the recent reappraisal of the role of CD8 T cells in auto-immunity, our results suggest that disrupting B and CD8 T cell interactions could contribute to the dramatic efficacy of RTX in AAV. Furthermore, since CD8 TEMRA cells expansion have been linked to immunosenescence and cardiovascular risk, our findings also raise the question whether the benefit of B cell depletion therapy could extend to a reduction of cardiovascular comorbidities of AAV patients.

Material and Methods

Inclusion criteria

Inclusion criteria were a diagnosis of MPA or GPA according to 2012 Chapel Hill criteria (1). Patients with active disease (at diagnosis or relapse) received no treatment (including steroids). Patients in remission received either no treatment (n=9), maintenance Rituximab (RTX) (500mg every 6 months; n=18) or oral immunosuppressant (IS; n=14), namely azathioprine (AZA), methotrexate (MTX) or mycophenolate mofetil (MMF), +/- low dose corticosteroids (<10mg/d) for >3 months. Cases were categorized as GPA or MPA according to the EMEA algorithm **(7)**. EGPA patients and those that received renal replacement therapy were excluded. Disease activity was assessed using the Birmingham Vasculitis Activity Score (BVAS) (8). Active disease was defined by BVAS>3 that required therapeutic intervention. Remission was defined by a BVAS=0 for 3 months with no evidence of systemic inflammation, that allowed stable or tapered maintenance treatment. Clinical data of remission patients are reported in **Table 1**. Clinical data of patient with active disease are reported in **supplementary Table 1**. The study was performed in accordance with Helsinki declaration and with Nantes University Hospital Ethics Committee approval. Written informed consent was obtained from each subject.

For Peer Review

Blood Sample and CMV status

PBMCs were separated on a Ficoll gradient layer and frozen in 10% DMSO autologous serum. The presence of anti-CMV IgG, ie latent CMV infection, was determined by chemiluminescent immunoassay (DiaSorin) on frozen sera.

Polychromatic Flow Cytometry

Cells were analyzed with an LSRII flow cytometer (BD Immunocytometry Systems); frozen PBMCs were surface-stained with 4 different antibodies panels. CD4 T cell immunophenotyping panel included CD3 (OKT3; Brilliant Violet 605), CD4 (L200; PerCP-Cy5.5), CD45RA (T6D11; APC-Vio770), CCR7 (3D12; PE-Cy7), CCR4 (1G1; PE), CCR5 (2D7/CCR5; FITC) and CD161 (191B8; PE-Vio770). Regulatory T cell immunophenotyping panel included CD3 (OKT3; Brilliant Violet 605), CD4 (RPA-T4; Pacific Blue), CD25 (BC96; Brilliant Violet 421), CD127 (A019D5; PE), FoxP3 (236A/E7; Alexa Fluor 647), Helios (22F6; Alexa Fluor 488), CD45RA (T6D11; APC-Vio770) and CD161 (191B8; PE-Vio770). For CD8 T cell analysis, 2 panels were used, which included CD3 (BW264/56; VioBlue), CD8 (BW135/80; VioGreen), CD45RA (T6D11; APC-Vio770), CCR7 (3D12; PE-Cy7), CD27 (L128; Brilliant Violet 605), CD28 (CD28.2; PE-CF594) and either CD57 (TB03; FITC) and T-bet (O4–46; PE) or GZM-B (GB11; Alexa Fluor 700) and PERF (B-D48; PE). Yellow LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) was used to exclude dead cells from analysis. BD CompBeads stained separately with individual mAbs were used to define the compensation matrix. Data were analyzed using FlowJo Version 9.0.1 (TreeStar). All the antibodies were purchased from BD Biosciences, except for anti-CD3, -CD8, -CD45RA, -CD161 and -CD57 mAbs (Miltenyi), anti-Helios, Brilliant violet anti-CD3 and -CD25 (BioLegend), anti-FoxP3 (eBioscience) and anti-PERF mAb (Diaclone).

FACS sorting, cell culture and multiplex cytokine production measurement

PBMCs were thawed and rested overnight in TexMacs medium (Miltenyi). PBMCs were then stained with anti-CD3 (UCHT1; FITC), -CD4 (RPA-T4, APC), -CD8 (RPA-T8 ; PE) mAbs (BD Biosciences). CD3^{+}CD8^{+} were FACS-sorted using a FACSAria

For Peer Review

(BD Biosciences; purity greater than 95%) and then stimulated for 4 hours with 100ng/ml_Phorbol myristate acetate (PMA; Sigma-Aldrich) and 1µg/ml Ionomycine (Sigma-Aldrich) in 96-well round bottom plates (concentration: $5x10^6$cells/ml ; volume : 100µl) at 37°C in 5% CO_2. The production of 34 cytokines and chemokines was measured in culture supernatant using a multiplex Luminex immunoassay (ProcartaPlex Human Cytokine & Chemokine Panel 1A; Affymetrix).

Statistical Analysis

For Peer Review

Univariate analysis involved χ^2 or Fischer exact test as appropriate to compare categorical variables and the nonparametric Mann-Whitney test to compare continuous variables. One-way analysis of variance was carried out using non-parametric Kruskal–Wallis test and Dunn's post-test for multiple comparisons.

Results

Cohort characteristics

A total of 53 patients were included. During this 2011-2013 period, the decision to recourse to RTX or IS as maintenance treatment was not standardized but depended upon available evidence as well as physician preferences. There were 26 men and 27 women. Mean age was 67 years (IQR: 61-79). Mean estimated CKD-EPI glomerular filtration rate (eGFR) was 58 ml/min (IQR: 26-88). Thirty-one patients (58%) had GPA and 22 (42%) MPA, 27 (51%) anti-PR3 ANCA and 25 (47%) anti-MPO ANCA. One patient had ANCA negative biopsy-proven GPA. Among 60 samples, 20 were obtained from patients with untreated active disease (at diagnosis in 18 cases) and 40 from patients in remission under maintenance treatment with either RTX (n=17) or CIS (n=14, AZA in 10, MTX in 2 and MMF in 2), or during drug

free remission (n=9). Seven patients were analyzed at 2 time points, first while untreated (during active disease in 6) and then in remission under RTX or CIS. All RTX-treated patients had received a 500mg infusion the past 6 months and had undetectable B cells (<5/mm3). All other remission patients had never received rituximab.

Most key clinical data were comparable among patients treated with RTX vs CIS, including demographics, disease and ANCA type, past exposure to cyclophosphamide and renal function (**Table 1**). Patients treated with RTX tended to have suffered more relapses as compared to patients receiving CIS (**Table 1**), despite this difference was not statistically significant (**Supplementary Figure 1**). By contrast, drug free patients had more prolonged remission duration as compared to CIS or RTX treated patients (median: 46 vs 10 and 7 months, respectively, p=0.01 and 0.001). Furthermore, most of these Long Term Remission Off Therapy (LTROT) patients had suffered a unique disease flare before entering remission, whereas patients treated with CIS or RTX had a more frequent history of relapse (70%, for RTX, 12% for LTROT, p=0.01).

Patients in remission with conventional Immunosuppressant or rituximab exhibit similar CD4 T cells subsets distributions.

We first assess whether the disease activity and/or the maintenance therapy impact the distribution of the different CD4+ T cell subsets. CCR7 and CD45RA expression were used to identify naïve ($CD45RA^+CCR7^+$; Tn), central memory ($CD45RA^-CCR7^+$; Tcm), effector memory ($CD45RA^-CCR7^-$; Tem) and late differentiated effector memory ($CD45RA^+CCR7^-$; TEMRA) cells (9). Patients in remission had a decreased proportion of naïve CD4 T cell (**Figure 1A**; p=0.002) and an increase in effector

For Peer Review

memory cells (**Figure 1A**; p=0.001) as compared to patients with active disease. When the analysis was narrowed to remission patients, we found that RTX, CIS and LTROT patients had comparable distributions of CD4+ T cell subsets (**Figure 1B**).

The expression of CCR5, CCR4 and CD161 was then monitored as surrogate markers for Th1(10), Th2 (10) and Th17(11) cells respectively. As expected, CCR5, CCR4 and CD161 were mainly expressed on effector memory cells (**Figure 1C; upper panel**). Their level of expression was analyzed on total CD4+ cells as well as on Tn, Tcm, Tem and TEMRA subsets. No difference was observed between CIS, RTX and LTROT patients (**Fig 1C; lower panel**). In conclusion, RTX had no marked effect on the CD4+ T cell phenotype when compared to conventional treatments.

Rituximab has no significant impact on regulatory T cells subsets distributions.

Several groups have reported on quantitative and/or functional Treg deficiency in AAV (12–15). Several studies have also reported an increase in Treg frequency and/or suppressive function following Rituximab treatment in autoimmune diseases (6,16–19), albeit conflicting results have also been reported (20–23). A recent study suggested that RTX increased Treg frequency in patients with GPA (6). In our cohort, the frequency of $CD25^{hi}CD127^{lo}FoxP3+$ Tregs was not significantly different in CIS, RTX and LTROT patients (**Figure 2,** p=0.195).

Several Treg cell subsets have been identified in the recent years. According to the expression of CD45RA and Foxp3, 3 populations of FoxP3 expressing CD4 T cells have been described, which exhibit distinct functional properties as well as Foxp3 locus methylation level (24). $CD45RA^{+}$ cells and $CD45RA^{-}FoxP3^{hi}$ cells are

For Peer Review

considered as *bona-fide* Treg cells (naïve and memory, respectively) whereas the CD45RA$^-$FoxP3^{lo} population consists of non-suppressor cells with pro-inflammatory cytokines secretion potential. The expression of CD161 has also been reported to identify a subset of Treg cell capable of producing pro-inflammatory cytokines, including IL-17 (25,26). Similarly, cytokine-producing Tregs have been reported to lack the expression of Helios. The latter marker has been controversially claimed to identify thymus-derived as opposed to peripheral-derived Treg, despite controversy remains (27–29). The frequencies of the aforementioned subsets of Treg were similar between patients with active disease and patients in remission as well between RTX, CIS and LTROT patients (**Figure 2**, p>0.05). Furthermore, Treg cell frequency did not increase following treatment with RTX. Overall, RTX had no distinctive impact on the frequency of Treg cells and their subsets as compared to CIS.

For Peer Review

CD8 T cell subsets variations according to maintenance treatment group

We then analyzed the frequency of CD8 T cell subsets according to disease activity and maintenance treatment. Unlike for CD4 T cells, the distribution of CD8 T cell subsets (Tn, Tcm, Tem, TEMRA) was similar in patients with active disease as compared to those in remission (**Supplementary Figure 2**). However, when focusing on remission patients, we observed significant variations in CD8 T cell subsets frequencies in CIS, RTX and LTROT patients (**Figure 3A**, p<0.05). LTROT patients had a higher proportion of naïve T cells as compared to patients receiving maintenance treatment. Interestingly, even though they had more relapsing diseases, patients receiving RTX had a CD8 memory T cell compartment that more closely resembled to the one of LTROT as opposed to that of CIS patients. Indeed, RTX and

LTROT patients had less TEMRA and more Tem cells than CIS patients. The profound imbalance between CD8 TEMRA and Tem was further highlighted by the monitoring of TEMRA/Tem ratio (**Figure 3A**). LTROT and RTX patients had a strikingly lower TEMRA/Tem ratio as compared to CIS patients (0.72 and 0.69 vs 2.98 respectively; $p<0.05$; **Figure 3A**). We then assessed whether the phenotypic features of TEMRA CD8 T cells were similar across the 3 groups of patients in remission. As expected (10,30,31), TEMRA CD8 cells were predominantly $CD28^-$ $CD27^-$ with a high expression of perforin, granzyme B, CD57, and Tbet as compared to $CD45RA^-$ effector memory cells (**Figure 3B**). No significant differences among treatment groups were seen for the expression of prototypic markers of TEMRA CD8 T cells (**Figure 3B,** $p>0.05$).

Hence, LTROT patients and those receiving with RTX exhibited a lower proportion of TEMRA CD8 cells as compared to CIS treated patients, with no significant change of TEMRA phenotype.

B cell depletion therapy and conventional immunosuppressant have opposite effect on the CD8 TEMRA pool, irrespective of CMV status.

Previous studies performed in older adults have demonstrated that both age and latent CMV infection concur to TEMRA expansion in healthy individuals, both of which being interrelated (32,33). Two recent studies have also revealed that CD4 and CD8 TEMRA frequency is critically influenced by latent CMV infection in AAV (34,35). Accordingly, we found that latent CMV infection had a major impact on CD8 T cell subsets in our patients and was associated with an increased proportion of TEMRA cells (**Figure 4A**). Indeed, among the entire cohort, the frequency of TEMRA CD8 cells was 20.5% and 48.5% in CMV^- and CMV^+ patients respectively ($p<0.001$;

For Peer Review

Figure 4A). A weak positive correlation was also observed between the TEMRA frequency and age, regardless the CMV status. Given the heterogeneous frequency of TEMRA in patients in remission according to the maintenance therapy, we investigate whether the increase of TEMRA CD8 T cells observed in CIS group was related to a higher prevalence of CMV infection or the age of patients. In the CIS, RTX and LTROT groups, the frequency of latent CMV infection was similar (8/14 (57%), 6/17 (35%) and 2/8 (25%), respectively) as well as patients' age (**Table 1**). Hence, neither age nor CMV status could explain the difference of TEMRA cell frequency observed across treatment groups.

For Peer Review

Unlike previous reports (36,37), we found no correlation between disease duration and TEMRA frequency in our cohort (**Figure 4B**, p=0.35). Furthermore, disease phenotype, ANCA type, past exposure to cyclophosphamide, number of previous relapses, or renal function did not correlate with TEMRA frequency (**Figure 4B**). We then analyzed the frequency of TEMRA CD8 T cells according to the CMV status in the 3 groups of patients in remission. We found that CMV^+ individuals receiving RTX had lower TEMRA cell frequency as compared to those receiving a CIS (median: 47.2 vs 69.5 %, respectively, p=0.035; **Figure 4C**). On the other hand, there were not significant differences in the CMV negative individuals. In order to better understand the respective impact of RTX and CIS on TEMRA expansion and the role of CMV infection, we performed a longitudinal analysis of 7 patients before and after introduction of the 2 types of maintenance therapeutic regimen (CIS or RTX). All patients receiving RTX as maintenance therapy exhibit a decrease in the frequency of TEMRA CD8 T cells irrespectively to their CMV status. In contrast, an increase of TEMRA CD8 T cell was observed after initiation of CIS (**Figure 4C**), despite these patients were CMV negative. Collectively, these results demonstrate that irrespective

of CMV status, RTX inhibits TEMRA expansion in AAV patients, whereas CIS have the opposite effect. Further, CIS and CMV infection have synergistic effects on TEMRA expansion.

B cell depletion therapy decreases CD8 T cell cytokine production as opposed to conventional immunosuppressants.

In order to determine whether the opposite effect of RTX and CIS on the CD8 T cells compartment had functional consequences, we assessed the *in vitro* cytokine and chemokine production of purified CD8 T cells stimulated with PMA and ionomycin, using a 34plex immunoassay. We found that CD8 T cells from patients receiving RTX produced lower levels of pro-inflammatory cytokine IFN-γ and chemokines (MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, IP-10/CXCL10, SDF-1α/CXCL12a, Eotaxine) compared to patients with active disease an/or those receiving a CIS (**Figure 5A**). Importantly, CD8 cells from patients in remission under CIS produced similar levels of cytokine/chemokine when compared with those of patients with untreated active disease.

IFN-γ expression strongly correlated with different cytokine and chemokine expression (**Figure 5B**). IFN-γ production could thus be used as a prototypic marker of inflammatory response and the correlation between its levels and the CMV status, age or renal function was tested. Of interest, none of these factors correlated with the level of pro-inflammatory cytokine IFN-γ. Finally, the frequency of TEMRA CD8 T cells did not correlate with the production of IFN-γ, suggesting that the effect of RTX on the CD8 T cell compartment lies beyond the limitation of TEMRA expansion (**Fig 5B**). These finding demonstrate that, unlike CIS, B cell depletion therapy effectively inhibits the pro-inflammatory potential of CD8 T cells in AAV patients.

Discussion

Herein we report the result of a cross sectional study of the T cell compartment phenotype in patients with AAV. The main objective of our study was to compare the impact of RTX and CIS on CD4 T cells, regulatory T cells and CD8 T cells.

The first finding of our study was that patients receiving RTX or CIS had similar CD4 and regulatory T cells subsets. Several investigators have reported that AAV patients' regulatory T cells had a decreased suppressive function (12–15), even in remission and in the absence of any immunosuppressant (13). As for B cell depletion therapy effects on Treg cells, contradictory data have been reported in studies performed in various settings such as cryoglobulinemic vasculitis (16), rheumatoïd arthritis (20,23), systemic lupus (18,19), autoimmune thrombocytopenia (17). Importantly, most of the mentioned studies were based on uncontrolled longitudinal studies using simple staining strategies for Treg cell identification. Furthermore, functional assay have been rarely performed (17,18). Herein, we report the result of an in-depth phenotypic analysis of Treg cells of AAV patients including Helios^{+}, CD161^{+}, resting and memory subsets. We found no clear impact of B cell depletion therapy as compared to CIS. Recently, Zhao et al. compared the frequency of Treg cells and CD45RA/CD25 expression defined subsets in GPA patients under various conditions (active vs inactive, CIS vs RTX). They found that the frequency of Tregs in GPA patients following RTX was similar to that of healthy controls, while GPA patients receiving CIS had a reduced frequency of Treg cells (6). They also found a trend for Treg frequencies to increase following RTX. By contrast, we found no significant difference in Treg cell frequency in patients receiving RTX or CIS, and RTX did not increase following RTX in our patients. Of note, the increase in Treg cells (CD4^{+}CD25^{+}CD127^{-} T cells) noticed by Zhao et al. was mainly driven by

For Peer Review

CD45RA^{-}CD25^{+} cells, which are probably not *bona fide* Tregs (24). Interestingly, a recent study demonstrated that in tumor bearing mice, RTX could even dampen Treg expansion (38). Overall, further studies are needed to determine whether RTX impact the Treg cell compartment in patients with autoimmune diseases.

The main finding of our study was that RTX and CIS had opposite effects on the CD8 T cell compartment. TEMRA cells were expanded by CIS but decreased under RTX. These cells had a typical TEMRA phenotype with a high cytotoxic potential as reflected by the strong expression of Perforin and Granzyme B, which may be implicated in tissue damage. Indeed, both anti-CD8 and anti-perforin antibody therapies have been shown to reduce glomerular lesions in a murine model pauci-immune glomerulonephritis (39,40). In AAV patients, CD8 T cells are present in granulomatous (41), glomerular (42,43) and muscle vasculitic lesions (personal observation) but until recently, little data were available regarding the role of CD8 T cells in AAV. In 2008, McKinney *et al.* analyzed the prognostic value of the transcriptional profiling of neutrophils, monocytes, CD8 T cells, CD4 T cells and B cells in AAV (44). Unexpectedly, they found that the CD8 T cell transcriptome identified two subject groups and predicted relapse risk. Recently, they demonstrated that the good prognosis CD8 T cell signature reflected T cell exhaustion and predicted a favorable outcome in a wide range of autoimmune disease but reduced viral clearance in chronic viral infection (45). These findings suggest that CD8 T cell effector functions have a deleterious role in AAV and other autoimmune diseases. Interestingly, we found that B cell depletion therapy reduced AAV patients' CD8 T cell cytokine production, whereas CIS did not. In other words, a B cell targeted therapy had more impact on CD8 T cells cytokine production than an immunosuppressant that directly impact T cell biology. This paradoxical finding suggest that B cells play a

For Peer Review

key role in the CD8 T cell response, which may contribute to the high efficacy of RTX in AAV. Further studies are needed to determine how B cell impact the CD8 T cell response and whether B cell depletion therapy could promote CD8 T cell exhaustion.

Additionally, we found that latent CMV infection had a major impact on CD4 and CD8 T cell compartment in AAV, by promoting TEMRA expansion, which is consistent with two recent studies (34,35). Whether this phenomenon impact patients' outcome remains to be determined. In RA, where CMV also drives TEMRA expansion, a cross-sectional study suggested that latent CMV infection was associated with more severe joint destruction (46). However, since CMV seroprevalence increases with age, disease duration can be an important source of bias in a cross-sectional analysis (47). A subsequent prospective study found that a profile of PBMC cytokine production in response to CMV/EBV lysates correlated with response to therapy, whereas latent CMV infection itself did not (48) but the impact of anti-CMV response on chronic inflammation/autoimmunity and *vice-versa* remains to be fully established. As for AAV, the impact of latent CMV infection and/or anti-CMV response on disease presentation and relapse risk is unknown.

TEMRA expansion is one of the hallmarks of human immunosenescence, in which latent CMV infection plays a key role. This phenomenon has been linked to an increased risk of mortality, infection susceptibility and decreased efficacy of vaccines in the very elderly (49). More recently, latent CMV infection has been linked to an increased cardiovascular mortality in healthy older adults (>65y) (50). In HIV infection, which is also characterized by a state of chronic immune activation and T cell senescence, CMV infection is an independent risk factor for cardiovascular diseases (50,51). Furthermore, HIV patients with higher frequencies of CD4 or CD8 $CD28^{-}CD57^{+}$ T cells exhibit increased carotid artery stiffness (53). In AAV patients,

For Peer Review

Morgan *et al.* found that a higher frequency of $CD4^{+}CD28^{-}$ cells at presentation increased the risk of infection and mortality, whereas latent CMV infection itself did not (35). Of note, $CD4^{+}CD28^{-}$ cells expanded under conventional immunosuppressant's, consistently with our findings. Our observation that B cell depletion therapy has the opposite effect raises the question whether its benefit could extend beyond disease control, and include reduced cardio-vascular risk, which is an increasing matter of concern in patients with AAV (2,54). Only long-term observation of RTX versus CIS treated patient could answer this question.

In conclusion, we found that B cell depletion therapy had a profound impact on the CD8 T cell compartment. Unlike conventional immunosuppressant, B cell depletion therapy dampens TEMRA expansion and reduces CD8 T cell cytokine production. Along with recent data regarding the role of CD8 T cell in auto-immunity, our result raises the question whether the disruption of B cell help to CD8 T cell could contribute to the dramatic efficacy of RTX. Further studies are needed to clarify the impact of long term B cell depletion therapy on immunosenescence and cardiovascular risk in AAV patients.

Acknowledgments

The French Internal Medicine Society (Société Nationale Française de Médecine Interne) and Groupama Loire Bretagne - Fondation Groupama pour la santé supports A Néel. The International Fellowship Grant from ProGreffe Fondation supports M Yap. This work was supported by the FP7 VISICORT, project that has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 602470. Authors thank Matthieu Hanf for insightful help with statistical analysis.

For Peer Review

References

1. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, et al. 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheum.* 2013;65(1):1-11.

2. Flossmann O, Berden A, de Groot K, Hagen C, Harper L, Heijl C, et al. Long-term patient survival in ANCA-associated vasculitis. *Ann Rheum Dis.* 2011;70(3):488-94.

3. Stone JH, Merkel PA, Spiera R, Seo P, Langford CA, Hoffman GS, et al. Rituximab versus cyclophosphamide for ANCA-associated vasculitis. N Engl J Med. 2010;363(3):221-32.

4. Guillevin L, Pagnoux C, Karras A, Khouatra C, Aumaître O, Cohen P, et al. Rituximab versus azathioprine for maintenance in ANCA-associated vasculitis. *N Engl J Med.* 2014;371(19):1771-80.

5. Liossis SN, Sfikakis PP. Rituximab-induced B cell depletion in autoimmune diseases: potential effects on T cells. *Clin Immunol.* 2008;127(3):280-5.

6. Zhao Y, Lutalo PMK, Thomas JE, Sangle S, Choong LM, Tyler JR, et al. Circulating T follicular helper cell and regulatory T cell frequencies are influenced by B cell depletion in patients with granulomatosis with polyangiitis. *Rheumatology.* 2014;53(4):621-30.

7. Watts R, Lane S, Hanslik T, Hauser T, Hellmich B, Koldingsnes W, et al. Development and validation of a consensus methodology for the classification of the ANCA-associated vasculitides and polyarteritis nodosa for epidemiological studies. *Ann Rheum Dis.* 2007;66(2):222-7.

8. Mukhtyar C, Lee R, Brown D, Carruthers D, Dasgupta B, Dubey S, et al. Modification and validation of the Birmingham Vasculitis Activity Score (version 3). Ann Rheum Dis. 2009;68(12):1827-32.

9. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature.* 1999;401(6754):708-12.

10. Appay V, van Lier RA, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A.* 2008;73(11):975-83.

11. Cosmi L, De Palma R, Santarlasci V, Maggi L, Capone M, Frosali F, et al. Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. *J Exp Med.* 2008;205(8):1903-16.

12. Abdulahad WH, Stegeman CA, van der Geld YM, Doornbos-van der Meer B, Limburg PC, Kallenberg CGM. Functional defect of circulating regulatory CD4+ T cells in patients with Wegener's granulomatosis in remission. *Arthritis Rheum.* 2007;56(6):2080-91.

13. Rimbert M, Hamidou M, Braudeau C, Puéchal X, Teixeira L, Caillon H, et al. Decreased numbers of blood dendritic cells and defective function of regulatory T cells in antineutrophil cytoplasmic antibody-associated vasculitis. *PloS One.* 2011;6(4):e18734.

14. Morgan MD, Day CJ, Piper KP, Khan N, Harper L, Moss PA, et al. Patients with Wegener's granulomatosis demonstrate a relative deficiency and functional

impairment of T-regulatory cells. *Immunology*. 2010;130(1):64-73.

15. Free ME, Bunch DO, McGregor JA, Jones BE, Berg EA, Hogan SL, et al. Patients with antineutrophil cytoplasmic antibody-associated vasculitis have defective Treg cell function exacerbated by the presence of a suppression-resistant effector cell population. *Arthritis Rheum*. 2013;65(7):1922-33.

16. Saadoun D, Rosenzwajg M, Landau D, Piette JC, Klatzmann D, Cacoub P. Restoration of peripheral immune homeostasis after rituximab in mixed cryoglobulinemia vasculitis. *Blood*. 2008;111(11):5334-41.

17. Stasi R, Cooper N, Del Poeta G, Stipa E, Laura Evangelista M, Abruzzese E, et al. Analysis of regulatory T-cell changes in patients with idiopathic thrombocytopenic purpura receiving B cell-depleting therapy with rituximab. *Blood*. 2008;112(4):1147-50.

18. Vigna-Perez M, Hernández-Castro B, Paredes-Saharopulos O, Portales-Pérez D, Baranda L, Abud-Mendoza C, et al. Clinical and immunological effects of Rituximab in patients with lupus nephritis refractory to conventional therapy: a pilot study. *Arthritis Res Ther*. 2006;8(3):R83.

19. Vallerskog T, Gunnarsson I, Widhe M, Risselada A, Klareskog L, van Vollenhoven R, et al. Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE. *Clin Immunol*. 2007;122(1):62-74.

20. Feuchtenberger M, Müller S, Roll P, Waschbisch A, Schäfer A, Kneitz C, et al. Frequency of regulatory T cells is not affected by transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis. *Open Rheumatol J*. 2008;2:81-8.

21. Kamburova EG, Koenen HJ, van den Hoogen MW, Baas MC, Joosten I, Hilbrands LB. Longitudinal analysis of T and B cell phenotype and function in renal transplant recipients with or without rituximab induction therapy. *PloS One*. 2014;9(11):e112658.

22. Abdulahad WH, Meijer JM, Kroese FG, Meiners PM, Vissink A, Spijkervet FK, et al. B cell reconstitution and T helper cell balance after rituximab treatment of active primary Sjögren's syndrome: a double-blind, placebo-controlled study. *Arthritis Rheum*. 2011;63(4):1116-23.

23. Díaz-Torné C, Ortiz de Juana MA, Geli C, Cantó E, Laiz A, Corominas H, et al. Rituximab-induced interleukin-15 reduction associated with clinical improvement in rheumatoid arthritis. *Immunology*. 2014;142(3):354-62.

24. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity*. 2009;30(6):899-911.

25. Afzali B, Mitchell PJ, Edozie FC, Povoleri GA, Dowson SE, Demandt L, et al. CD161 expression characterizes a subpopulation of human regulatory T cells that produces IL-17 in a STAT3-dependent manner. *Eur J Immunol*. 2013;43(8):2043-54.

26. Pesenacker AM, Bending D, Ursu S, Wu Q, Nistala K, Wedderburn LR. CD161 defines the subset of FoxP3+ T cells capable of producing proinflammatory cytokines. *Blood*. 2013;121(14):2647-58.

27. Verhagen J, Wraith DC. Comment on "Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally

induced Foxp3+ T regulatory cells." *J Immunol.* 2010;185(12):7129.

28. Takatori H, Kawashima H, Matsuki A, Meguro K, Tanaka S, Iwamoto T, et al. Helios Enhances Treg Cell Function in Cooperation With FoxP3. *Arthritis Rheumatol.* 2015;67(6):1491-502.

29. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol.* 2010;184(7):3433-41.

30. Strioga M, Pasukoniene V, Characiejus D. CD8+ CD28- and CD8+ CD57+ T cells and their role in health and disease. *Immunology*. 2011;134(1):17-32.

31. Yap M, Boeffard F, Clave E, Pallier A, Danger R, Giral M, et al. Expansion of highly differentiated cytotoxic terminally differentiated effector memory CD8+ T cells in a subset of clinically stable kidney transplant recipients: a potential marker for late graft dysfunction. *J Am Soc Nephrol.* 2014;25(8):1856-68.

32. Wertheimer AM, Bennett MS, Park B, Uhrlaub JL, Martinez C, Pulko V, et al. Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T cell subsets in humans. *J Immunol.* 2014;192(5):2143-55.

33. Vescovini R, Fagnoni FF, Telera AR, Bucci L, Pedrazzoni M, Magalini F, et al. Naïve and memory CD8 T cell pool homeostasis in advanced aging: impact of age and of antigen-specific responses to cytomegalovirus. *Age (Dordr).* 2014;36(2):625-40.

34. Eriksson P, Sandell C, Backteman K, Ernerudh J. Expansions of CD4+CD28- and CD8+CD28- T cells in granulomatosis with polyangiitis and microscopic polyangiitis are associated with cytomegalovirus infection but not with disease activity. *J Rheumatol.* 2012;39(9):1840-3.

35. Morgan MD, Pachnio A, Begum J, Roberts D, Rasmussen N, Neil DA, et al. CD4+CD28- T cell expansion in granulomatosis with polyangiitis (Wegener's) is driven by latent cytomegalovirus infection and is associated with an increased risk of infection and mortality. *Arthritis Rheum.* 2011;63(7):2127-37.

36. Iking-Konert C, Vogl T, Prior B, Wagner C, Sander O, Bleck E, et al. T lymphocytes in patients with primary vasculitis: expansion of CD8+ T cells with the propensity to activate polymorphonuclear neutrophils. *Rheumatology (Oxford)*. 2008 May;47(5):609–16.

37. Vogt S, Iking-Konert C, Hug F, Andrassy K, Hänsch GM. Shortening of telomeres: Evidence for replicative senescence of T cells derived from patients with Wegener's granulomatosis. *Kidney Int.* 2003;63(6):2144-51.

38. Deligne C, Metidji A, Fridman W-H, Teillaud J-L. Anti-CD20 therapy induces a memory Th1 response through the IFN-γ/IL-12 axis and prevents protumor regulatory T-cell expansion in mice. *Leukemia*. 2015;29(4):947-57.

39. Fujinaka H, Yamamoto T, Feng L, Nameta M, Garcia G, Chen S, et al. Anti-perforin antibody treatment ameliorates experimental crescentic glomerulonephritis in WKY rats. *Kidney Int.* 2007;72(7):823-30.

40. Reynolds J, Norgan VA, Bhambra U, Smith J, Cook HT, Pusey CD. Anti-CD8 monoclonal antibody therapy is effective in the prevention and treatment of

experimental autoimmune glomerulonephritis. *J Am Soc Nephrol.* 2002;13(2):359-69.

41. Coulomb-L'Hermine A, Capron F, Zou W, Piard F, Galateau F, Laurent P, et al. Expression of the chemokine RANTES in pulmonary Wegener's granulomatosis. *Hum Pathol.* 2001;32(3):320-6.

42. Weidner S, Carl M, Riess R, Rupprecht HD. Histologic analysis of renal leukocyte infiltration in antineutrophil cytoplasmic antibody-associated vasculitis: importance of monocyte and neutrophil infiltration in tissue damage. *Arthritis Rheum.* 2004;50(11):3651-7.

43. Cunningham MA, Huang XR, Dowling JP, Tipping PG, Holdsworth SR. Prominence of cell-mediated immunity effectors in "pauci-immune" glomerulonephritis. *J Am Soc Nephrol.* 1999;10(3):499-506.

44. McKinney EF, Lyons PA, Carr EJ, Hollis JL, Jayne DR, Willcocks LC, et al. A CD8+ T cell transcription signature predicts prognosis in autoimmune disease. *Nat Med.* 2010;16(5):586-91.

45. McKinney EF, Lee JC, Jayne DR, Lyons PA, Smith KGC. T-cell exhaustion, co-stimulation and clinical outcome in autoimmunity and infection. *Nature.* 2015;523(7562):612-6.

46. Pierer M, Rothe K, Quandt D, Schulz A, Rossol M, Scholz R, et al. Association of anticytomegalovirus seropositivity with more severe joint destruction and more frequent joint surgery in rheumatoid arthritis. *Arthritis Rheum.* 2012;64(6):1740-9.

47. van Bergen J, van Gaalen FA, van Dongen H, Huizinga TW, Feltkamp MC, Toes RE, et al. Anticytomegalovirus seropositivity in rheumatoid arthritis is not associated with the presence of severe extraarticular complications: comment on the article by Pierer et al. *Arthritis Rheum.* 2012;64(8):2803-4.

48. Davis JM, Knutson KL, Strausbauch MA, Green AB, Crowson CS, Therneau TM, et al. Immune response profiling in early rheumatoid arthritis: discovery of a novel interaction of treatment response with viral immunity. *Arthritis Res Ther.* 2013;15(6):R199.

49. Pawelec G. Immunosenenescence: role of cytomegalovirus. *Exp Gerontol.* 2014;54:1-5.

50. Roberts ET, Haan MN, Dowd JB, Aiello AE. Cytomegalovirus antibody levels, inflammation, and mortality among elderly Latinos over 9 years of follow-up. *Am J Epidemiol.* 2010;172(4):363-71.

51. Parrinello CM, Sinclair E, Landay AL, Lurain N, Sharrett AR, Gange SJ, et al. Cytomegalovirus immunoglobulin G antibody is associated with subclinical carotid artery disease among HIV-infected women. *J Infect Dis.* 2012;205(12):1788-96.

52. Lichtner M, Cicconi P, Vita S, Cozzi-Lepri A, Galli M, Lo Caputo S, et al. Cytomegalovirus coinfection is associated with an increased risk of severe non-AIDS-defining events in a large cohort of HIV-infected patients. *J Infect Dis.* 2015;211(2):178-86.

53. Kaplan RC, Sinclair E, Landay AL, Lurain N, Sharrett AR, Gange SJ, et al. T cell activation predicts carotid artery stiffness among HIV-infected women. *Atherosclerosis.* 2011;217(1):207-13.

54. Cohen Tervaert JW. Cardiovascular disease due to accelerated atherosclerosis

For Peer Review

in systemic vasculitides. *Best Pract Res Clin Rheumatol.* 2013;27(1):33-44.

For Peer Review

Figures

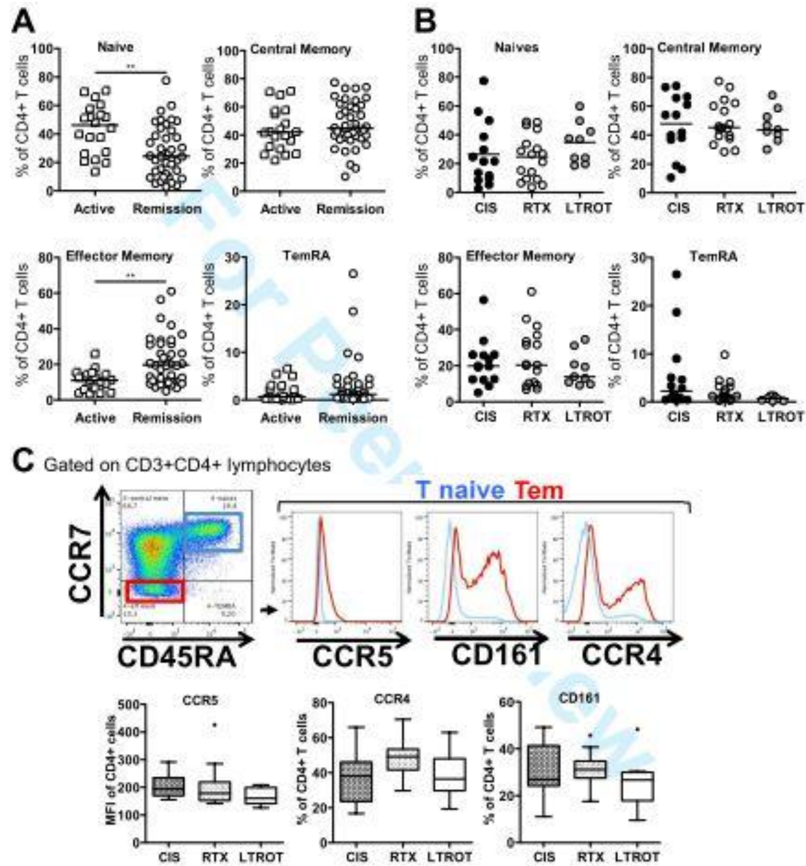


Figure 1 : During remission, maintenance treatment type has no impact on CD4+ T cell subsets or on CD4+ helper T cell markers.

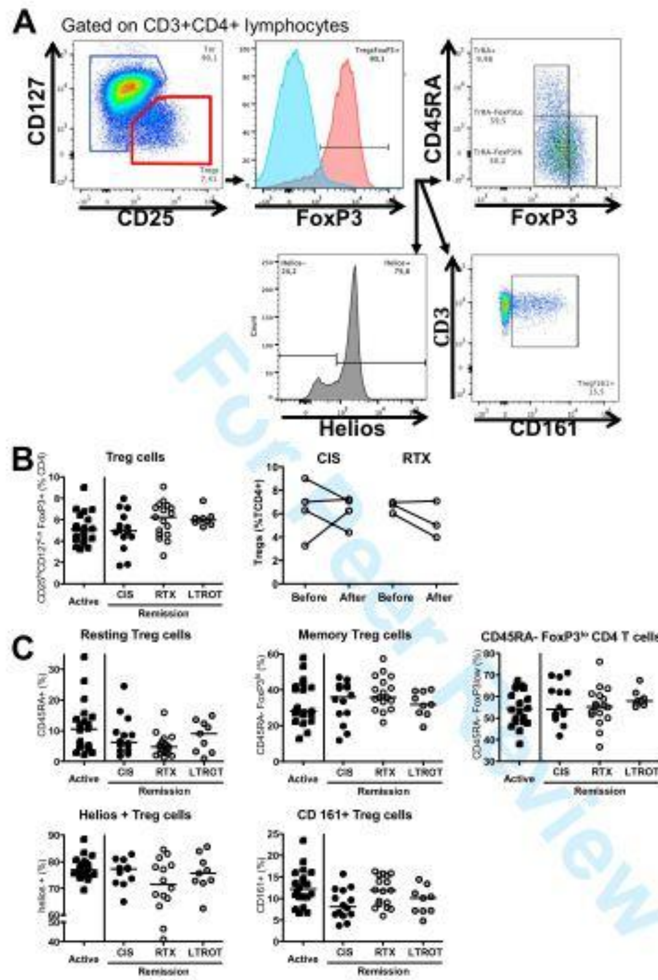


Figure 2. Frequency of Treg cell and Treg cell subsets is not influenced by maintenance treatment type.

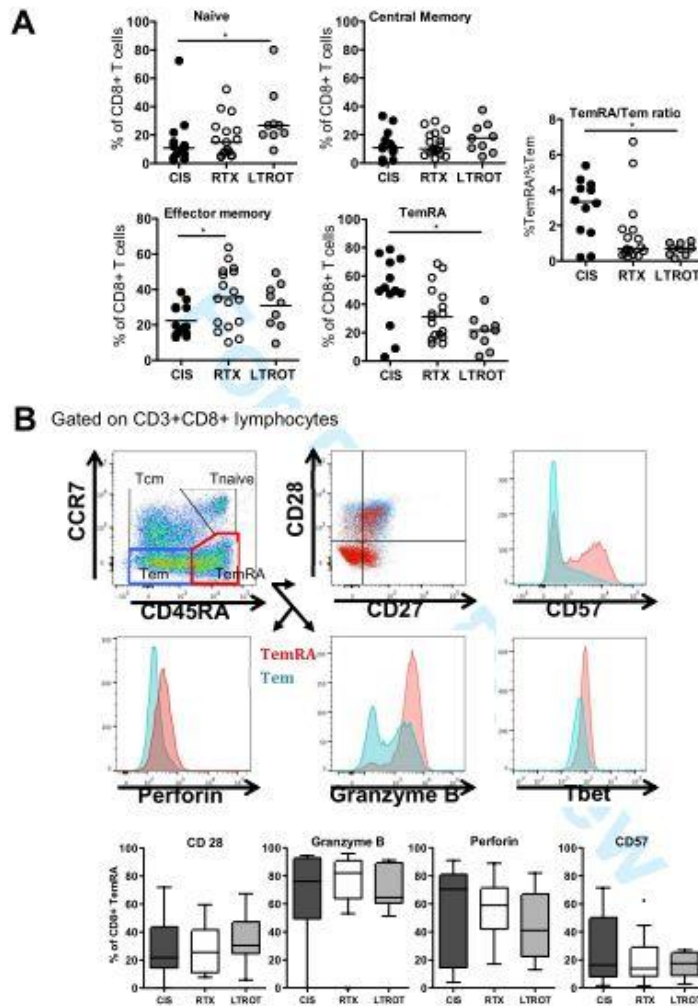


Figure 3. Variation of TEMRA/Tem CD8 balance according to maintenance treatment type and confirmation of the prototypic TEMRA phenotype of CD45RA⁺CCR7⁻ cells.

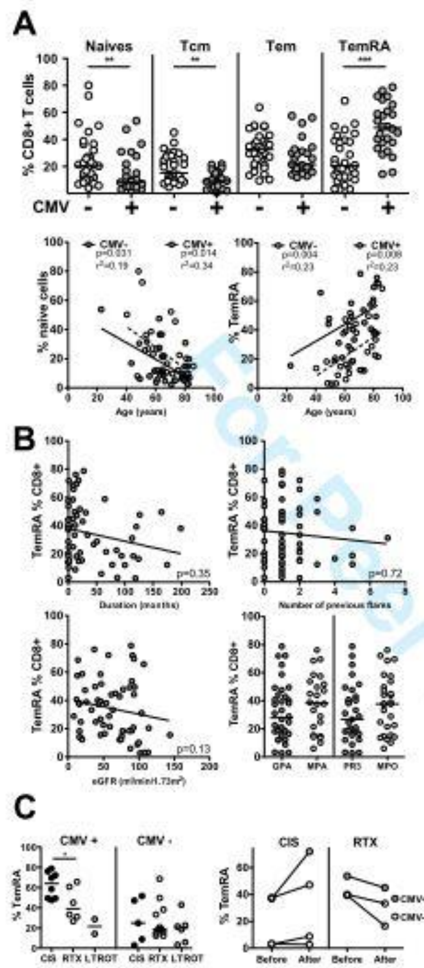


Figure 4. TEMRA CD8 T cell frequency is influenced by maintenance treatment type and CMV status.

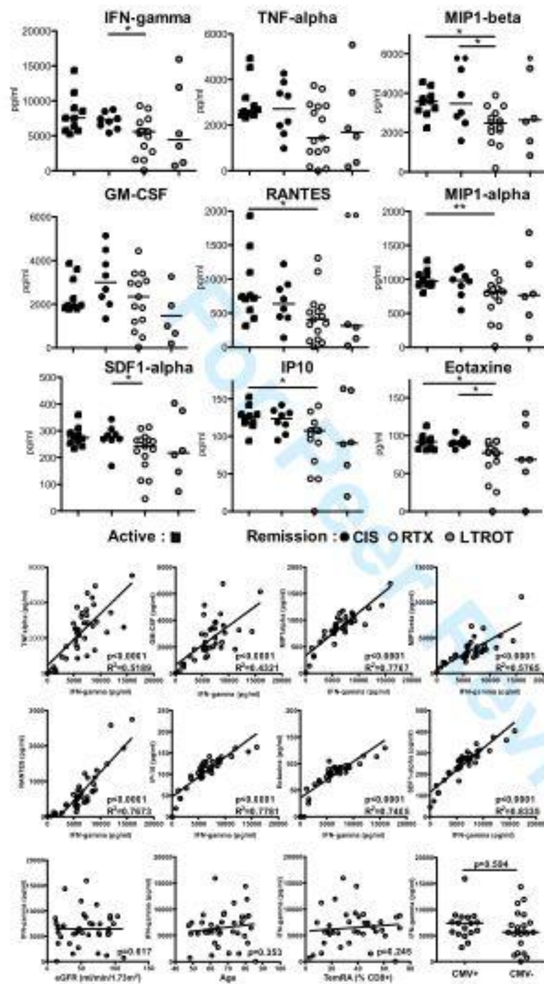


Figure 5. B cell depletion therapy efficiently inhibits cytokine and chemokine production by *in vitro* stimulated CD8 T cells, whereas conventional immunosuppressants do not.

Figure Legends

Figure 1. During remission, maintenance treatment type has no impact on CD4+ T cell subsets or on CD4+ helper T cell markers. CCR7 and CD45RA expression were used to identify naïve ($CD45RA^{+}CCR7^{+}$; Tn), central memory ($CD45RA^{-}CCR7^{+}$; Tcm), effector memory ($CD45RA^{-}CCR7^{-}$; Tem) and late differentiated effector memory ($CD45RA^{+}CCR7^{-}$; TEMRA) CD4 cells. T cells subsets frequencies were analyzed according to (**A**) disease activity and (**B**) maintenance treatment. (**C**) The expression of CCR5, CCR4 and CD161 were measured at the cell surface of CD4 T cells and used as surrogates for Th1, Th2 and Th17 response, respectively. The expression of these markers was compared between Tn and Tem (C, upper panel) and across the 3 groups of patients in remission (C, lower panel). CIS: conventional immunosuppressant, RTX: Rituximab, LTROT: Long term remission off therapy. Horizontal bar within dot plots represent the median. Boxplot were done using the Tukey method. 1A; **$p<0.01$, Mann-Whitney test, B and C: $p>0.05$, Kruskal-Wallis.

For Peer Review

Figure 2. Frequency of Treg cell and Treg cell subsets is not influenced by maintenance treatment type. (A) Gating strategy for the identification of Tregs ($CD3^{+}CD4^{+}CD25^{hi}CD127^{lo}FoxP3^{+}$) and Tregs subsets. (B) Total Treg cells frequency was assessed according to maintenance treatment type (left panel) and measured prospectively in 7 patients started on CIS or RTX (right panel): no differential effect of RTX vs CIS was found. (A and C) Resting and activated Treg cells were defined as $CD45RA^{+}$ and $CD45RA^{-}FoxP3^{hi}$ Tregs, respectively. The frequency of $CD161^{+}$, $helios^{+}$, resting, memory Tregs and $CD45RA^{-}FoxP3^{lo}$ cells were not significantly different among CIS, RTX and LTROT patients (All $p>0.05$, Kruskal-Wallis). CIS: conventional immunosuppressant, RTX: rituximab, LTROT: Long term remission off therapy. Horizontal bars indicate median.

Figure 3. Variation of TEMRA/Tem CD8 balance according to maintenance treatment type and confirmation of the prototypic TEMRA phenotype of CD45RA$^+$CCR7$^-$ cells. (A) CCR7 and CD45RA expression were used to identify naïve (CD45RA$^+$CCR7$^+$; Tn), central memory (CD45RA$^-$CCR7$^+$; Tcm), effector memory (CD45RA$^-$CCR7$^-$; Tem) and late differentiated effector memory (CD45RA$^+$CCR7$^-$; TEMRA) CD8 cells. CD8 T cells subsets frequencies were analyzed according to maintenance treatment regimen. (**B**) Gating strategy and representative flow cytometry plots for the phenotypic characterization of TEMRA cells. (**C**) TEMRA phenotype was compared across remission patients and no relationship with maintenance treatment type was found. CIS: conventional immunosuppressant, RTX: Rituximab, LTROT: Long term remission off therapy. Horizontal bar within dot plots represent the median. Boxplot were done using the Tukey method. *p<0.05, Kruskal-Wallis with Dunn's post test.

For Peer Review

Figure 4. TEMRA CD8 T cell frequency is influenced by maintenance treatment type and CMV status. (**A**) Upper panel: Impact of CMV infection on naïve, central memory, effector memory and TEMRA CD8 T cells frequency among the entire cohort. Lower panel: correlation between age and frequency of naïve and TEMRA CD8 T cells according to CMV status. (**B**) Lack of correlation between TEMRA CD8 T cells frequency and disease characteristics including disease duration, number of previous flare, renal function, clinical phenotype and ANCA specificity. (**C**) TEMRA frequency according to CMV status and maintenance treatment type; left panel: cross-sectional analysis of remission patients; right panel: longitudinal analysis of 7 patients started on CIS or RTX showing their opposite effects on TEMRA frequency. CIS: conventional immunosuppressant, RTX: Rituximab, LTROT: Long term remission off therapy, eGFR: estimated glomerular filtration rate. Horizontal bar within dot plots represent the median. Boxplot were done using the Tukey method. (A and C) *p<0.05, **p<0.01, ***p<0.001, Mann-Whitney. (A and B) Lines within dot plots represent linear regression. Statistical significance was tested using Spearman's rank correlation.

Figure 5. B cell depletion therapy efficiently inhibits cytokine and chemokine production by *in vitro* stimulated CD8 T cells, whereas conventional immunosuppressants do not. (**A**) FACS-sorted CD8 T cells were stimulated with PMA ionomycin for 4 hours and cytokines/chemokines production was assessed using a 34plex immunoassay. CD8 T cells from RTX treated patients produced lower levels of pro-inflammatory cytokines/chemokines than CIS treated patients and/or those with active untreated disease (*p<0.05,**p<0.01, Kruskal-Wallis with Dunn's post test [Active vs CIS vs RTX]). Differentially expressed cytokines/chemokines included IFN-γ, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, IP-10/CXCL10, SDF-1α/CXCL12a and Eotaxine. A similar trend was observed with TNF-α and GM-CSF. Low amounts of IL4, IL5, IL8, IL10, IL12p70, IL13, IL18, IL21, IL22, IL27, MCP1 and GROα were also detectable, without any difference across groups. Levels of IL1-α, IL1-β, IL1Ra, IL6, IL7, IL9, IL15, IL17A, IL23, IL31, IFN-α and TNF-β were below the detection threshold, whereas IL2 production was to high to confidently quantify. (**B**) IFN-γ production correlate with other differentially expressed cytokines, but neither with age, estimated glomerular filtration rate (eGFR), TEMRA frequency nor CMV status. Lines within dot plot represent linear regression. Statistical significance was tested using Spearman's rank correlation. CIS: conventional immunosuppressant, RTX: Rituximab, LTROT: Long term remission off therapy. Horizontal bars represent medians.

For Peer Review

Table 1: Comparison of clinico-biological characteristics of patients in remission according to maintenance treatment.

	B Cell Depletion Therapy (RTX, N=17)	Conventional Immunosuppressant (CIS, N=14)	Remission Off Therapy (LTROT, N=9)
Male	12 (70%)	5 (36%)	2 (22%)
Age	66 (56-75)	68 (59-82)	63 (62-73)
GPA	12 (70%)	7 (50%)	6 (66%)
Anti-MPO	6 (35%)	7 (50%)	4 (44%)
eGFR (ml/min/1,73m^2)	54 (26-83)	67 (44-93)	59 (34-82)
Disease duration	34 (11-119)	19 (13-53)	58 (13-86)
Past CYC exposure	15 (88%)	11 (79%)	7 (78%)
Remission duration	7 (4-9)	10 (6-17)	46 (20-73)^A
Single flare	5 (30%)	8 (57%)	8 (88%)^B
Previous flares (n)	2 (1-4.5)^C	1 (1-2)	1 (1-1)

Figures indicate ratios (percentage) or median (interquartile range). eGFR : estimated glomerular filtration rate; CYC: cyclophosphamide ; LTROT: Long Term Remission Off Therapy. ^{A} p=0.0019 vs RTX, ^{B} p=0.01 vs RTX; ^{C} p=0.0056 vs LTROT, Mann-Whitney test.

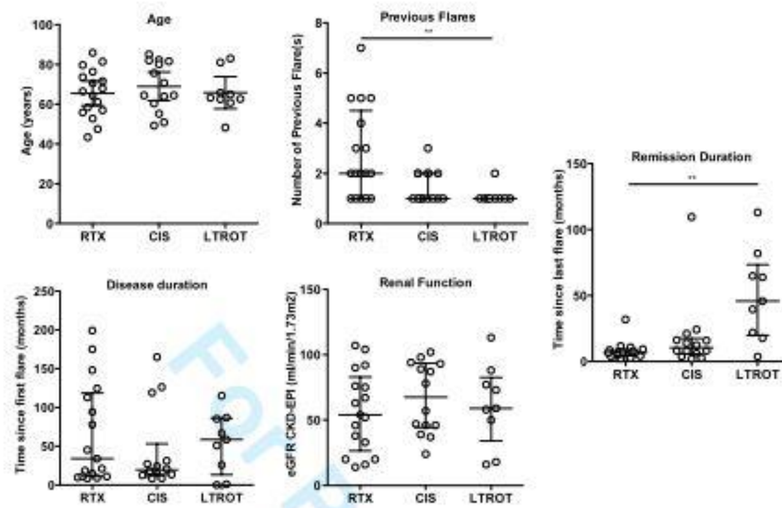
For Peer Review

Supplementary data

Supplementary Table 1. Clinical characteristics of patients with active disease

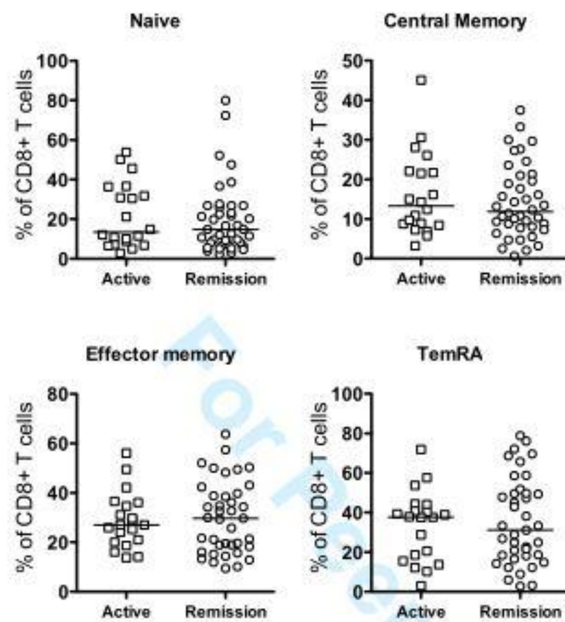
	Active disease (N=20)
Male	13 (65%)
Age	66 (55-78)
GPA	11 (55%)
Anti-MPO	11 (55%)
eGFR ($ml/min/1,73m^2$)	53.5 (16.5-92.5)
First flare	18 (90%)
BVAS	14 (10.5-16.5)

For Peer Review



Supplementary Figure 1. Clinical characteristics of patients in remission according to maintenance treatment. CIS: conventional immunosuppressant, RTX: Rituximab, LTROT: Long term remission off therapy. Horizontal bars represent medians and interquartile range. **p<0.01, Kruskal-Wallis with Dunn's post test.

For Peer Review



Supplementary Figure 2. Naïve and memory CD8 T cells subsets frequencies are not influenced by disease activity. CCR7 and CD45RA expression were used to identify naïve ($CD45RA^{+}CCR7^{+}$; Tn), central memory ($CD45RA^{-}CCR7^{+}$; Tcm), effector memory ($CD45RA^{-}CCR7^{-}$; Tem) and late differentiated effector memory ($CD45RA^{+}CCR7^{-}$; TEMRA) CD4 cells. T cells subsets frequencies were analyzed according to disease activity. All p>0.05, Mann-Withney.

For Peer Review

6.4 Publications

Expansion of Highly Differentiated Cytotoxic Terminally Differentiated Effector Memory CD8^{+} T Cells In a Subset of Clinically Stable Kidney Transplant Recipients: A Potential Marker for Late Graft Dysfunction; Yap M, Boeffard F, Clave E, Pallier A, Danger R, Giral M, Dantal J, Foucher Y, Guillot-Gueguen C, Toubert A, Soulillou J, Brouard S, and Degauque N, Journal of the American Society of Nephrology, 2014 Aug; 25(8): 1856-68.

Targeting CD8 T Cell Metabolism in Transplantation; Yap M. Brouard S, Pecqueur C, and Degauque N, Frontiers in Immunology, 2015 October; 6:547; doi: 10.3389/fimmu.2015.00547.

The Benefits of Using CD45RA and CD28 to Investigate CD8 Subsets in Kidney Transplant Recipients; Yap M, Tilly G, Giral M, Brouard S and Degauque N, American Journal of Transplantation, *In Press*.

6.5 Orals Presentations

Rapid effector function of TEMRA CD8 T cells in healthy volunteers and in immune-stimulated patients requires sustained glycolytic switch; European Society of Transplantation, Brussels, Belgium, September 2015.

CD8 T cell biomarkers improves the capacities of the Kidney Transplant Failure Score for the long-term prognostic of kidney graft failures; European Society of Transplantation, Brussels, Belgium, September 2015.

Sustained glycolytic switch required for rapid effector function of TEMRA CD8 T cells in healthy volunteers and in immune-stimulated patients; Federation of Clinical Immunolgy Societies Meeting, San Diego, USA, June 2015.

Comprehensive study of 3 nomenclatures to discriminate CD8 subsets in healthy volunteers and in kidney transplant recipients; Societé Française d'Immunologie, Lille, France, November 2014.

6.6 *Poster Presentations*

Multiple sclerosis and kidney allotransplantation induce antigen-experienced CD8 T cells with different metabolic profiles; European Congress of Immunology, Vienna, Austria, September 2015.

Sustained glycolytic switch required for rapid effector function of TEMRA CD8 T cells in healthy volunteers and in immune-stimulated patients; European Congress of Immunology, Vienna, Austria, September 2015.

Comprehensive study of 3 nomenclatures to discriminate CD8 subsets in healthy volunteers and in kidney transplant recipients; Federation of Clinical Immunolgy Societies Meeting, San Diego, USA, June 2015.

Comprehensive study of 3 nomenclatures to discriminate CD8 subsets in healthy volunteers and in kidney transplant recipients; American Transplant Congress, Philadelphia, USA, May 2015.

Rapid effector function of TEMRA CD8 T cells in healthy volunteers and in immune-stimulated patients requires sustained glycolytic switch; American Transplant Congress, Philadelphia, USA, May 2015.

CD8 T cell biomarkers improves the capacities of the Kidney Transplant Failure Score for the long-term prognostic of kidney graft failures; American Transplant Congress, Philadelphia, USA, May 2015.

TEMRA CD8 T cells are highly cytopathic cells that escape from costimulatory based-therapy; World Transplant Congress, San Francisco, USA, July 2014.

A Subset of Patients Accumulated Highly Differentiated CD4 & CD8 T Memory Cells Despite Having Long-Term Stable Graft Function; World Transplant Congress, San Francisco, USA, July 2014.

TEMRA CD8 T cells are highly cytopathic cells with a unique metabolic signature; Nantes Actualités Transplantation, Nantes, France, June 2014.

An increase of highly differentiated TEMRA CD8 T cells with potent effector function in kidney transplant recipients with stable graft function revealed patients with at-risk of long-term graft function; Basic Science Meeting, Paris, France, November 2013.

Expansion of cytotoxic CD127- CD57+T-bet^{hi} TEMRA CD8 T cells with an Altered TCR Vb repertoire define a population at-risk of long-term kidney graft dysfunction; European Society of Transplantation, Vienna, Austria, September 2013.

Heterogeneity in the CD8 T cell Poplulation of Long Term Stable kidney Graft Recipients; Nantes Actualités Transplantation, Nantes, France, June 2013.

6.7 Awards

Young Investigator Award, American Transplant Congress, Philadelphia, PA, May 2015

TTS-SFT Mentee-Mentor Award, Basic Science Meeting, Paris, France November 2013

l'u nam l'université nantes angers le mans
COMMUNAUTÉ D'UNIVERSITÉS ET ÉTABLISSEMENTS

UNIVERSITÉ DE NANTES

Thèse de Doctorat

Michelle YAP

The characterization of CD8 T lymphocyte subset differentiation and their immunometabolic programming in kidney transplantation

Caractérisation de la différentiation des sous populations des lymphocytes T CD8 et de leur profiles immunométaboliques en transplantation rénales

Résumé

Malgré l'efficacité des immunosuppresseurs, les patients transplantés rénaux sont confrontés à une perte tardive de leur greffon. Comprendre les mécanismes aboutissant au rejet et identifier des biomarqueurs prognostiques sont deux enjeux clefs pour améliorer la survie des greffons. En étudiant des patients transplantés recrutés plus de 5 années après greffe, nous avons montré que l'accumulation de cellules T CD8 de type TEMRA constituait un facteur de sur-risque de dysfonction chronique (Article 1). Le suivi de la fréquence des LT CD8 (12 mois post-transplantation) combiné à des marqueurs cliniques simples permet de définir un score composite de perte du greffon dans les 6 années suivant la greffe (Article 2). Après avoir établi une combinaison optimale de marqueurs de différenciation pour l'étude des cellules T CD8 (Article 3) et étant donné le rôle des LT CD8 dans le rejet chronique, nous avons comparé le métabolisme des CD8 TEMRA avec celui des cellules naïves et effectrices mémoires (EM) (Article 4). Chez les volontaires sains, le métabolisme des cellules T CD8 de type TEMRA présentent de grande similitude avec celui des cellules EM avec notamment un niveau basal élevé de glycolyse et de fortes quantités d'ATP par rapport à ceux des cellules naives. L'activité de glycolyse et de respiration mitochondriale est maintenue dans le temps après activation. Enfin, la glycolyse et la glutaminolyse sont essentielles pour la production de cytokines inflammatoires. Ces résultats viennent remettre en cause le caractère sénescent des cellules TEMRA et nous amènent à définir les propriétés immuno-métaboliques des LT CD8 chez les patients transplantés selon leur devenir clinique.

Mots clés
Transplantation, Lymphocyte T CD8, Immunométaboliques, Biomarquers

Abstract

Despite the advancements in immunosuppresssion therapy, long term allograft dysfunction is still a main barrier to successful kidney transplantation. Therefore, biomarkers that can guide patient care are important to extending the life of a kidney allograft. The aim of this thesis was to investigate the role of CD8 T cells in kidney transplant patients, evaluate the use of these cells as possible biomarkers, and to examine the metabolic programming and machinery used by these cells in healthy volunteers and transplant patients.
Kidney transplant patients who have stable graft function one year post-transplantation and have an increased frequency of late-differentiated TEMRA CD8 T cells were more at risk of late graft dysfunction compared to stable kidney transplant recipients with lower levels of these cells. Furthermore, using CD8 cells as biomarkers and additional parameters in a revised Kidney Transplant Failure Score (KTFS) resulted in better classification of at-risk patients.
We also compared the metabolic machinery used by TEMRA CD8 cells to those of naive and effector memory (EM) CD8 subsets. In healthy volunteers, EM and TEMRA cells have higher basal glycolytic activity compared to naive cells, and after activation, EM and TEMRA can sustain higher levels of glycolysis and mitochondrial respiration compared to naive cells. Additionally, TEMRA and EM cells have a higher basal concentration of stored ATP. Furthermore, glycolysis and glutaminolysis are essential for CD8 production of proinflammatory cytokines. Overall, these results show that the immunometabolic properties of CD8 T cells have a promising role in future clinical therapies of transplant patients.

Key Words
Transplantation, CD8 T Lymphocytes, Immunometabolics, Biomarkers