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INHIBITION DU PROTEASOME OU DU RECEPTEUR DE L'INTERLEUKINE-7 DANS LA TRANSPLANTATION D'ORGANE

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LIST OF ABBREVIATIONS

5-LO	5-lipoxygenase
AchR	acetylcholine receptor
AMR	antibody-mediated rejection
ANCA	anti-neutrophil cytoplasmic antibodies
APC	antigen-presenting cell
APRIL	a proliferation-inducing ligand
ASCT	autologous stem cell transplant
ATF6	activating transcription factor 6
BAFF	B-cell activating factor
Bcl-2	B-cell lymphoma 2
BCMA	B-cell maturation protein
BCR	B-cell receptor
Bim	Bcl-2-interacting mediator of cell death
Blys	B lymphocyte stimulator
BMT	bone marrow transplantation
C/EBP	cAMP response element-binding transcription factor
CCL19/MIB-3β	C-C motif chemokine 19/macrophage inflammatory protein-3 beta
CCL2, 5	chemokine (C-C motif) ligand 2, 5
CCR7	chemokine receptor type 7
CFSE	carboxyfluorescein diacetate succinimidyl diester
CHOP	C/EBP homologous protein
CNS	central nervous system
COX-2	cyclooxygenase-2
CTLA-4	cytotoxic T-lymphocyte antigen 4
CX3CL1	chemokine (C-X3-C motif) ligand 1
DC-SIGN	dendritic cell- specific intercellular adhesion molecule-3- grabbing non-integrin (CD209)
DRiPs	defective ribosomal products
DSA	donor-specific antibody
DSA	donor-specific antibody

eIF2α	eukaryotic initiating factor 2α
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
ERS	endoplasmic reticulum stress
FDA	Food and Drug Administration
FoxP3	forkhead box P3
GFR	glomerular filtration rate
GRP78	glucose-regulated protein of 78 kDa
GVHD	graft-versus-host disease
ICAM-1	intercellular adhesion molecule 1 (CD54)
IFN-γ	interferon gamma
IKK	IκB kinase
IL-7	interleukin-7
IL-7Rα	interleukin-7 receptor alpha
IMPDH	inosine monophosphate dehydrogenase
iNOS	inducible nitric oxide synthase
IRE1	inositol-requiring enzyme 1
IVIg	intravenous immunoglobulin
IκBα, β, or ε	inhibitor of NF-κB α, β, or ε
JAK	Janus kinase
JNK	c-jun N-terminal kinase
LPM1	low-molecular mass polypeptide 1
MECL1	multicatalytic endopeptidase complex-like 1
MHC	major histocompatibility complex
MMF	mycophenolate mofetil
MPA	mycophenolic acid
MPO	myeloperoxidase
NEMO	NF-κB essential modifier
NF-AT	nuclear factor of activated T-cells
NF-κB	nuclear factor kappa B

NK cell	natural killer cell
NO	nitric oxide
PERK	pancreatic ER kinase (PKR)-like ER kinase
PI(s)	proteasome inhibitor(s)
PI3K	phosphatidylinositol 3-kinase
PR-3	proteinase 3
PSMA-1	proteasome subunit alpha type-1
PSMB-1	proteasome subunit beta type-1
RAG1	recombination activating gene 1
RANK	receptor activator of nuclear factor κ B
rATG	rabbit anti-thymocyte globulin
SCID	severe combined immunodeficiency
SLE	systemic lupus erymathosus
STAT	signal transducers and activators of transcription
TACI	transmembrane activator and calcium modulator and cyclophilin ligand activator
TAP	transporter associated with antigen presentation
TAP	transporter associated with antigen processing
TCR	T-cell receptor
TLR	toll-like receptor
TNFR1	tumor necrosis factor receptor 1
TNF-α	tumor necrosis factor alpha
TRAF2	TNF receptor-associated factor 2
TRAIL	tumor necrosis factor-related apoptosis inducing ligand
TSLP	thymic stromal lymphopoietin
UPR	unfolded protein response
VCAM	vascular cell adhesion molecule
XBP1	X box-binding protein 1

RÉSUMÉ EN FRANÇAIS

Grâce aux avancées dans les domaines de l'immunologie, de la chirurgie et des soins et suivis post-greffes, la survie des greffes d'organes a été nettement améliorée. Cependant, le rejet d'allogreffe reste un problème majeur en transplantation d'organe. Le rejet d'allogreffe est classé en deux catégories : le rejet dépendant des cellules T et le rejet dépendant des anticorps, en sachant que ces deux mécanismes sont souvent associés chez le patient. Bien que la majorité des rejets réponde aux traitements actuels, une proportion non-négligeable des rejets est réfractaire à toutes combinaisons de médicaments et la perte du greffon reste inévitable à long-terme. De plus, les immunosuppresseurs actuels ont de nombreux effets indésirables. Dans ce contexte, le but de nos recherches est de trouver de nouveaux médicaments efficaces pour la prévention et le traitement des rejets d'allogreffe.

Ma thèse se divise en deux parties. Dans la première, nous avons étudié l'efficacité du Bortézomib, le prototype des inhibiteurs de protéasome dans la prévention du rejet aigu et dans le traitement du rejet chronique dépendant des anticorps dans un modèle d'allogreffe cardiaque chez le rat. Nous avons aussi rapporté nos expériences sur l'utilisation du Bortézomib pour traiter le rejet dépendant des anticorps chez quelques patients transplantés rénaux. Dans la deuxième partie de ce travail, nous avons démontré l'efficacité du blocage du récepteur de l'interleukine-7 par un anticorps monoclonal dans la prolongation de la survie du greffon dans des modèles d'allogreffe de peau et d'îlots pancréatiques chez la souris. L'effet du blocage du récepteur de l'interleukine-7 en transplantation est lié principalement à une diminution du nombre de toutes les sous-populations de lymphocytes T périphériques ainsi que dans les organes lymphoïdes.

PARTIE 1: INHIBITION DU PROTÉASOME EN TRANSPLANTATION D'ORGANE

INTRODUCTION

1. Rejet d'allogreffe – Classification, mécanismes et traitement :

La classification actuelle du rejet d'allogreffe d'organe est basée sur des réunions organisées à Banff au Canada tous les 2-3 ans entre les spécialistes mondiaux en transplantation et en anatomopathologie, d'où vient le nom de «classification Banff» (Solez et al, 1993, Racusen et al, 1999, Racusen et al, 2003, Solez et al, 2007, Solez et al, 2008, Sis et al, 2010). Ici nous limitons notre discussion à la classification de Banff du rejet d'allogreffe rénale, ce qui est de loin la greffe d'organe la plus réalisée. Selon cette classification, le rejet du greffon rénal se divise en

deux catégories : le rejet dépendant des cellules T et le rejet dépendant des anticorps. Chaque catégorie se divise encore en rejet aigu et rejet chronique.

Le rejet dépendant des cellules T est initié par le signal 1, c'est à dire l'interaction entre les récepteurs des cellules T (TCR) et les molécules du complexe majeur d'histocompatibilité (CMH) sur les cellules présentatrices d'antigènes. Pourtant, l'activation des lymphocytes T a besoin d'un deuxième signal, c'est la costimulation crée principalement par l'interaction entre les molécules CD80 et CD86 sur les cellules B et la molécule CD28 sur les cellules T (Poirier et al, 2010). Après reconnaissance, les cellules T s'activent, prolifèrent et secrètent des cytokines et chémokines permettant ainsi le recrutement des autres lymphocytes et monocytes. Ceci conduit à une infiltration massive des cellules mononuclées entrainant la destruction du greffon sans traitement efficace.

Les mécanismes d'action du rejet dépendant des anticorps sont moins bien connus. De nombreuses études cliniques ont corrélé la présence d'anticorps spécifiques anti-CMH du donneur à la diminution de la survie du greffon (Worthington et al, 2003, Terasaki et al, 2004, Hourmant et al, 2005, Mizutani et al, 2005, Mao et al, 2007, Terasaki et al, 2007, Lachmann et al, 2009, Everly et al, 2010, Cooper et al, 2011). En parallèle, les expériences dans les modèles d'animaux ont aussi montré que les anticorps anti-CMH du donneur peuvent induire par eux-mêmes des lésions typiques du rejet dépendant des anticorps (Uehara et al, 2007, Jindra et al, 2008, Hirohashi et al, 2010).

La plupart des immunosuppresseurs actuels agissent sur les lymphocytes T, peu parmi eux peuvent cibler les cellules B et/ou les anticorps. Le traitement du rejet dépendant des anticorps est basé aujourd'hui sur les trois thérapies : la plasmaphérèse pour éliminer rapidement les anticorps anti-donneur, le Rituximab ou anticorps monoclonal anti-CD20 pour dépléter les lymphocytes B, et les immunoglobulines intraveineuses dont l'effet est complexe, affectant les réponses immunitaires cellulaire et humorale. Cependant, certains patients ne répondent pas à ces traitements.

De nouveaux anticorps monoclonaux comme l'ocrelizumab et l'ofatumumab (anti-CD20) ou l'epratuzumab (anti-CD22) ont ainsi été développés afin d'optimiser la capacité déplétante sur les cellules B. Une autre piste est de cibler les facteurs de survie et de différentiation des cellules B comme BAFF (B-cell activating factor) et APRIL (a proliferation-inducing ligand) avec les deux nouveaux médicaments biologiques : belimumab (anticorps monoclonal anti-BAFF) et atacicept ou TACI-Ig (TACI : transmembrane activator and calcium modulator and cyclophilin ligand interactor, un des récepteurs de BAFF et APRIL) (Vincenti et al, 2010). Cependant, aucun

de ces médicaments n'a l'effet sur les plasmocytes, les cellules qui sécrètent les anticorps. La seule classe de médicaments qui peut induire l'apotose des plasmocytes et ainsi diminuer la formation des alloanticorps est celle des inhibiteurs du protéasome, ce qui sera le sujet de la première partie de ma thèse.

2. La voie ubiquitine-protéasome :

Une balance stricte entre la synthèse et la dégradation des protéines est cruciale pour la survie des cellules et de l'organisme. La dégradation des protéines intracellulaires est assurée en grande partie par la voie ubiquitine-protéasome. Ainsi, le prix Nobel de chimie en 2004 a été conjointement attribué à trois scientifiques : Aaron Ciechanover, Avram Hershko et Irwin Rose pour leur découverte de l'ubiquitinylation des protéines et son rôle dans la protéolyse intracellulaires.

L'ubiquitine est une protéine hautement conservée composée de 76 acides aminés avec 7 résidus lysine et la glycine en position carboxy terminale. L'ubiquitine est attachée à la protéine cible via une liaison entre sa glycine C-terminale et un résidu lysine sur la protéine cible. Une chaîne de polyubiquitine est alors assemblée via les liaisons d'isopeptide entre un résidu lysine en position 29 ou 48 d'une molécule et la glycine C-terminale d'une autre molécule d'ubiquitine afin de former une bande hydrophobe qui est déterminante pour l'interaction avec le protéasome.

Le protéasome 26S est une organelle intracytoplasmique en forme de baril composée d'un complexe régulateur 19S et d'un cœur catalytique 20S. Le cœur catalytique est formé de quatre anneaux, deux anneaux externes contenant chacun 7 sous-unités alpha, numérotées de α-1 à α-7 et deux anneaux internes contenant chacun 7 sous-unités beta, numérotées de β-1 à β-7. L'activité enzymatique du protéasome réside dans trois sous-unités beta : β-1, β-2 et β-5, avec des activités protéolytiques de type caspase, trypsine et chymotrypsine, respectivement. Dans les cellules du système immunitaire, ces trois sous-unités β-1, β-2 et β-5 du protéasome sont remplacées par trois sous-unités de l'immunoprotéasome β-1i, β-2i et β-5i, respectivement pour augmenter l'activité catalytique. La fonction la plus connue du protéasome et de l'immunoprotéasome dans le système immunitaire est la génération des peptides qui seront liés aux molécules du CMH de classe I pour la présentation d'antigène aux cellules T CD8 (Strehl et al, 2005).

3. Inhibition du protéasome :

Comme la protéolyse est essentielle pour le métabolisme des cellules, l'inhibition du protéasome perturbe différentes fonctions cellulaires et peut causer la mort de la cellule.

L'inhibition du protéasome induit l'apoptose par deux mécanismes principaux.

Le premier mécanisme est l'inactivation de la voie de signalisation du facteur NF-κB . NF-κB est un facteur de transcription composé de 5 sous-unités : RelA (p65), RelB, c-Rel, NF-κB1 (p50 et son précurseur p105) et NF-κB2 (p52 et son précurseur p100) (Jost et al, 2007). Ces sous-unités sont maintenues sous la forme dimérique dans le cytoplasme et sont liées avec des protéines inhibitrices du facteur NF- κB, IκB. L'activation d' NF-κB par la voie classique ou alterne doit passer par une phosphorylation d'IκB par IκB kinase (IKK), IκB phosphorylé est ensuite ubiquitinylé et dégradé par le protéasome, libérant NK-κB dimérique (le plus souvent p65/p50 hétérodimère) qui va entrer dans le noyau pour initier la transcription des gènes cibles. L'inhibition du protéasome empêche la dégradation d'IκB, NK-κB reste alors fixé à sa molécule chaperonne IκB dans le cytoplasme et ne peut pas entrer dans le noyau pour exercer sa fonction. Certains types de cellules telles que les plasmocytes sont dépendants de la voie de signalisation d'NK-κB pour leur survie et sont par conséquent plus sensible à l'apoptose induite par l'inhibition du protéasome.

Le deuxième mécanisme et probablement le plus important est l'augmentation du stresse du réticulum endoplasmique lié à l'accumulation de protéines mal repliées dans le réticulum. [endoplasmic reticulum stress (ERS) et unfolded protein response (UPR)]. Quand il y a une accumulation des protéines mal repliées dans le réticulum endoplasmique comme dans le cas de l'hypoxie ou lors d'une infection virale, la voie UPR est activée. L'aactivation de cette voie entraine l'augmentation de l'expression des molécules chaperones pour faciliter le repliement des protéines, l'augmentation de la dégradation des protéines mal repliées dans le réticulum endoplasmique via un processus qui s'appelle ERAD (endoplasmic reticulum-associated degradation) et le ralentissement de la synthèse de nouvelles protéines (Szegezdi et al, 2009). La voie UPR a pour but de restaurer l'homéostasie et maintenir la vie cellulaire. Cependant, l'inhibition du protéasome cause une sur-accumulation de protéines mal repliées dans le réticumum entrainant le débordement de la voie UPR et l'augmentation de l'expression des protéines proapoptotiques comme Bim (Bcl-2-interacting mediator of cell death), TRAIL (tumor necrosis factor-related apoptosis inducing ligand) et TRAF2 (TNF receptor-associated factor 2). La phase finale est l'activation des caspases qui induit la mort cellulaire. Les plasmocytes qui synthétisent des milliers de molécules d'immunoglobulines par seconde sont dépendants de la voie UPR pour leur survie, ce qui explique leur sensibilité à l'inhibition du protéasome.

4. Inhibiteurs du protéasome dans l'hémato-oncologie :

Peu après sa découverte dans les années 80, le protéasome a été reconnu comme une cible thérapeutique potentielle. Plusieurs inhibiteurs du protéasome dont le Bortézomib (Velcade®, Millennium Pharmaceuticals) ont été synthétisés et testés dans différents types de cancer. Le Bortézomib ou l'acide boronique inhibe sélectivement l'activité enzymatique de type chymotrypsine de la sous-unité β5 du protéasome. Le Bortézomib a été approuvé par le FDA pour le traitement du myélome multiple en 2005 (Kane et al, 2006) et du lymphome à cellule du manteau en 2010 (Goy et al, 2010). Le Bortézomib a aussi été testé dans le traitement des autres types de cancer mais les résultats n'ont pas été encourageants.

Suite au succès de Bortézomib, des inhibiteurs du protéasome de seconde génération de structures chimiques différentes ont été développés. On peut citer parmi eux le carfilzomib, le marizomib et le MN9708. Ces nouveaux inhibiteurs du protéasome pourraient être plus efficace et moins toxique et sont actuellement testés dans des essais cliniques de phase I ou II.

5. Inhibition du protéasome dans l'immunologie et transplantation :

En plus de ses applications confirmées dans l'hémato-oncologie, les inhibiteurs du protéasome ont aussi été étudiés dans le domaine d'immunologie. Le Bortézomib inhibe la fonction de plusieurs types de cellules du système immunitaire, dont les lymphocytes T (Berges et al, 2009), les lymphocytes B (Cascio et al, 2008), les cellules dendritiques (Zinser et al, 2009) et les plasmocytes (Lang et al, 2010).

Plusieurs études ont montré l'efficacité du Bortézomib et de certains autres inhibiteurs du protéasome dans différents modèles animaux de maladies autoimmunes comme la polyarthrite rhumatoïde (Palombella et al, 1998, Muchamuel et al, 2009), le lupus érythémateux disséminé (Neubert et al, 2008), la glomérulonéphrite associée aux ANCA (anti-neutrophil cytoplasmic antibodies) (Bontscho et al, 2011), l'encéphalite autoimmune expérimentale (Fissolo et al, 2008), la myasthénie gravis (Gomez et al, 2011) et la colite expérimentale (Basler et al, 2010).

Concernant la transplantation d'organe, deux études ont montré que l'acide boronique prolongeait la survie du greffon dans des modèles de greffe cardiaque (Lu et al, 2001) et d'îlots pancréatiques (Wu et al, 2004). Cependant, les mécanismes d'action du Bortézomib n'ont pas été étudiés en détail.

Récemment, le Bortézomib a été utilisé dans le traitement du rejet dépendant des anticorps chez quelques malades greffés rénaux avec des résultats contradictoires. Everly et al ont traité six patients avec du Bortézomib en combinaison avec la plasmaphérèse, l'injection

d'immunoglobulines par voie intraveineuse et le Rituximab et sont arrivés à stopper le rejet et diminuer les anticorps spécifiques du donneur malgré le fait que deux de ces six patients ont finalement perdu leur greffon rénal moins d'un an après la greffe (Everly et al, 2008). Au contraire, Sberro-Soussan et al ont utilisé le Bortézomib en monothérapie chez quatre malades souffrant de rejet sous-aigu dépendant des anticorps et n'ont montré aucune réduction des anticorps anti-donneurs (Sberro-Soussan et al, 2009).

Dans ce contexte, nos études contribuent à clarifier le rôle du Bortézomib dans le traitement et la prévention du rejet d'allogreffes dans un modèle animal et chez quelques malades transplantés rénaux.

PARTIE EXPÉRIMENTALE

1. Résultats :

Les résultats de nos études ont été publiés dans deux articles dans Kidney International 2010 et Clinical Transplant 2009 (voir article 1 page … et article 2 page …, respectivement).

2. Discussion et perspectives :

Dans l'article 1, dans un modèle de greffe cardiaque chez le rat, nous avons démontré pour la première fois que le Bortézomib en monothérapie permettait de réduire la formation des anticorps spécifiques anti-donneur et atténuer certains signes histologiques de rejet chronique. Un autre groupe a montré aussi l'efficacité du Bortézomib dans un modèle de greffe rénale avec rejet chronique chez le rat en combinaison avec le sirolimus (Vogelbacher et al, 2010).

Dans l'article 2, nous avons rapporté nos expériences avec le traitement du rejet dépendant des anticorps par Bortézomib chez trois patients greffés rénaux, dont deux ont subi un rejet aigu et un a subi un rejet chronique. Seul une patiente souffrant de rejet aigu a répondu au traitement avec une diminution des anticorps anti-donneur et une amélioration de la fonction rénale. Actuellement, plusieurs équipes de transplantation dans le monde ont essayé le Bortézomib chez leurs malades. En général, les patients souffrant de rejets aigus répondent mieux que ceux souffrant de rejets chroniques, et les patients avec rejets aigus qui surviennent moins de 6 mois après la greffe répondent mieux que les patients présentant des rejets aigus plus tardifs (Everly, 2009). Dans tous les cas, le Bortézomib doit être combiné avec d'autres thérapies ciblant les cellules B et les anticorps comme le Rituximab, la plasmaphérèse et l'administration d'immunoglobulines intraveineuses pour optimiser son efficacité.

Dans l'avenir, nous souhaitons poursuivre nos études sur les mécanismes d'action des inhibiteurs du protéasome (notamment de deuxième génération) dans des modèles animaux ainsi que dans

les essais cliniques afin de trouver des nouvelles thérapies applicables dans le rejet dépendant des anticorps. En parallèle, nous avons envisagé les projets de recherche suivants :

- *Inhibiteurs du protéasome de deuxième génération dans des modèles de transplantation d'organe*. Nous discutons avec plusieurs sociétés pharmaceutiques afin d'obtenir leurs nouveaux inhibiteurs du protéasome et les tester dans nos modèles de greffes allogéniques chez le rat et la souris. Certains nouveaux inhibiteurs du protéasome pourraient avoir un meilleur ratio efficacité-toxicité.

- *Radioimmunothérapie ciblant les plasmocytes dans des modèles de transplantation chez la souris*. Nous coopérons avec l'équipe du Pr Chérel à l'Institut de Biologie à Nantes pour tester un anticorps monoclonal anti-CD138 de souris marqué avec du Bismuth-213 radioactif afin de tuer les plasmocytes et ainsi diminuer les anticorps anti-donneur.

- *Le Bortézomib en combinaison avec la plasmaphérèse, l'administration d'immunoglobulines intraveineuses (IVIg) et la méthylprednisolone dans le traitement du rejet chronique dépendant des anticorps chez les patients transplantés rénaux.* Cette étude sera réalisée via le réseau Centaure avec la participation de trois centres de transplantation Lyon, Nantes et Necker Paris et sera coordonnée par Dr Snanoudj à l'Hôpital Necker, Paris. Les patients souffrant d'un rejet chronique dépendant des anticorps diagnostiqué selon la classification Banff seront randomisés en deux groupes. Le groupe 1 recevra un ajustement du traitement d'immunosuppression de maintenance avec du tacrolimus, de l'acide mycophenolique et de la prednisone. Le groupe 2 recevra le même ajustement du traitement d'immunosuppression de maintenance auquel s'ajouteront le bortézomib, la plasmaphérèse, les IVIg et la méthylprednisolone en IV. 50 patients seront recrutés dans chaque groupe pendant 2 ans. Les critères d'évaluation primaire seront une réduction des anticorps anti-donneur d'au moins 50% et une stabilisation des lésions histologiques. Nous espérons que cette étude permettra de clarifier le rôle du Bortézomib dans le traitement du rejet chronique dépendant des anticorps.

- *Le Belimumab pour la diminution des anticorps anti-donneur produits de novo chez les patients transplantés rénaux*. Le Belimumab (Benlysta®, Glaxo-SmithKline) est un anticorps monoclonal dirigé contre le facteur BAFF approuvé récemment par la FDA dans le traitement du lupus érythémateux disséminé. BAFF (B-cell activating factor) est nécessaire pour la différentiation et l'activation des lymphocytes B. L'anticorps anti-BAFF diminue le taux des auto-anticorps chez les patients lupiques et pourrait aussi

diminuer les anticorps anti-donneur chez les patients greffés. Cette étude sera un essai de phase II avec la participation des centres de transplantation de Nantes, Angers et Tours et sera coordonnées par le Pr Dantal à Nantes. Trente patients greffés rénaux présentant des anticorps anti-donneur produits de novo seront randomisés en deux groupes. Le premier groupe recevra un ajustement du traitement d'immunosuppression de maintenance avec du tacrolimus, de l'acide mycophénolique et de la prednisone. Le deuxième groupe recevra le même ajustement du traitement d'immunosuppression de maintenance plus belimumab pendant 6 mois. Le critère d'évaluation primaire sera une réduction des anticorps anti-donneur d'au moins 50%. Le critère d'évaluation secondaire sera l'évolution histologique. Si le résultat est positif, cette étude ouvrira une nouvelle voie de traitement pour le rejet chronique dépendant des anticorps.

PARTIE 2: INHIBITION DU RÉCEPTEUR DE L'INTERLEUKINE-7 EN TRANSPLANTATION D'ORGANE

Tandis que la première partie de ma thèse concerne principalement le rejet dépendant des anticorps, la deuxième partie de ma thèse est consacrée à l'étude d'une nouvelle voie thérapeutique ciblant principalement les lymphocytes T. Même si les anticorps anti-donneur attirent de plus en plus l'attention des chercheurs et des cliniciens travaillant dans le domaine de la transplantation d'organes, ce sont les lymphocytes T qui jouent le rôle crucial dans le rejet d'allogreffe. L'interleukine-7 (IL-7) est connue comme le facteur de croissance le plus important des lymphocytes T. En inhibant le récepteur de l'interleukine-7 (IL-7R) par un anticorps monoclonal avec ou sans combinaison avec d'autres anticorps lympho-déplétants, nous avons induit une lymphopénie et ainsi prolongé la survie du greffon dans des modèles d'allogreffe chez la souris.

1. L'interaction IL-7/IL-7R est essentielle pour la lymphopoïèse :

L'IL-7 et son récepteur ont été découverts au début des années 90 (Goodwin et al, 1989, Goodwin et al, 1990). L'IL-7 est produit principalement par les cellules stromales du thymus et de la moelle osseuse (Komschlies et al, 1995, Kim et al, 2011). Le récepteur de l'IL-7 est présent sur les cellules T, pré-B et dendritiques (Rochman et al, 2009). Il est composé de deux chaînes, la chaînes gamma (γc) est commune à plusieurs cytokines : IL-2, IL-4, IL-7, IL-9, IL-15 et IL-21, tandis la chaîne alpha (IL-7Rα) est utilisée par le récepteur de l'IL-7 et de TSLP (thymic stromal lymphopoietin). Ces deux chaînes n'ont pas d'activités enzymatiques en elles-mêmes, mais sont liées aux deux Janus kinases JAK1 et JAK3, respectivement. L'interaction de l'IL-7 et son récepteur entraine une phosphorylation de ces deux kinases, ce qui à son tour entrainent une

phosphorylation des STATs (signal transducers and activators of transcription) qui entrent dans le noyau cellulaire pour réguler l'expression des gènes.

Le knockout du gène de l'IL-7 (von Freeden-Jeffry et al, 1995) ou de l'IL-7R (Peschon et al, 1994) ainsi que le blocage de l'IL-7 (Bhatia et al, 1995) ou de l'IL-7R (Sudo et al, 1993) par des anticorps monoclonaux chez la souris induisent une lymphopénie affectant les cellules T, B, mais pas les cellules NK. Chez l'homme, la mutation de la chaîne alpha du récepteur de l'IL-7 est une des causes du déficit immunitaire combiné sévère (DICS) (severe combined immunodeficiency ou SCID en anglais) (Macchi et al, 1995). Les nourrissons atteints des mutations de l'IL-7Rα ont une lymphopénie sévère affectant les cellules T, tandis que le nombre des cellules B et NK est normal ou augmenté. Ces enfants ont besoin d'une greffe de moelle le plus tôt possible, faute de quoi ils risquent de succomber à des infections répétitives. L'interleukine-7 est donc une cytokine non-redondante pour la lymphopoïèse normale.

Plusieurs études utilisant des souris lymphopéniques ont montré que l'IL-7 est aussi nécessaire pour l'homéostasie des lymphocytes T CD4 et CD8 naïves et mémoires (Schluns et al, 2000, Tan et al, 2002). De façon intéressants, l'IL-7Rα ou CD127 n'est pas exprimé ou exprimé à un faible niveau par les cellules T régulatrices CD4+CD25+FOXP3+. CD127 est donc le marqueur extracellulaire utilisé plus spécifiquement que le marqueur intracellulaire FOXP3 pour isoler des cellules T régulatrices dans les études fonctionnelles (Liu et al, 2006, Seddiki et al, 2006, Michel et al, 2008). Plus important encore, si on a un anticorps déplétant anti-IL-7Rα, on peut dépléter les cellules T effectrices en respectant les cellules T régulatrices.

2. IL-7 et IL-7R comme cible thérapeutique :

Étant donnant le rôle crucial de l'IL-7 dans la lymphopoïèse, l'IL-7 recombinant humaine (rhIL-7) est actuellement développé afin d'augmenter le taux des lymphocytes chez les patients HIV ou les patients lymphopéniques secondairement à une chimiothérapie pour cancers. Deux études de phase I chez les patients atteints des cancers réfractaires ont montré que l'rhIL-7 augmente significativement le nombre des lymphocytes T CD4 et CD8 (Sportès et al, 2008, Rosenberg et al, 2006). Une étude de phase I/II chez les patients VIH positifs a aussi montré que l'rhIL-7 augmente significativement le nombre de lymphocytes T CD4 (Levy et al, 2009). Une étude de phase II est en cours.

A l'inverse, l'inhibition de l'IL-7 ou de l'IL-7R a été testée aussi dans plusieurs modèles animaux. Le blocage du récepteur de l'IL-7 atténue l'anémie et prolonge la survie dans un modèle d'anémie hémolytique auto-immune chez la souris (Hoyer et al, 2007). L'inhibition de l'IL-7 ou de l'IL-7R améliore les scores cliniques dans un modèle d'encéphalite auto-immune

expérimentale chez la souris via l'induction de l'apoptose des cellules T_H17 (Liu et al, 2010). Le blocage du récepteur de l'IL-7 par un anticorps anti-IL-7Rα à faible dose réduit le taux de mortalité et de morbidité liée à la maladie du greffon contre l'hôte dans un modèle de greffe de moelle chez la souris (Chung et al, 2007).

En transplantation d'organe, une seule étude a montré l'efficacité d'un anticorps polyclonal anti-IL-7 en combinaison avec un blocage de costimulation dans la prolongation de la survie du greffon dans un modèle de greffe cardiaque chez la souris (Wang et al, 2006). Dans cette partie de ma thèse, nous avons étudié l'efficacité d'un anticorps bloquant anti-IL-7Rα (clone A7R34) utilisé seul ou en combinaison avec des anticorps déplétants anti-CD4 et anti-CD8 dans des modèles d'allogreffe chez la souris.

PARTIE EXPÉRIMENTALE

1. Résultats :

Les résultats de notre étude sont présentés sous forme d'un article en préparation. Brièvement, nous avons trouvé que le blocage du récepteur de l'IL-7 par un anticorps anti-IL-7Rα (clone A7R34) donné à partir du jour de la greffe ne prolonge pas la survie des îlots pancréatiques chez les souris présentant un diabète induit par la streptozotocine. Par contre, quand le traitement est commencé trois semaines avant la greffe et continué jusqu'à jour 90 post-greffe, nous avons induit une survie du greffon à long terme au-delà de 180 jours. Ensuite, nous avons montré que les souris traitées par l'anticorps anti-IL-7Rα après une déplétion des cellules T par une combinaison de l'anticorps anti-CD4 et anti-CD8 présentent une survie de la greffe de peau significativement prolongée par rapport aux souris ayant reçu seulement une déplétion des cellules T par la même combinaison d'anti-CD4 et anti-CD8. Le modèle d'allogreffe de peau est plus « strigent » que le modèle de greffe d'îlots chez les souris diabétiques induits par la streptozotocine. Les mécanismes principaux du blocage du récepteur de l'IL-7 dans la prolongation de la survie du greffon sont la diminution du nombre de presque toutes les sous-populations de lymphocytes, T, T CD4, T CD8, B et T mémoires définis comme $CD44^{hi}CD62L^{lo}$ et l'augmentation relative du pourcentage des cellules T régulatrices CD4+CD25+FOXP3+. Dans ces deux modèles d'allogreffe, les souris traitées avec l'anti-IL-7Rα ont produit moins d'anticorps anti-donneur que souris contrôles.

2. Perspectives :

Nous allons étudier les mécanismes de cette prolongation de survie du grefon en culture mixe lymphocytaire, Elispot, et étudier l'effet du transfert des cellules T régulatrice dans ces modèles.

Nous avons également commencé à tester le même protocole de blocage du récepteur de l'IL-7R après une déplétion de cellules T dans un modèle de greffe d'îlots chez les souris NOD (non obese diabetic). Ce modèle est aussi un modèle « stringent » puisque les îlots greffés sont attaqués à la fois par l'allo-immunité et l'auto-immunité, une situation plus proche de celle de l'homme.

Nous sommes coopérons avec l'industrie pour développer un anticorps monoclonal anti-IL-7Rα humaine et allons le tester dans un modèle d'allogreffe chez les primates non-humains. Si son efficacité est confirmée, cet anticorps pourrait ouvrir une nouvelle voie thérapeutique en transplantation d'organe.

PREFACE

Human as well as animals are armed with an immune system allowing them to combat innumerable types of infection and cancer that they may encounter during life. The human immune system comprises an innate immune system and an adaptive immune system. The innate immune system has a cellular component including mainly granulocytes, monocytes, macrophages, and natural killer cells as well as a humoral component including the complement system and various inflammatory mediators. The adaptive immune system likewise also has a cellular component including T lymphocytes, B lymphocytes, and plasma cells as well as a humoral component composed of antibodies. Innate immunity provides a strong, immediate, but non-specific reaction to infections, giving the time for the adaptive immune system to mount a specific immune response that helps to eliminate the pathogens more effectively and confers long-term protection against these pathogens through immune memory. Dendritic cells (and to a lesser extent macrophages) are professional antigen-presenting cells that link the innate to the adaptive immune system.

One of the principal mechanisms of action of the adaptive immune system is the recognition and subsequent elimination of foreign or non-self antigens, which consist of two main types: the first one includes proteins, polysaccharides, and glycolipids of microbiological pathogens, and the second one consists of major and minor histocompatibility antigens (MHC and mHAg, respectively). Whereas the recognition of microbiological antigens is essential for immune defense, the recognition of MHC and mHAgs, or alloimmunity is the major hurdle to organ transplantation, leading to graft rejection.

Although innate immunity also has an important role, the adaptive immune system is central to the pathogenesis of allograft rejection. Allograft rejection is currently classified into two main categories: T-cell mediated or antibody-mediated rejection (AMR), although these two mechanisms usually coexist in the same patient. Most of the therapies used in organ transplantation target T cells, only few of them can directly decrease alloantibody formation. Moreover, current immunosuppressive drugs have important side effects, and rejection may still occur or be refractory to treatment leading to graft failure despite the combination of currently available drugs. Therefore, the aim of our study is to find new drugs that target each of these two main mechanisms of graft rejection.

The first part of my thesis is dedicated to the study of bortezomib, a prototype of proteasome inhibitors, a novel class of drugs which has been used mainly in the treatment of multiple myeloma. Bortezomib, besides its proapoptotic effects on myelomatous plasma cells, can also

target non-malignant plasma cells, leading to a decrease in alloantibody formation, which is essential for the treatment of antibody-mediated rejection.

In the second part of my thesis, we explored a new therapeutic pathway to target T cells through the blockade of the interleukin-7 receptor (IL-7R) using a monoclonal antibody. Since interleukin-7 is critical for normal lymphopoiesis, IL-7R blockade will profoundly affect T cell homeostasis in a way that may be potentially beneficial for allograft survival.

Although the two parts of my thesis seem unrelated at the first glance, I think that they address two major and coexisting mechanisms of allograft rejection: the antibody-mediated and T cell-mediated rejection. Both mechanisms must be taken into account in order to effectively treat or prevent these two types of rejection to assure a better long-term graft survival.

PART 1

PROTEASOME INHIBITION IN ORGAN TRANSPLANTATION

INTRODUCTION

1. Allograft rejection:

Organ transplantation is the only treatment for patients with end-stage heart or liver failure and the treatment of choice for patients with end-stage renal failure. The development of novel effective immunosuppressive therapies over the past few decades has improved both the short-term and long-term outcome of transplant patients. Nevertheless, allograft rejection still remains the principal concern in solid organ transplantation.

1.1. Classification of allograft rejection:

Recent advances in renal pathology and transplant immunology have provided us with a detailed and internationally accepted classification of renal allograft rejection which is known as the Banff classification. This classification originated from a meeting of a group of renal pathologists and transplant physicians held in Banff, Canada in 1991 (Solez et al, 1993). The classification has been welcome by the transplant community and clinically validated in numerous studies. Subsequent meetings were held in Banff every two years to refine the classification (Racusen et al, 1999, Racusen et al, 2003, Solez et al, 2007, Solez et al, 2008, Sis et al, 2010).

Although Banff classifications also exist for liver, cardiac, and pancreatic allograft pathology, we will limit our subsequent discussion to kidney transplantation, which is by far the most common type of organ transplantation. According to the last Banff classification (Sis et al, 2010), renal allograft rejections are divided into T-cell-mediated and antibody-mediated, although these two categories are not mutually exclusive.

Acute T-cell mediated rejection is characterized by significant interstitial infiltration of more than 25% of parenchyma associated with foci of moderate (grade IA) or severe (grade IB) tubulitis. It is also characterized by mild to moderate (grade IIA) or severe (grade IIB) intimal arteritis. Rejection is most severe (grade III) when there are transmural arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic infiltration.

Chronic active T-cell mediated rejection is diagnosed when there is a chronic allograft arteriopathy defined by arterial intimal fibrosis with mononuclear cell infiltration in fibrosis and formation of neo-intima.

Acute antibody-mediated rejection is diagnosed by a triad of (1) presence of circulating

antidonor antibodies, (2) peritubular capillary C4d deposition, and (3) morphologic evidence of acute tissue injury, such as acute tubular necrosis (ATN)-like minimal inflammation (grade I), capillary and/or glomerular inflammation and/or thromboses (grade II), and transmural arteritis (grade III).

Chronic active antibody-mediated rejection is characterized by a triad of (1) presence of circulating antidonor antibodies, (2) peritubular capillary C4d deposition, and (3) morphologic evidence of chronic tissue injury, such as glomerular double contours and/or peritubular capillary basement membrane multilayering and/or interstitial fibrosis/tubular atrophy and/or fibrous intimal thickening in arteries.

Several points in this classification need to be clarified. First of all, although the terms acute and chronic may denote a temporal relationship to the time of transplant, it is not always the case. Although acute rejection occurs most often in the first few months post-transplant, it can happen years after transplantation. Conversely, histologic signs of chronic rejection can be detected as early as a few months post-transplantation. Secondly, T-cell mediated and antibody-mediated rejection can coexist in the same patient. Thirdly, the term hyperacute rejection is not mentioned in the newer Banff classifications, although it was used in the older versions. Hyperacute rejection is associated with preformed anti-donor HLA antibodies (Patel et al, 1969). It occurs within minutes to a few days post-transplant and is characterized by severe transmural arteritis, widespread glomerular capillary thrombosis, and in the most severe form, renal cortical necrosis. Graft loss is almost inevitable despite treatment. Fortunately, these preformed antibodies can readily be detected by lymphocytotoxicity crossmatches which are performed routinely before transplant, as a result, hyperacute rejection become rare nowadays. Finally, chronic rejection is one of the main causes, but not the only cause of late graft loss. Other etiologic factors, including hypertension, calcineurin inhibitor toxicity, chronic obstruction, bacterial pyelonephritis, and viral interstitial nephritis should be carefully sought when reading renal transplant biopsies (Solez et al, 2007).

1.2. Mechanisms of allograft rejection:

Although the innate immune system including complement, Toll-like receptors, and NK cells also plays important roles (LaRosa et al, 2007), the adaptive immune system including T cells and antibodies is central in the pathogenesis of allograft rejection.

1.2.1. *T cell-mediated rejection:*

The interaction between T-cell receptors (TCR) on T cells and MHC molecules on antigen-presenting cells (APC) delivers the first signal to initiate allorecognition. T cells recognize

alloantigens through three pathways: the direct pathway, whereby recipient T cells recognize intact allogenic MHC-peptide complexes expressed by donor APC, the indirect pathway, whereby T cells recognize peptides derived from allogenic MHC proteins presented by self APC, and the semidirect pathway, whereby recipient dendritic cells (DC) acquire intact allogenic MHC-peptide complexes from donor cells and present them to recipient T cells (Safinia et al, 2010).

Besides the TCR-MHC interaction, T cell activation requires second signals, or costimulatory signals, which are generated mainly by the interaction between CD80 (B7-1) or CD86 (B7-2) expressed on APCs and CD28 or CTLA-4 (cytotoxic T-lymphocyte antigen-4) displayed on T cells. The binding of CD80 or CD86 to CD28 stimulates T cells, whereas the binding of these B7 molecules to CTLA-4 delivers an inhibitory signal (Poirier et al, 2010).

Following alloantigen recognition, T cells become activated, proliferate, and differentiate into subtypes with characteristic cytokine profiles (Nankivell et al, 2010). Type 1 helper T cells (Th1), which mainly secrete IFN-γ and IL-2, induce cellular immune response, type 2 helper T cells (Th2), which mainly secrete IL-4, 5 and 13, produce humoral immune response. The third type of helper T cells, type 17 helper T cells (Th17), which is characterized by the secretion of IL-17, 21, and 22 also play a role in allograft rejection (Heidt et al, 2010). On the other hand, a fraction of T cells differentiate to regulatory T cells, which are characterized by the expression the transcription factor FOXP3 (folkhead box P3), secrete IL-10 and TGF-β, and can limit rejection response (Li et al, 2010).

In acute T cell-mediated renal allograft rejection, T cells use adhesion molecules, including leukocyte-function-associated antigen 1 (LFA-1) to attach to the endothelium and migrate across the peritubular capillaries to enter the graft. Graft-infiltrating T cells have been cloned and shown to be able to recognize donor-specific HLA molecules (Moreau et al, 1986, Bonneville et al, 1988, Soulillou et al, 1990). In the graft, T cells secrete tumor necrosis factor (TNF) α and β, express Fas ligand (FasL), and release cytotoxic granules containing granzyme B and perforin, all these molecules can induce apoptosis of tubular cells. In addition, the secretion of numerous chemokines such as chemokine (C-C motif) ligand 2 (CCL2), CCL3, 4, 5, and chemokine (C-X3-C motif) ligand 1 (CX3CL1) in the graft undergoing rejection attracts more mononuclear cells which increase inflammation and destroy the graft if left untreated (Cornell et al, 2008).

1.2.2. *Antibody-mediated rejection*:

The association between the appearance of donor specific antibody (DSA) and refractory kidney allograft rejection has been known long time ago (Soulillou et al, 1978). However, antibody-

mediated rejection has been widely recognized as a distinct clinicopathologic entity only in the past decade (Racusen et al, 2003). The diagnosis of antibody-mediated rejection is facilitated by two major advances in technology. The first one is the immunohistological detection of C4d complement fragment in peritubular capillaries pioneered by Feucht and confirmed by other groups (Feucht et al, 1993, Colvin, 2007). C4d is a fragment of C4b, an activating product of the classic complement pathway. C4d has no known function by itself, but it remains bound to tissue after immunoglobulin and C1 have been released and serves as an evidence of the previous fixation of anti donor antibodies onto the capillary endothelium. The second one is the development of sensitive methods for the detection of circulating anti donor HLA antibodies, especially the luminex technology (Mizutani et al, 2007, Tait et al, 2010).

Numerous clinical observational studies have shown the association between the formation of donor specific antibodies and poor graft outcome (Worthington et al, 2003, Terasaki et al, 2004, Hourmant et al, 2005, Mizutani et al, 2005, Mao et al, 2007, Terasaki et al, 2007, Lachmann et al, 2009, Everly et al, 2010, Cooper et al, 2011). In parallel, several experimental studies attempt to answer the question whether and through what mechanisms alloantibodies per se can cause allograft rejection.

Anti-HLA class I can activate endothelial exocytosis resulting in the release of von Willebrand Factor (vWF) and P-selectin which in turn promote leukocyte recruitement and cause vascular inflammation (Yamakuchi et al, 2007). HLA class I ligation on endothelial cells also induce cytoskeleton rearrangement and cellular proliferation through activation of the GTP-binding protein RhoA (Coupel et al, 2004). Anti-HLA class I antibodies induce proliferation of endothelial cells and vascular smooth muscle cells via two major pathways: the mammalian target of rapamycin (mTOR) pathway and the mitogen-activated protein kinase (MAPK) signaling pathway (Li et al, 2009). In experimental models in which cardiac allograft is transplanted to recombinant activating gene-1 knockout (RAG1-/-) mice, the passive transfer of anti-HLA class I alloantibodies can cause typical lesions of antibody-mediated rejection such as capillary dilatation with intracapillary macrophages, C4d deposition, and chronic transplant arteriopathy (Uehara et al, 2007, Jindra et al, 2008, Hirohashi et al, 2010). Since RAG1-/- knockout mice are devoid of both T cells and B cells, these studies demonstrate that anti-HLA class I antibodies per se can cause allograft rejection.

However, little is known about the role of anti-HLA class II antibodies in the pathogenesis of antibody-mediated rejection. Anti-human HLA DR antibodies failed to trigger apoptosis of vascular endothelial cells, whereas anti-HLA class II ligation induced cell death in antigen-presenting cells (Le Bas-Bernardet et al, 2004).

2. Treatment of antibody-mediated rejection (AMR):

Most of the immunosuppressive drugs currently used in clinical organ transplantation target T cells (Halloran, 2004, Taylor et al, 2007). For example, calcineurin inhibitors including cyclosporine and tacrolimus block the dephosphorylation and nuclear translocation of nuclear factor of activated T-cells (NF-AT), thereby inhibit T cell cytokine gene transcription and T cell activation. Mammalian target of rapamycin (mTOR) inhibitors including sirolimus and everolimus affect the phosphatidylinositol 3-kinase (PI3K) signaling pathway which is required for cell-cycle progression, thereby inhibiting T-cell proliferation. Anti-IL-2 receptor monoclonal antibodies (basiliximab or daclizumab) inhibit T-cell activation. Thymoglobulin, the polyclonal antiserum against human thymocytes, as well as OKT3, the murine monoclonal antibody directed against human CD3 depletes T cells and is used as induction therapy or in the treatment of severe acute rejection. Another depleting monoclonal antibody used in induction therapy, the anti-CD52 alemtuzumab (Campath-1H) profoundly depletes T cells, although B cells and monocytes are also partially depleted because they also express CD52. Only mycophenolic acid (MPA) can decrease antibody formation because it inhibits the proliferation of both T and B lymphocytes through inhibition of inosine monophosphate dehydrogenase (IMPDH), an enzyme required for the de novo synthesis of guanosine. Whereas other cells can reuse purines from the turnover of nucleic acids through the salvage pathway, lymphocytes are critically dependent on the de novo synthesis of purines for their proliferation (Jain et al, 2004).

Unfortunately, antibody-mediated rejection (AMR) is relatively unresponsive to treatments targeting T cells. Although the process of B-cell antigen recognition, proliferation, and differentiation into antibody-secreting plasma cells requires the interaction with helper T cells, profound T cell lymphopenia following induction therapy by T cell depletion therapy by alemtuzumab could not completely prevent the occurrence of acute AMR (Knechtle et al, 2003, Flechner et al, 2005, Willicombe et al, 2011).

2.1. Current treatment of AMR:

There are only a few therapies that direct against antibodies and/or B cells: (1) plasmapheresis or plasma exchange that rapidly removes circulating alloantibodies, (2) rituximab, a monoclonal anti-CD20 antibody that depletes B cells (Pescovitz, 2006), and (3) intravenous immunoglobulin (IVIg), whose immune-modulatory actions are complex and affect both humoral and cell-mediated immunity (Jordan et al, 2009).

Several case-series studies showed the beneficial effects of IVIg, plasmapheresis, and rituximab alone or in combination in the treatment of acute AMR (Rocha et al, 2003, Becker et al, 2004).

In a recent comparative study, combination therapy with plasmapheresis plus high dose IVIg and rituximab was shown to be superior to high dose IVIg alone in terms of DSA removal and kidney graft survival (Lefaucheur et al, 2009). Another study also showed that treatment with rituximab plus plasmapheresis was associated with better kidney graft survival than plasmapheresis alone (Kaposztas et al, 2009). In fact, the current therapy of acute AMR relies on the combination of these three treatment modalities and the choice of a specific regimen is center-dependent.

IVIg alone (Glotz et al, 2002, Jordan et al, 2003) or in combination with rituximab (Vo et al, 2008, Vo et al, 2010) was also used as desensitization therapy for patients awaiting renal transplantation but having preformed alloantibodies and positive crossmatches to multiple potential donors. These treatments decreased alloantibody levels and allowed renal transplantation although acute rejection occurred in up to 50% of patients (Vo et al, 2008) and graft loss at 2 year post-transplant was up to 16% (Vo et al, 2010).

Another approach for patients with preformed DSA is the use prophylactic therapy directed against antibodies/B cells at the time of renal transplantation. A recent study compared two prophylactic therapies, one combining IVIg, rituximab, and plasmapheresis, and the other using only IVIg given at the time of transplant in patients with preformed DSA. All patients also received quadritherapy including induction with thymoglobulin or basiliximab and maintenant immunosuppression with tacrolimus or cyclosporine (Neoral), mycophenolate mofetil (MMF), and corticosteroid. Although the acute AMR rates were not different between the two treatment groups, the combined therapy with IVIg/rituximab/plasmapheresis was associated with a lower rate of chronic AMR, a greater reduction of DSA, and a higher glomerular filtration rate (GFR) at 1 year post-transplant (Loupy et al, 2010).

Recently, in a large single-center study, Montgomery et al used a desensitization therapy with plasmapheresis and low-dose IVIg for patients having available live-donor kidneys but having either positive crossmatch or negative crossmatch but positive DSA on multiplex bead assay. This treatment allowed live-donor kidney transplantation in 211 of 215 patients. Patients undergoing live-donor kidney transplantation after this desensitization therapy had much better estimated patient survival compared to two carefully matched control groups of patients, one group remained always on dialysis and the other group continued dialysis and underwent transplantation whenever HLA-compatible kidneys were available. This is the first large study that demonstrates patient survival benefit of desensitization therapy, although longer followup is necessary to confirm these results since haft of the patients in the cohort were followed up for less than 3 years (Montgomery et al, 2011).

2.2. Novel therapies for AMR:

Although the current treatment for acute AMR is generally effective, a non-negligible proportion of patients with acute AMR do not respond to treatment, and patients having had desensitization or acute AMR have reduced long-term graft survival compared to unsensitized patients or patients who have not experienced AMR episodes (Webber et al, 2011). More importantly, there is virtually no effective treatment for chronic AMR, despite anecdotal reports of successful treatment of chronic AMR by rituximab combined with IVIg (Billing et al, 2008, Fehr et al, 2009). As a result, novel therapies directed against B cells and alloantibodies are being actively sought.

2.2.1. *New B-cell depleting monoclonal antibodies:*

Since rituximab is a chimeric monoclonal antibody (mAb), new humanized anti-CD20 mAbs such as ocrelizumab, ofatumumab, and AME-133 are under development and tested in preclinical models and early clinical studies for the treatment of lymphoma and systemic lupus erythematosus (SLE). These humanized mAbs have lower immunogenicity and higher potency in depleting B cells as compared with rituximab as they can more strongly activate the antibody-dependent cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) pathways. These new agents may be useful for the treatment of AMR (Vincenti et al, 2010).

Another B cell surface marker, CD22 remains expressed on B cells as they mature and become committed to antibody secretion whereas CD20 has disappeared. Therefore, epratuzumab, a humanized mAb anti-CD22 may be a more effective strategy than using anti-CD20 mAbs (Traczewski et al, 2011).

2.2.2. *Therapies targeting B-cell survival factors:*

Other potential targets for controlling humoral immunity include the B-cell activating factors (BAFF), also known as B-lymphocyte stimulator (BLyS) and a proliferation-inducing ligand (APRIL). BAFF and APRIL belong to the tumor necrosis factor (TNF) ligand family and act as antiapoptotic factors which are critical for the survival and maturation of the B-cell lineage (Bossen et al, 2006, Tangye et al, 2006, Mackay et al, 2009). BAFF signals through three receptors present on B cells: the BAFF receptor (BAFF-R), the B-cell maturation protein (BCMA), and the transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI). APRIL signals through the latter two. The downstream effect is the activation of nuclear factor kappa b (NF-κB) which then stimulates activation and differentiation of B cells.

There are currently two biologics that target the BAFF/APRIL signaling pathway. The first one

is belimumab, a humanized anti-BAFF mAb that block the binding of BAFF to its receptors on B cells. Belimumab has recently been approved by FDA for the treatment of SLE after a successful phase III clinical trial (Navarra et al, 2011). A phase II trial of belimumab in the desensitization of patients with preformed alloantibodies awaiting kidney transplantation is under way (ClinicalTrials.gov). The second one is atacicept, a TACI-Ig fusion protein that inhibits B-cell stimulation by blocking both BAFF and APRIL ligands. TACI-Ig acts mainly on B cells at later stages of differentiation but does not affect B-cell progenitors in the bone marrow. In a phase Ib trial, atacicept treatment reduced B cell number and immunoglobulin levels in SLE patients (Pena-Rossi et al, 2009). This drug may be a promising agent in the desensitization and treatment of AMR but currently there is no undergoing clinical trial in transplant patients.

2.2.3. *Inhibition of complement activation:*

Since complement activation is an important mechanism in AMR, targeting the complement system may have a role in the treatment of AMR. Eculizumab, a humanized anti-C5 mAb used in the treatment of paroxysmal nocturnal hemoglobinuria (PNH) (Hillmen et al, 2006) and atypical hemolylic uremic syndrome (Noris et al, 2009) has been shown to reduce the incidence of AMR in the first month following kidney transplantation in patients with preformed high-titer DSA (Stegall et al, 2010). However, 6 of the 16 treated patients later developed chronic AMR (Cornell et al, 2010) suggesting that the effect of eculizumab is short-term and it should be combined with treatment that lowers DSA levels.

2.2.4. *Proteasome inhibition – a novel therapy that targets plasma cells:*

The major setback of all the aforementioned treatments is that none of them affects plasma cells, which are the antibody-producing cells. The anti-CD20 and anti-CD22 antibodies deplete B cells but not plasma cells because CD20 and CD22 no longer express when B cells terminally differentiate to plasma cells. Similarly, targeting the BAFF signaling pathway does not deplete plasma cells because BAFF is necessary for the survival of murine but not human plasma cells (Tangye et al, 2006).

Recently, bortezomib, which belongs to a novel class of drugs – the proteasome inhibitors – has emerged as a potential therapeutic agent for AMR (Everly et al, 2008). Bortezomib has been approved for the treatment of multiple myeloma and is indeed a major advance in the treatment of this disease. Through inhibition of cellular protein degradation by proteasome, bortezomib induces apoptosis not only of myelomatous plasma cells, but also of normal plasma cells and thereby abrogates alloantibody production.

I am interested in this new class of drugs and have devoted more than half of my PhD research studying the role of bortezomib in the treatment of acute and chronic AMR in animal models as well as in kidney transplant patients.

In order to explain the rational for the choice of proteasome inhibition as a potential target in organ transplantation, in the following sections, I will discuss in detail the basic knowledge about the ubiquitin-proteasome system, the development proteasome inhibitors and their clinical use in cancer treatment as well as their preclinical and pilot clinical use in immunology and transplantation. Then I will present and discuss my two published papers on the role of the proteasome inhibitor bortezomib in organ transplantation in rat models (article 1) and in kidney transplant patients (article 2).

3. The ubiquitin-proteasome system:

3.1. Overview:

All cells and organisms require a regulated balance between protein synthesis and degradation for the maintenance of life. After the discovery of lysosome in the 1950s, this organelle was once believed to be responsible for all cellular protein degradation within the body. However, lysosomal inhibitors were found to be ineffective in preventing basal protein breakdown. Subsequently, the ubiquitin-proteasome pathway was found to be a tightly regulated system which is responsible for the degradation of the majority of intracellular proteins permitting the adaptation of cells to different physiologic conditions. Indeed, the Nobel Prize in Chemistry 2004 was awarded jointly to Aaron Ciechanover, Avram Hershko and Irwin Rose “for the discovery of ubiquitin-mediated protein degradation”.

Degradation of intracellular proteins occurs in the following situations: 1) Proteins are not synthesized correctly, for example, misincorporation of an amino acid can produce proteins with folding defects, which should be quickly recognized and destroyed. 2) Environmental agents such as heat and oxidation can damage proteins, which should be repaired or degraded. 3) Many short-live functional proteins such as cyclins, transcription factors, and enzymes are no longer required although they are not damaged. The timely degradation of these functional proteins is essential to maintain cellular homeostasis.

In order to accomplish these important functions, cells are armed with the ubiquitin-proteasome system which is a pathway for degrading proteins with a very high degree of specificity. To be degraded, the protein must first be tagged with ubiquitin, or ubiquitinated.

3.1.1. *Ubiquitin:*

Ubiquitin, as indicated by its name, is ubiquitous in all eukaryotic cells. Indeed, it is one of the most conserved proteins ever known since human and yeast ubiquitin share 96% sequence identity. It is a small, highly compact protein containing 76 amino acids with 7 lysine residues, several small hydrophobic patches on the surface, and a highly reactive carboxy terminus (Figure 1). The ubiquitination of target protein requires 3 key enzymes. 1) E1: ubiquitin-activating enzyme that binds 2 ubiquitins via a thioester bond. E2: ubiquitin-conjugating enzyme which mediates the transfer of ubiquitin from E1 to E3. 3) E3: ubiquitin-protein ligase which binds the ubiquitin to the protein substrate via an isopeptide bond between the carboxy-terminal glycine (glycine-76) and a lysine side-chain on the target protein (substrate). However, the attachment of a single ubiquitin will not promote protein degradation. The target protein must be attached to a chain of multiple ubiquitin molecules in order to be degraded. The multiubiquitin chain is assembled via an isopeptide bond between a lysine residue on one ubiquitin to the carboxy-terminal glycine of another ubiquitin. Although each molecule of ubiquitin has 7 lysine residues, a chain of ubiquitin that promote protein degradation can only be assembled using lysine-29 and lysine-48. The reason is that a chain of ubiquitin assembled in that way leads to the alignment of the small hydrophobic patches on each ubiquitin to form an extended hydrophobic stripe, which is the primary determinant for promoting interaction with the proteasome (Figure 2A and 2B).

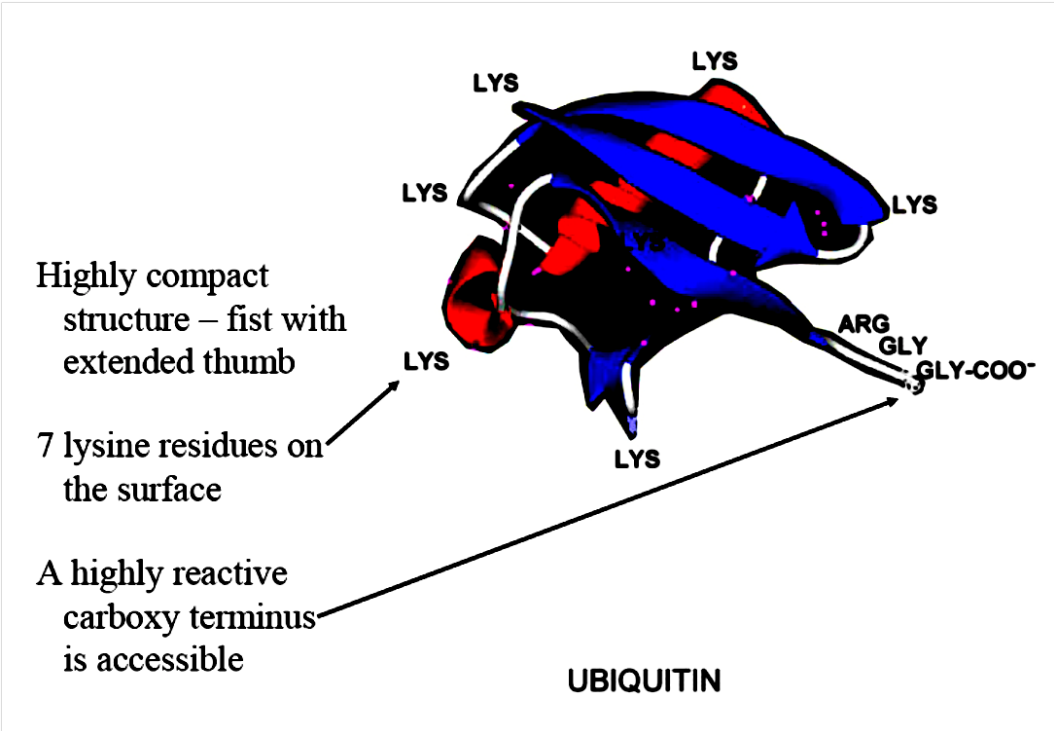


Figure 1: Tertiary structure of ubiquitin. Ubiquitin is a highly compact protein composed of 76 amino acids including 7 lysine residues and a highly reactive carboxy terminus.

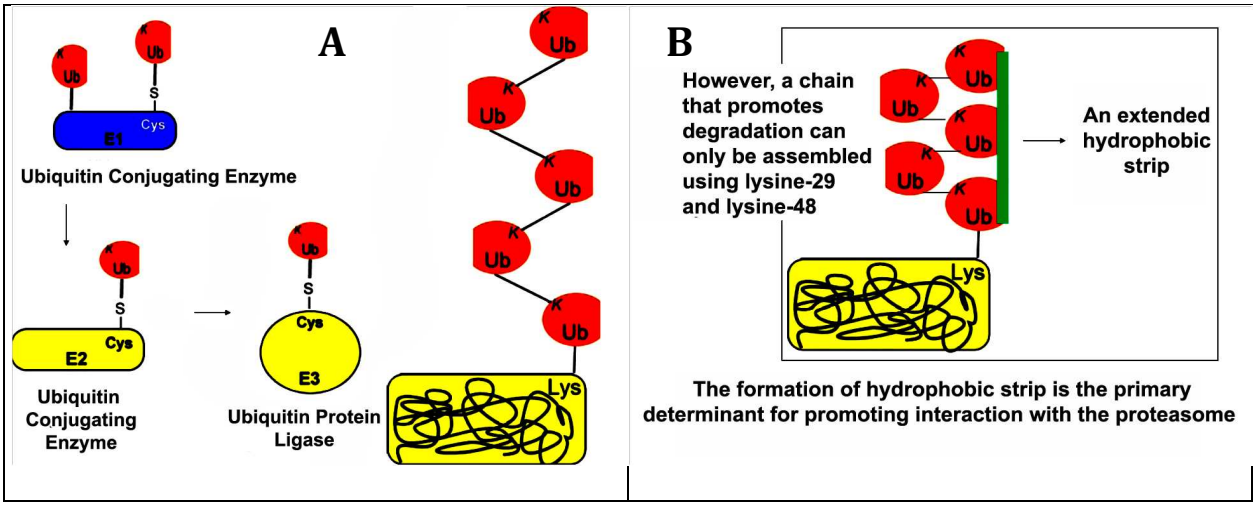


Figure 2 : (A) Ubiquitination of target proteins. The ubiquitination of target protein requires 3 key enzymes: E1: ubiquitin-activating enzyme, E2: ubiquitin-conjugating enzyme, and E3: ubiquitin-protein ligase. The target protein must be attached to a chain of multiple ubiquitin molecules in order to be degraded. **(B) Formation of an extended hydrophobic stripe.** A chain of ubiquitin that promote protein degradation can only be assembled using lysine-29 and lysine-48 leading to the alignment of the small hydrophobic patches on each ubiquitin to form an extended hydrophobic stripe, which promotes interaction with the proteasome.

3.1.2. *Proteasome:*

The proteasome is a large, multiprotein, hollow cylindrical particle present in both the cytoplasm and the nucleus of all eukaryotic cells and accounts for approximately 1 to 2% of cell mass (Voges et al, 1999). The most common form of proteasome is the 26S proteasome, composed of two functional components: a 20S central catalytic complex and an outer 19S regulatory subunit. The aforementioned multiubiquitinated protein will be recognized by a receptor on the 19S complex. The multiubiquitin chain is then released for reuse and the target protein is unfolded in order to be able to enter the hollow 20 S catalytic core and be ready for degradation.

The 20S complex is composed of four heptameric protein rings assembled like four doughnuts comprising two identical outer α-rings and two identical inner β-rings, each containing seven subunits, α1-α7 and β1-β7, respectively. The nomenclature of the α- and β-subunits is rather complicated because each subunit can be called by 3-4 different names with confusing numerical system (Table 1) (Nandi et al, 2006).

Table 1 : Nomenclature of 20S constitutive proteasome subunits in mammals

Subunit	Alternative names	Subunit	Alternative names
α1	PSMA6/Iota/LMP11	β1	PSMB6/Y/delta/LMP19
α2	PSMA2/C3/LMP8	β2	PSMB7/Z/LMP9
α3	PSMA4/C9/LMP14	β3	PSMB3/C10/LMP5
α4	PSMA7/C6/XAPC7/LMP16	β4	PSMB2/C7/LMP6
α5	PSMA5/Zeta/LMP1	β5	PSMB5/X/MB1/LMP17
α6	PSMA1/C2/LMP13	β6	PSMB1/C5/LMP15
α7	PSMA3/C8/LMP18	β7	PSMB4/N3/beta/LMP3

PSMA1, 2, 3, etc… : proteasome subunit alpha type-1, 2, 3, etc…

PSMB1, 2, 3, etc… : proteasome subunit beta type-1, 2, 3, etc…

LMP1, 2, 3, etc… : low-molecular mass polypeptide-1, 2, 3, etc…

The α-subunits form a selective barrier between the catalytic chamber and the cytoplasm. They are the sites for the binding of various regulatory particles and selectively permit the entry and exit of substrates. On the hand, β-subunits harbor the catalytic sites. In fact the proteolytic activities of proteasome reside only on three β-subunits, β1, β2, and β5 with caspase-like (cleavage after acidic amino acids), trypsin-like (cleavage after basic amino acids), and chymotrypsin-like (cleavage after hydrophobic amino acids) activities, respectively. Thus, inside the catalytic chambers, the target protein is surrounded by six protease-active sites (three on each β-subunit ring). Proteins processed by proteasome are reduced to small polypeptides 3 to 22 residues in length (Nussbaum et al, 1998).

Proteolysis by the 26S proteasome is an essential metabolic process, and complete blockade of proteasome activities results in the death of cells and organisms. Proteasome has been shown to be responsible for the degradation of various functional proteins such as cyclins, transcription factors, and enzymes.

Table 2 presents some examples of important functional proteins which are degraded by the proteasome. Many of them are cell-cycle regulatory proteins, as a result, proper proteasome functioning is necessary for cell proliferation. On the contrary, proteasome inhibition sensitizes cells to apoptosis. Moreover, by degrading IκB (inhibitor of NF-κB), proteasome activates NF-κB, and inhibition of proteasome results in abrogation of NF-κB activity. This is one of the key mechanisms of action of proteasome inhibitors and will be discussed further in the sections that follow.

Table 2: Examples of important functional proteins degraded by the proteasome

Class of proteins	Protein	Protein function
Cyclins and related proteins	Cyclins A, B, D, E Cyclin dependent kinase (CDK) inhibitors	Cell-cycle progression Regulation of cyclin activity
Tumor suppressor	p53	Transcription factor
Oncogenes	c-fos/c-jun, c-myc, N-myc	Transcription factor
Inhibitory proteins	IκB p130	Inhibitor of NF-κB Inhibitor of E2F-1
Enzymes	cdc25 phosphatase Tyrosine amino transferase (TAT)	CDK1/cyclin B phosphatase Tyrosine metabolism
Others	Ki-67	Cell proliferation

***From Adams, 2002*. IκB: inhibitor of NF-κB, E2F: elongation factor 2, cdc25: cell division cycle 25**

Another important function of proteasome is the processing of peptides for antigen presentation by MHC class I (Rock et al, 1994). Whereas MHC class II mainly presents peptides of exogenous origin processed by endosomal and lysosomal proteases, MHC class I mainly presents peptides resulted from the proteolysis of endogenously synthesized proteins by proteasome (Strehl et al, 2005, Li et al, 2005). Interestingly, one of the main sources of peptides for MHC class I antigen presentation comes from defective ribosomal products (DriPs). These are newly synthesized defective proteins that are ubiquitinated and made ready for proteasome degradation. Therefore, proteasome can at the same time clear cells from these unwanted nascent proteins and provide peptide antigens for MHC class I presentation without having to wait until the end of a protein's life. However, the majority of the peptides generated by proteasome will be further degraded by cytosol aminopeptidases to single amino acids in order to be reused for protein synthesis. Only a small proportion of these peptides are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). Within the ER, peptides are trimmed to the appropriate size of 8 to 10 amino acids by ER aminopeptidase-1 (ERAP-1) in order to bind to MHC class I molecules. The peptide-loaded MHC molecules are then translocated via the Golgi apparatus to the cell surface to present the antigen to CD8+ T cells (Figure 3).

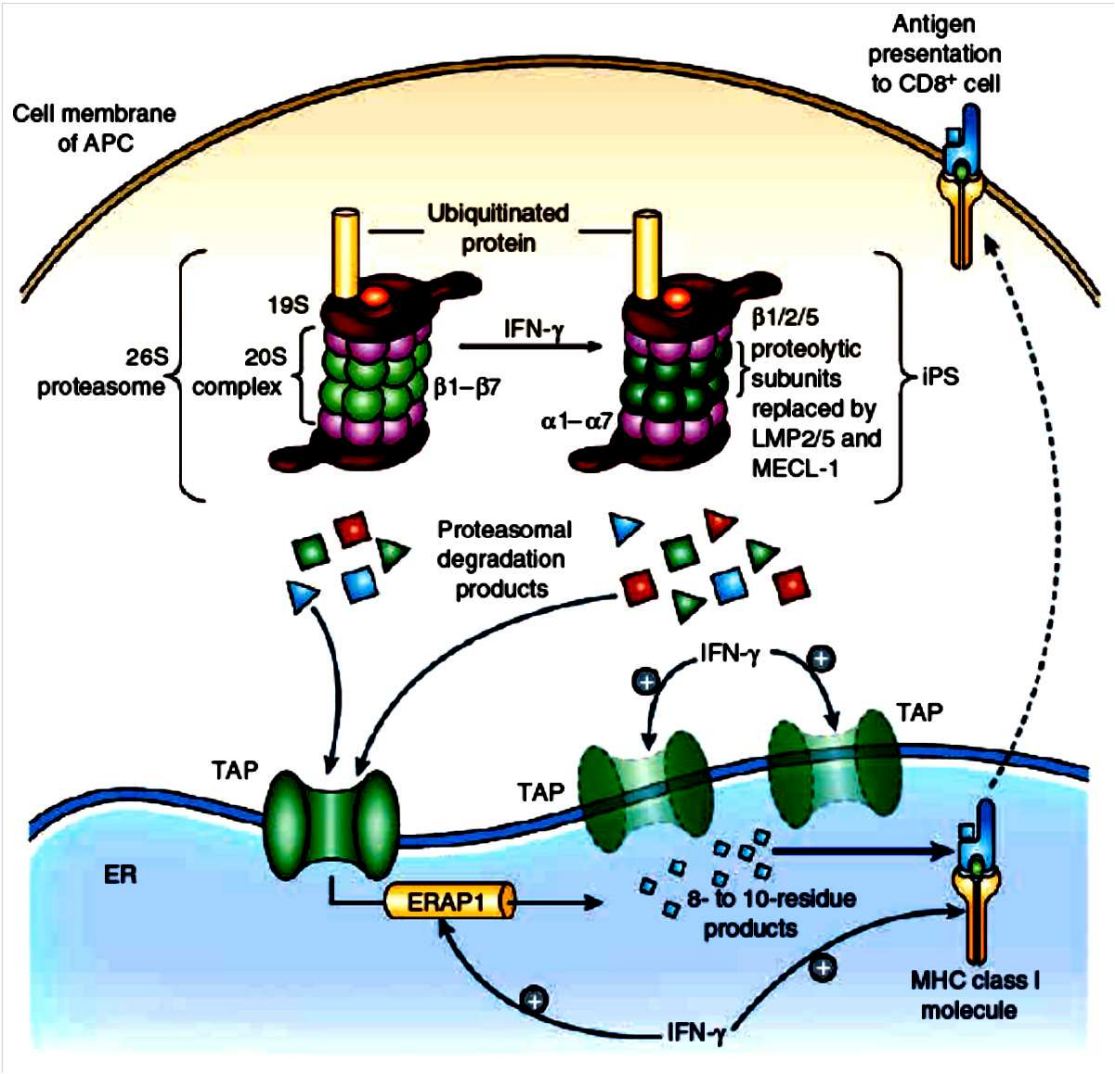


Figure 3: Proteasome and immunoproteasome in protein degradation and MHC class I antigen presentation (*from Tang et al, 2009*). Short-lived nascent proteins are ubiquitinated and degraded by proteasome to small peptides which are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). Within the ER, a small proportion of these peptides are trimmed to the appropriate size of 8 to 10 amino acids by ER aminopeptidase-1 (ERAP-1) in order to bind to MHC class I molecules. The peptide-loaded MHC molecules are then translocated via the Golgi apparatus to the cell surface to present the antigen to CD8+ T cells. In immune cells, IFN-γ produced during inflammation increases the synthesis of immunoproteasome subunits and upregulates TAP, ERAP-1, and MHC expression. Together, these changes lead to enhanced antigen presentation by MHC molecule as a response to inflammatory conditions.

Proteasome composed of the α1-α7 and β1-β7 subunits exists in all cell types and are called constitutive proteasome. On the other hand, in cells of the immune system such as lymphocytes, plasma cells, or dendritic cells, there is another form of proteasome called immunoproteasome, which differs from constitutive proteasome in three catalytic β-subunits. The β1, β2, and β5 in constitutive proteasome are replaced by β1i, β2i, and β5i, respectively, in immunoproteasome (Table 3).

Table 3: Examples of important functional proteins degraded by the proteasome

Constitutive proteasome		Immunoproteasome	
Subunit	Alternative names	Subunit	Alternative names
β1	PSMB6/Y/delta/LMP19	β1i	PSMB9/ /LMP2
β2	PSMB7/Z/LMP9	β2i	PSMB10/ /LMP10/MECL1
β5	PSMB5/X/MB1/LMP17	β5i	PSMB8/ /LMP7

MECL1: multicatalytic endopeptidase complex-like 1

In immune cells, IFN-γ produced during inflammation increases the synthesis of immunoproteasome, which is more potent than constitutive proteasome. At the same time, IFN-γ upregulates TAP, ERAP-1, and MHC expression. Together, these changes lead to enhanced antigen presentation by MHC molecule as a response to inflammatory conditions.

3.2. Proteasome inhibition:

As proteolysis by proteasome is fundamental for the metabolism of cells and organisms, proteasome inhibition affects many cell functions and can induce apoptosis. However, not all cell types respond in the same way to proteasome inhibition. In general, cells that synthesize large amount of proteins such as plasma cells (including myelomatous and normal plasma cells), rapidly proliferating cells such as tumor cells, and activated immune cells are more sensitive to the proapoptotic effect of proteasome inhibition. Proteasome inhibition can induce apoptosis through many pathways, among which there are two major mechanisms: inhibition of NF-κB activation and induction of endoplasmic reticulum stress (ERS) and terminal unfolded protein response (UPR).

3.2.1. *NF-κB signaling pathway:*

NF-κB is not a single protein but a family of inducible transcription factors. In mammals, the NF-κB family consists of five subunits: RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) (Jost et al, 2007, Siebenlist et al, 2005). These proteins share a 300 amino acid sequence in the N-terminal region, known as the Rel homology domain, which mediates DNA binding, protein dimerization, and nuclear translocation. These proteins form various homodimers and heterodimers and are kept inactive in the cytoplasm by binding to inhibitory proteins, which include mainly IκBα, Iκβ, Iκγ, and IκBε (inhibitor of NF-κB α, β, γ, and ε), as well as the p105 and p100 precursors of p50 and p52, respectively.

Activation of NF-κB can occur through either the canonical or alternative pathway (Figure 4).

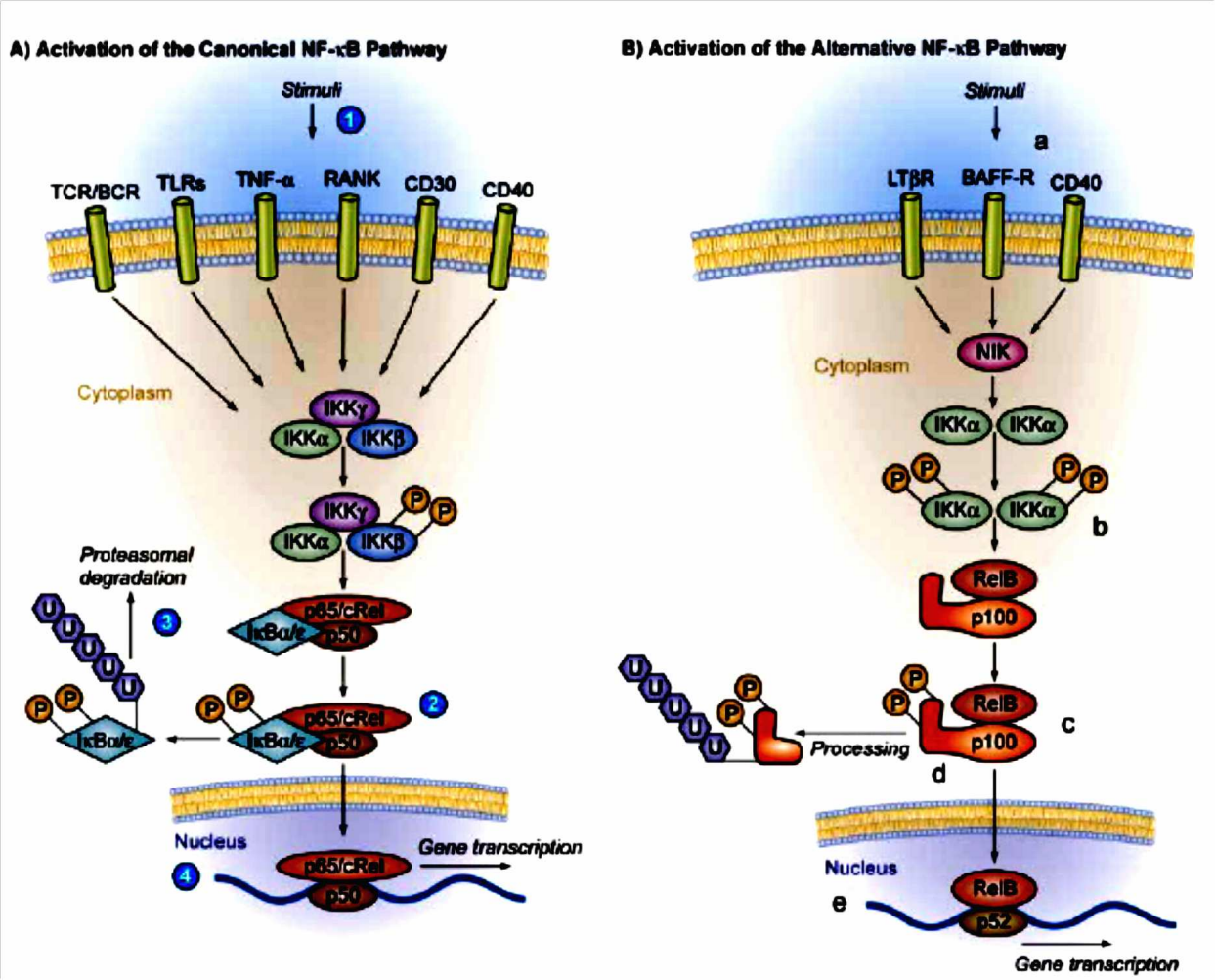


Figure 4: Canonical and alternative pathway of NK-κB activation (*from Jost et al, 2007).* **(A) Activation of the canonical NF-B pathway.** A series of stimuli activate the canonical pathway of NF-κB activation through TCR/BCR, TLRs, RANK, CD30, and CD40 (1). Activated IKK phosphorylates IκB proteins on 2 conserved serine residues and induces IκB polyubiquitinylation (2), which in turn induces their recognition by the proteasome and causes successive proteolytic degradation (3). Following the IκB degradation, the cytoplasmic NF-κB dimers are released and translocate into the nucleus, where gene transcription is activated (4). **(B) Activation of the alternative NF-κB pathway.** The alternative pathway of NF-κB activation is engaged by a restricted set of cell-surface receptors that belong to the TNF receptor superfamily, including CD40, the lymphotoxin β receptor, and the BAFF receptor (a). This pathway culminates in the activation of IKKα (b), which can directly phosphorylate NF-κB2/p100 (c), inducing partial proteolysis of p100 to p52 by the proteasome (d). The p52 protein lacks the inhibitory ankyrin repeats and preferentially dimerizes with RelB to translocate into the nucleus (e).

The canonical pathway can be initiated by various stimuli, including proinflammatory cytokines especially TNF-α and IL-1, pathogen-associated molecular patterns that bind to Toll-like receptors (TLRs), T-cell receptor (TCR) and B-cell receptor (BCR) signaling. The alternative pathway is important in mature B cells and is activated through CD40, lymphotoxin β receptor, and BAFF (B-cell activating factor) receptor. Activation of either pathway leads to phosphorylation of IκB proteins by the IκB-kinase (IKK). In the canonical pathway, the IKK

complex is composed of two catalytic subunits IKKα (IKK1) and IKKβ (IKK2) and a regulatory subunit IKKγ (or NEMO for NF-κB essential modifier). Activated IKK complex phosphorylates IκB proteins, phosphorylated IκB is then ubiquitinated and degraded by proteasome, releasing NF-κB dimer (most commonly the p65/p50 heterodimer) which translocates into the nucleus and binds to NF-κB binding motifs on DNA to induce transcription of target genes. In the alternative pathway, IKKα directly phosphorylates p100, inducing the partial proteolysis of p100 by proteasome to p52, which preferentially dimerizes with RelB and migrates into the nucleus (Palombella et al, 1994)

Proteasome inhibition abrogates NF-κB activation by precluding the degradation of IκB proteins, which remain bound to NF-κB proteins and retain them in the cytoplasm (Adams, 2002). As NF-κB activation induces the transcription of a large variety of genes, including proinflammatory cytokines (e.g. TNF, IL-1, IL-6), stress response enzymes (COX2, NO, 5-LO), cell adhesion molecules (ICAM, VCAM, E-selectin), and anti-apoptotic proteins (the Bcl-2 family), NF-κB inactivation via proteasome inhibition affects the functions and induces apoptosis of various cell types. Several tumor cells, especially myelomatous plasma cells are dependent on NF-κB signaling for survival, explaining why these cells are particularly sensitive to proteasome inhibition.

3.2.2. *Endoplasmic reticulum stress (ERS) and unfolded protein respond (UPR):*

The endoplasmic reticulum (ER) is an eukaryotic organelle composed of a complex membranous network. The rough ER which has ribosomes attached on its surface synthesizes proteins, whereas smooth ER has various metabolic functions such as lipid and steroids synthesis and carbohydrate metabolism. The ER is the place where the secretory and membrane proteins synthesized along the rough ER undergo folding and pass onto the Golgi apparatus for post-translational modification. In order to accomplish its protein folding functions, the ER has high concentration of chaperone proteins which facilitate the correct folding of nascent proteins. Many chaperones are Ca^{2+}-dependent.

A wide variety of stimuli can cause disruption of ER functions, including expression of mutant proteins which cannot be correctly folded, lack of energy (resulting from hypoxia and/or glucose deprivation), viral infection and Ca^{2+} depletion. The disruption of protein folding capacity leads to the accumulation of unfolded and misfolded proteins within the ER, causing endoplasmic reticulum stress and activating the unfolded protein response (UPR). The UPR is mediated through the activation of three transmembrane stress sensors: pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1

(IRE1). In resting cells, all three stress receptors are maintained in an inactive state through their association with the ER chaperone glucose-regulated protein of 78 kDa (GRP78). An increase in the level of unfolded proteins within the ER redirects GRP78 to these proteins, liberating the ER stress sensors and triggering the UPR (Figure 5).

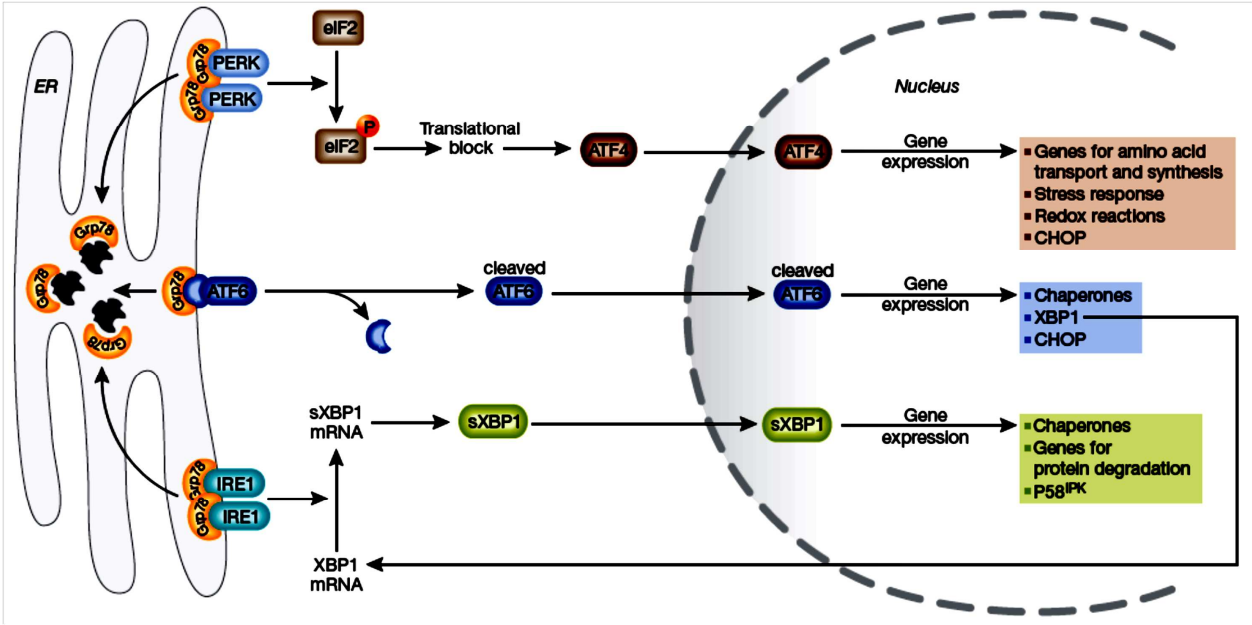


Figure 5: **The unfolded protein response** (*from Szegezdi et al, 2006*). On aggregation of unfolded proteins, GRP78 dissociates from the three endoplasmic reticulum (ER) stress receptors, pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1), allowing their activation.The activation of the receptors occurs sequentially,with PERK being the first, rapidly followed by ATF6,whereas IRE1 is activated last.Activated PERK blocks general protein synthesis by phosphorylating eukaryotic initiation factor 2α (eIF2α). This phosphorylation enables translation of ATF4, which occurs through an alternative, eIF2α-independent translation pathway.ATF4, being a transcription factor, translocates to the nucleus and induces the transcription of genes required to restore ER homeostasis.ATF6 is activated by limited proteolysis after its translocation from the ER to the Golgi apparatus.Active ATF6 is also a transcription factor and it regulates the expression of ER chaperones and X box-binding protein 1 (XBP1), another transcription factor.To achieve its active form, XBP1 must undergo mRNA splicing,which is carried out by IRE1. Spliced XBP1 protein (sXBP1) translocates to the nucleus and controls the transcription of chaperones, the co-chaperone and PERK-inhibitor P58IPK, as well as genes involved in protein degradation. This concerted action aims to restore ER function by blocking further build-up of client proteins, enhancing the folding capacity and initiating degradation of protein aggregates.CHOP, C/EBP homologous protein.

The activation of the UPR leads to: 1) increased expression of chaperones to enhance protein folding capacity, 2) enhanced degradation of misfolded proteins within the ER through a process called ER-associated degradation (ERAD), and 3) decreased global protein synthesis (Healy et al, 2009). These mechanisms together can restore ER homeostasis and maintain cell life.

The unfolded protein response is primarily a pro-survival response. However, if the prolonged

and severe ER stress is not resolved, the UPR will switch to initiation of apoptosis. In fact, certain components of the UPR are proapoptotic. For example, CHOP (C/EBP homologous protein), which is induced by all three arms of UPR through XBP1, ATF4, and ATF6 transcription factors, alters the balance between pro-survival and pro-apoptotic Bcl-2 family members thus promoting apoptosis through the mitochondrial pathway. CHOP causes upregulation of Bim (Bcl-2-interacting mediator of cell death), a pro-apoptotic BH3-only member of the Bcl-2 protein family (Puthalakath et al, 2007) and suppresses anti-apoptotic proteins. CHOP also promotes the extrinsic apoptotic pathway through the upregulation of TRAIL (tumor necrosis factor-related apoptosis inducing ligand) (Yamaguchi et al, 2004). Another proapoptotic mechanism involves the recruitment of TNF receptor-associated factor 2 (TRAF2), leading to activation of c-jun N-terminal kinase (JNK) which phosphorylates Bcl-2 proteins and enhances their proapoptotic potential (Szegezdi et al, 2009). The final executive phase of all these pathways is the activation of the caspase cascade leading to ER stress-induced cell death.

Proteasome inhibition, by blocking the principal mechanism for cellular protein degradation, causes the accumulation of misfolded proteins which overwhelms the ER-associated degradation (ERAD) leading to ER stress. As the ER stress induced by proteasome inhibition is severe and unresolved, the induced unfolded protein response rapidly switches to proapoptotic pathways and terminates in cell death. Many cell types which synthesize large amounts of proteins are dependent on the UPR for proper functioning and survival, such as normal plasma cells, and to a greater extent, myelomatous plasma cells or other secreting tumor cells. For example, myeloma cells produce thousands of antibody molecules per second; as a result, the UPR is already activated to maintain ER homeostasis and further ER stress induced by proteasome blockade will rapidly induced cell death (Obeng et al, 2006). This explains why these cells are particularly sensitive to proteasome inhibition.

4. Proteasome inhibitors and cancer treatment:

Following its discovery in the early 1980s, proteasome was recognized to be a potential therapeutic target and proteasome inhibitors (PIs) began to be developed. The most important step is the creation in 1992 of a biotech company named MyoGenics by Alfred L. Goldberg and his colleagues, who are also the pioneers in the discovery of the ubiquitin-proteasome system. Interestingly, the initial aim of MyoGenics, as indicated by its name, was to develop PIs which can be used to inhibit protein degradation in order to prevent muscle wasting seen in various disease states such as cancer cachexia. Several PIs were thus synthesized and distributed to

academic institutions for further studies that help us to understand the multiple functions of proteasome in cell physiology. As often seen in science, the most successful outcome turns out not to be the one initially expected. Cancers were soon recognized to be the most potential target for proteasome inhibition.

4.1. First generation proteasome inhibitor – bortezomib:

Among these early PIs, a molecule was synthesized in 1995 and named MG-341 (MG stands for MyoGenics), better known as PS-341 as the company's name changed to ProScript. PS-341, which is now known as bortezomib (Velcade®, Millennium) was approved by FDA in 2003 for the treatment of multiple myeloma (Bross et al, 2004). It is also the first PI approved for clinical use. The success story of bortezomib serves as an example of the collaboration between academic laboratories and scientists with pharmaceutical industries.

Bortezomib is a boronic acid dipeptide derivative which has a high degree of selectivity for the proteasome and does not inhibit many common proteases. It is a synthetic compound whose molecular weight is 384.24 Da. Bortezomib selectively inhibits the chymotrypsin-like activity of proteasome and immunoproteasome (β5 and β5i, respectively), and this inhibition is slowly reversible (Adams et al, 1999).

Initial screening using the National Cancer Institute's (NCI) tumor cell lines showed that bortezomib is active against a broad range of tumor types, including multiple myeloma cell lines. Studies in xenograft models confirmed its effects (Adams et al, 1999, Teicher et al, 1999), prompting several human phase I trials which established the tolerability of bortezomib (Adams, 2002). Two phase II trials with good results led to the accelerated approval by FDA in 2003 for the treatment of multiple myeloma in patients who have received at least two prior therapies and have demonstrated disease progression on the last therapy (Bross et al, 2004). Soon after that, in 2005, FDA have granted regular approval for bortezomib in the treatment of multiple myeloma based on a successful phase III clinical trial enrolling more than 600 patients (Kane et al, 2006). Many subsequent clinical trials have confirmed the efficacy of bortezomib as a frontline therapy in newly diagnosed multiple myeloma. Bortezomib-based regimens have now become standard therapy either as chemotherapy for patients ineligible for transplantation or as induction therapy before stem cell transplantation (Ludwig et al, 2010).

Recently, bortezomib has also been approved by FDA for the treatment of relapsed or refractory mantle cell lymphoma (MCL), one of the B-cell lymphomas with the worst prognosis (Goy et al, 2010). Furthermore, some clinical trials have shown that bortezomib in combination with rituximab is effective in the treatment of either newly diagnosed or relapsed/refractory

Waldenström macroglobulinemia, a distinct B-cell disorder resulting from the accumulation, predominantly in the bone marrow, of clonally related IgM-secreting lymphoplasmacytic cells (Ghobrial et al, 2010).

Besides hematologic malignancy, bortezomib has also been tested in phase II clinical trials in several types of advanced solid tumors such as non-small cell lung cancer (Scagliotti et al, 2010), ovarian cancer (Aghajanian et al, 2009), urothelial tract transitional cell carcinoma (Rosenberg et al, 2008), and metastatic breast cancer (Engel et al, 2007). Unfortunately, the results obtained so far are not encouraging.

4.2. Second generation proteasome inhibitors:

Following bortezomib, second-generation PIs with different chemical structures have been developed and currently tested for clinical use.

Carfilzomib was developed based on the natural product epoxomicin, a peptidyl epoxyketone first isolated from an Actinomycetes strain (Hanada et al, 1992). Carfilzomib selectively and irreversibly inhibits the chymotrypsin-like activity of proteasome and immunoproteasome (Demo et al, 2007). Its activity has been shown in preclinical models of multiple myeloma (Kuhn et al, 2007) and Waldenstrom macroglobulinemia (Sacco et al, 2011). A phase I dose escalation study confirmed its tolerability (O'Connor et al, 2009) and a phase II trial is underway.

Two other molecules derived from epoxomicin have also been developed by Onyx Pharmaceuticals (formerly Proteolix), the company that produces carfilzomib. The first one is PR-047, an orally active PI which also inhibits the chymotrypsin-like activity of proteasome and immunoproteasome (Zhou et al, 2009). The second one named PR-957 is a selective inhibitor of the chymotrypsin-like activity of the β5i subunit (or LMP7) of immunoproteasome, with minimal activities against other catalytic subunits at therapeutic doses (Muchamuel et al, 2009). However, this molecule has weak anticancer properties and is developed primarily for immunologic diseases (Basler et al, 2010).

Another natural PI named marizomib (NPI-0052, salinosporamide A) is developed by a company named Nereus Pharmaceuticals. Marizomib is a β-lactone-γ-lactam isolated from the marine Actinomycete *Salinispora tropica* (Feling et al, 2003). Marizomib inhibits all three catalytic activities of proteasome and immunoproteasome, the caspase-like (β1 and β1i), trypsin-like (β2 and β2i), and chymotrypsin-like (β5 and β5i) enzymatic activities at therapeutic concentrations. Marizomib has been shown to be effective against a wide variety of hematologic malignancies including multiple myeloma, Waldenström macroglobulinemia, lymphoma, and leukemia, as well as solid tumors including colon, pancreatic, and prostate cancer in preclinical studies (Potts

et al, 2011). Phase I clinical trials of marizomib are currently underway.

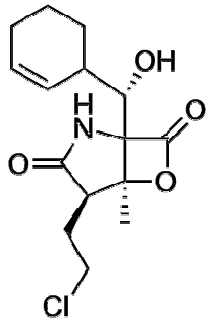
Like other drugs used in cancer chemotherapy, PIs have side effects which affect many organ systems, including the gastrointestinal (nausea, vomiting, diarrhea, and anorexia), hematological (thrombocytopenia, anemia, neutropenia), and neurological systems (peripheral neuropathy), as well as asthenia and pyrexia. These side effects are common for all PIs. Peripheral neuropathy is one of the most worrisome side effects because it may not be reversible even after cessation of PIs. Second generation PIs such as carfilzomib and marizomib are claimed to have fewer side effects than bortezomib, especially in terms of peripheral neuropathy, but more experiences are needed to confirm this because these new PIs have been used in far fewer patients than bortezomib.

Table 4 summarizes the profiles of PIs that are developed for clinical uses. Figure 6 presents the chemical structures of some of the commonly used PIs. For further discussion of all proteasome inhibitors, including the ones that are used in research only, please refer to a comprehensive review (De Bettignies et al, 2010).

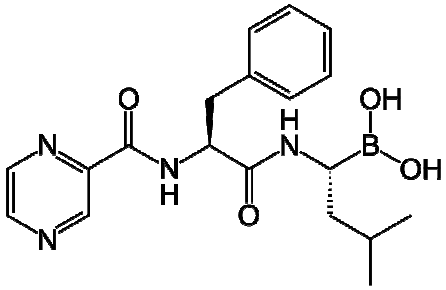
Table 4: Profiles and treatment regimens for proteasome inhibitors in clinical development

Proteasome inhibitor/ company	Struc-tural class	Pharmacodynamic profile			Develop-ment stage	Route of adminis-tration	Treatment schedule
		CT-L	T-L	C-L			
Bortezomib (Velcade®)/ Millennium Takeda	Peptide boronic acid	Slowly reversible		Sustained	Approved MM/MCL	IV (active SC in MM)	Day 1, 4, 8, 11 (21-day cycle)
Carfilzomib/ Onyx (Proteolix)	Peptide epoxy-ketone	Sustained			Phase 2	IV	Day 1,2,8,9,15,16 (28-day cycle)
Marizomib (NPI-0052, salinospora-mide A)	β-lactone -γ-lactam	Sustained	Sustained	Sustained	Phase 1b	IV (oral and SC efficacy)	Day 1, 8, 15 (28-day cycle) Day 1,4,8,11 (21-day cycle)
CEP-18770/ Cephalon	Peptide boronic acid	Slowly reversible			Phase 1	IV (oral efficacy)	Day 1, 4, 8, 11 (21-day cycle)
MLN9708/ Millennium Takeda	Peptide boronic acid	Reversible			Phase 1	IV/oral	Day 1,4,8,11 (21-day cycle) Day 1, 8, 15 (28-day cycle)
ONX-0912 (PR-047)/ Onyx (Proteolix)	Peptide epoxy-ketone	Sustained			Phase 1	Oral	Day 1,2,3,4,5 (14-day cycle)

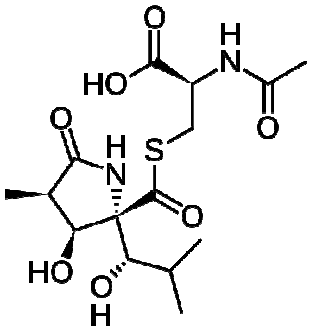
(*Adapted from Potts et al, 2011*)
CT-L: chymotrysin-like, T-L: trypsin-like, C-T: caspase-like, MM: multiple myeloma, MCL: mantle cell lymphoma, IV: intravenous, SC: subcutaneous.



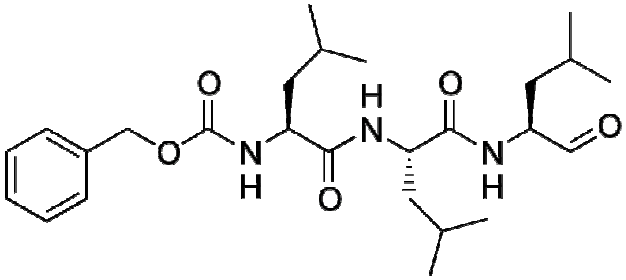
Marizomib
(β-lactone-γ-lactam)



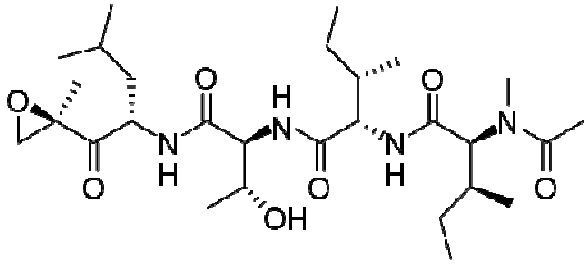
Bortezomib
(Peptide boronic acid)



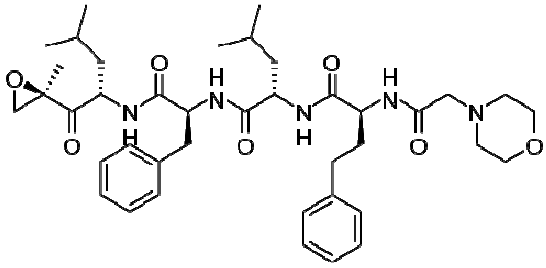
Lactacystin
(β-lactone)



MG132
(Peptide aldehyde)



Epoxomicin
(Peptide epoxyketone)



Carfilzomib
(Peptide epoxyketone)

Figure 6 : Chemical structures of some representative proteasome inhibitors

5. Proteasome inhibition in immunology:

Besides their established applications in oncology, proteasome inhibitors have been shown to affect the functions of various cell types of the immune system, including T cells, dendritic cells, B cells, and plasma cells. They have also been tested in various models of autoimmune diseases, including arthritis, lupus, anti-neutrophil cytoplasmic antibodies (ANCA)-associated glomerulonephritis, experimental autoimmune encephalitis (EAE), myasthenia gravis, colitis, and contact hypersensitivity.

5.1. In vitro effects of proteasome inhibition on different cell types of the immune system:

5.1.1. *T cells:*

As proteasome is the principal machinery that degrades cellular functional proteins, including many cytokines, transcription factors, and cyclins, it is not surprising that its activities are required for T cell activation and proliferation. The proteasome inhibitor lactacystin has been shown to repress the mitogen-induced T cell proliferation through blocking the G_0-to-G_1-phase transition (Wang et al, 1998). Moreover, both lactacystin and bortezomib induce apoptosis in activated T cells, but not resting T cells. The mechanisms of bortezomib-induced apoptosis in activated T cells include activation of caspase-9 and caspase-3, degradation of bcl-2, and activation of the mitochondrial pathway (Blanco et al, 2006, Berges et al, 2009). Both bortezomib and lactacystin decrease the expression of several markers of T cell activation such as CD25, CD69, and CD134, and reduce the production of both Th1 cytokines such as IFN-γ and IL-2 and Th2 cytokines such as IL-4 and IL-5 (Blanco et al, 2006, Berges et al, 2008). Interestingly, CD4+CD25+ regulatory T cells are resistant to the proapoptotic effects of bortezomib, and long-term culture of CD4+ T-cell in the presence of bortezomib promotes the emergence of regulatory T cells (Blanco et al, 2009).

5.1.2. *Dendritic cells:*

Bortezomib has been shown to impair several functions of human monocyte-derived dendritic cells (mDC) (Nencioni et al, 2006, Zinser et al, 2009). It prevents mDC maturation in response to inflammatory signals as evidenced by the reduced upregulation of DC maturation markers such as CD80, CD86, CD40, CD83, and DC-SIGN. Of note, CD83 and DC-SIGN are the major markers of DC maturation. DCs cultured in the presence of bortezomib produce less IL-12, the principal immunostimulatory cytokine secreted by mDCs. Bortezomib reduces the expression of the chemokine receptor CCR7, resulting in reduced migratory capacity of DC in response to the chemokine CCL19/MIB-3β – the ligand for CCR7. mDCs exposed to bortezomib display a reduced endocytic capacity as shown by the FITC-dextran uptake assay, associated with a decrease in the expression of CD206 (macrophage mannose receptor), which is required for the endocytosis of macromolecular antigens. Finally, as a result of the multiple aforementioned effects, bortezomib markedly impairs the capacity of DCs to stimulate allogeneic and autologous T cells (Naujokat et al, 2007).

Besides the effects on monocyte-derived dendritic cells, bortezomib has recently been shown to suppress the survival and immunostimulatory functions of human plasmacytoid dendritic cells

(pDC) (Hirai et al, 2011). pDCs differ from mDCs in two major characteristics, the former express TLR-2 and TLR-4 and produce IL-12, whereas the latter express TLR-7 and TLR-9 and produce IFN-α. In fact, pDC is most susceptible to the killing effects of bortezomib among immune cells in blood. Bortezomib induced apoptosis in pDC by disturbing endoplasmic reticulum (ER) homeostasis through the inhibition of XBP-1 (X-box binding protein 1) splicing to generate the active form of XBP-1, the transcription factor required for ER stress response. Bortezomib also strongly suppressed the production of IFN-α by pDC in response to TLR-7 and TLR-9 ligands. As pDC is involved in the pathogenesis of major autoimmune diseases such as lupus and psoriasis, the suppressive effects of bortezomib on pDC may have clinical implications.

5.1.3. *B cells:*

Similar to the case of T cells, activated B cells are more susceptible to the proapoptotic effects of bortezomib than resting B cells (Cascio et al, 2008). Interestingly, T-cell dependent antibody responses are impaired by bortezomib, whereas the early T-cell independent antibody responses remain unaffected (Lang et al, 2010). Using a mouse model, the authors have shown that the antibody response to immunization with dinitrophenyl (DNP)- keyhole limpet hemocyanin (KLH), a T-cell dependent antigen, was impaired by bortezomib treatment. On the other hand, the early antibody response to immunization with (4-hydroxy-3-iodo-5-nitrophenol)acetyl (NIP)-Ficoll, a T-cell independent antigen, was not reduced.

5.1.4. *Plasma cells:*

Proteasome inhibitors not only target neoplastic plasma cells, but they can also induce apoptosis of normal antibody-secreting plasma cells. Using the aforementioned mouse model, Lang et al have shown that bortezomib treatment decreased the percentage of plasma cells in the bone marrow and in the spleen (Lang et al, 2010). More importantly, the number of antibody secreting cells which produce IgG specific for DNP-KLH – the immunogen used in the model – was also decreased.

Similar results have been obtained for normal human plasma cells. Perry et al isolated plasma cells from bone marrow of kidney transplant recipients and cultured them in the presence of rATG (rabbit antithymocyte globulin), rituximab, IVIg (intravenous immunoglobulin), or bortezomib (Perry et al, 2008). Although the first three agents are the most commonly used drugs for the treatment of antibody-mediated rejection in transplant patients, none of them were able to induce apoptosis of plasma cells or to block antibody secretion. On the contrary, bortezomib at clinically obtainable concentrations induced apoptosis of more than 60 percent of plasma cells

and thereby blocked IgG secretion in vitro.

5.2. In vivo effects of proteasome inhibition in experimental models of autoimmune diseases:

Most of proteasome inhibitors were developed and tested in models of hematologic and solid organ cancers, which are up to the present time their main clinical applications. However, some proteasome inhibitors have also been tested in various models of autoimmune diseases.

5.2.1. *Rheumatoid arthritis:*

Early in its development, bortezomib has been tested in a rat model of Streptococcal cell wall-induced polyarthritis (Palombella et al, 1998). In this model, arthritis is initiated by an intraperitoneal injection of group A Streptococcal cell wall into female Lewis rat. Rats in this model typically exhibit a peripheral and symmetrical, biphasic polyarthritis with cycles of exacerbated recurrence and remission. The disease is clinically and histologically similar to rheumatoid arthritis. Treatment with bortezomib attenuated disease severity both clinically and histologically. The anti-inflammatory effects of bortezomib in this model were associated with an inhibition of IκBα degradation and NF-κB-dependent gene expression. This result has been reproduced in other models of rheumatoid arthritis, such as collagen-induced arthritis in mouse (Lee et al, 2009) and adjuvant-induced arthritis in rat (Yannaki et al, 2010).

Recently, PR-957, a novel selective inhibitor of the immunoproteasome subunit β5i, or low-molecular mass polypeptide-7 (LMP-7) has been tested in two mouse models of rheumatoid arthritis, namely the collagen antibody-induced arthritis (CAIA) and the collagen-induced arthritis (CIA) (Muchamuel et al, 2009). In both models, mice treated with PR-957 had less severe disease as evidenced by a lower clinical score, less inflammatory infiltration and bone erosion compared to vehicle-treated controls. The clinical and histological improvement was associated with a decrease in the mRNA expression of inflammatory mediators in affected joints such as TNF-α, IL-1β, IL-6, ICAM-1 (intercellular adhesion molecule-1), iNOS (inducible nitric oxide synthase), and Cox-2 (cyclooxygenase-2). Interestingly, the clinical improvement was better and autoantibody production was lower in mice treated with PR-957 than those treated with etanercept, a TNF inhibitor used clinically in patients with rheumatoid arthritis. Finally, it is noteworthy that PR-957 induced an anti-inflammatory response at doses less than one-tenth the maximal tolerated dose (MTD), whereas nonselective inhibitors such as bortezomib and carfilzomib could do so only at the MTD.

5.2.2. *Systemic lupus erythematosus (SLE):*

The beneficial effects of bortezomib on SLE have been demonstrated in the lupus-prone New Zealand Black/White (NZB/W) F1 mouse, a widely-used murine model of SLE (Neubert et al, 2008). Female NZB/W F1 mice have B cell hyperactivity and produce multiple autoantibodies, including anti-double-stranded DNA (anti-dsDNA) and other antinuclear antibodies. As a result, they develop immune complex-mediated glomerulonephritis resembling human lupus glomerulonephritis and almost all of them die from renal failure before 12 months of age. In these mice, long-lived plasma cells represent a large fraction of autoreactive antibody-producing cells in the spleen. These cells are resistant to cyclophosphamide, one of the principal drugs used in the treatment of lupus nephritis in human. In contrast, bortezomib depletes both short-lived and long-lived plasma cells, and thereby abrogates autoantibody production. As a result, early bortezomib treatment virtually prevented the lupus-like disease in NZB/W F1 mice, as evidenced by the diminution of anti-dsDNA titers, the absence of glomerulonephritis, and a markedly prolonged survival compared to the control group. Late bortezomib treatment in mice with established nephritis also reduced proteinuria and anti-dsDNA titers. Finally, bortezomib could be given twice per week for up to 40 weeks in this study without any overt toxicity (Neubert et al, 2008). Another study using the same mouse model confirmed the beneficial effects of bortezomib, however its effects were not superior to those of mycophenolate mofetil (MMF) combined to prednisolone (Lee et al, 2010).

5.2.3. *ANCA-associated glomerulonephritis:*

ANCA-associated glomerulonephritis is the most common form of crescentic, or rapidly-progressive glomerulonephritis. As indicated by its name, this form of glomerulonephritis is characterized by (1) the presence of autoantibodies in the serum directed against neutrophil cytoplasmic antigens, namely myeloperoxidase (MPO) or proteinase-3 (PR-3), (2) a pauci-immune glomerulonephritis (no or little glomerular deposition of immunoglobulins or complement components as seen by immunofluorescence) with variable percentage of glomeruli with necrosis and crescent formation, and (3) a rapid deterioration of renal functions over weeks or months rather than over years as in the majority of glomerulonephritides (Gómez-Puerta et al, 2009). The mainstay of treatment for ANCA-associated glomerulonephritis in the past two decades is cyclophosphamide combined with corticosteroid, although this treatment is associated with serious adverse effects. Recently rituximab has been shown to be not inferior to cyclophosphamide in inducing disease remission. However there was no difference in the rate of adverse events between the two treatment groups (Stone et al, 2010).

Using a mouse model in which ANCA-associated glomerulonephritis was induced by the transplantation of bone marrow containing plasma cells secreting specific antibodies to MPO, Bontscho et al have demonstrated that bortezomib can protect recipient mice from developing the disease (Bontscho et al, 2011). Bortezomib depleted total and MPO-specific plasma cells in the spleen and in the bone marrow, thereby abrogated anti-MPO autoantibody production, reduced proteinuria and hematuria, and decreased glomerular necrosis and crescent formation.

5.2.4. *Experimental autoimmune encephalitis (EAE):*

EAE is an animal model of the human central nervous system (CNS) demyelinating diseases, especially multiple sclerosis. EAE can be induced in a number of species including mice and rats by immunizing with different neuroantigens, most commonly myelin proteins or their peptides. Depending on the antigen used and the species and strains of the animal, rodents can display a monophasic EAE, a relapsing-remitting form, or chronic EAE. Rodents typically develop an ascending paralysis which begins from the tail and progresses to affect the hind limbs and finally the forelimbs.

Using a mouse EAE model in which C57BL/6 mice were immunized with Myelin Oligodendrocyte Glycoprotein (MOG) peptide 35-55, Fissolo et al have shown that bortezomib treatment reduced T cell response to the peptide antigen and ameliorated clinical score (Fissolo et al, 2008). The anti-inflammatory effect of bortezomib was attributed to the reduced activities of NF-κB in the CNS of the treated animals.

5.2.5. *Myasthenia gravis:*

Myasthenia gravis (MG) is an autoimmune neuromuscular disease caused by autoantibodies to acetylcholine receptors (AchRs). These antibodies block the effects of the neurotransmitter acetylcholine on AchRs at the post-synaptic neuromuscular junction, leading to muscle weakness, including limb and bulbar muscles (muscles innervated by the cranial nerve V, VII, IX-XII). If respiratory muscles are affected, the disease is potentially fatal, necessitating mechanical ventilation and plasma exchange to rapidly remove autoantibodies. Bortezomib, which can deplete plasma cells and abrogate antibody production, may be a therapeutic candidate.

Using an experimental MG model in which rat are immunized with *Torpedo* AchRs, Gomez et al have shown that bortezomib induced apoptosis of bone marrow plasma cells, reduced the rise of anti-AchR autoantibody titers, prevented ultrastructural damage of the postsynaptic membrane, improved neuromuscular transmission, and decreased myasthenic symptoms (Gomez et al, 2011).

5.2.6. *Experimental colitis:*

Inflammatory bowel disease (IBD), comprising Crohns'disease and ulcerative colitis, is characterized by chronic relapsing inflammation of the gut. A dysregulated activation of NF-κB leading to expression of many proinflammatory mediators commonly occurs in IBD. Enhanced proteasome activity, associated with the expression of immunosubunits, especially LMP7 (β5i), is involved in the sustained NF-κB-driven inflammation in IBD (Schmidt et al, 2010).

Recently, the LMP7-selective inhibitor, PR-957, has been tested in a mouse model of experimental colitis induced by dextran sulfate sodium (DDS). Treatment with PR-957 suppressed the expression of proinflammatory cytokines and mediators such as IL-1β, IL-23, iNOS, and COX-2, reduced inflammation and tissue destruction (Basler et al, 2010). Bortezomib has also been shown to be effective in the same model in a dose-dependent manner. However, high-dose bortezomib leaded to a surmortality of treated animals (Schmidt et al, 2010).

5.2.7. *Contact hypersensivity:*

Finally, the anti-inflammatory effect of proteasome inhibitors has also been tested in models of T-cell dependent delayed-type hypersensitivity (DTH). In these models, mice are immunized with a hapten, and then challenged with the same hapten at another skin site, leading to a local inflammation which is mediated by the hapten-primed T cells and manifested as a local skin induration and monocytic infiltration. The reaction usually peaks 24-48 hours after challenge and then decreases. Both bortezomib and epoxomicin have been shown to decrease inflammatory reaction in these models, associated with a reduction in the expression of pro-inflammatory cytokines such as IFN-γ and IL-6 in the skin lesions and in the draining lymph nodes (Meng et al, 1999, Yanaba et al, 2010).

Table 5 summarizes all the aforementionned experiments in which proteasome inhibition was tested in animal models of autoimmune diseases.

Table 5: Proteasome inhibition in experimental models of autoimmune diseases

Experimental model	Proteasome inhibitors	Main effects	References
Arthritis	Bortezomib PR-957	Decrease of clinical signs Decrease of histologic lesions Decrease of inflammatory mediators	Palombella, 1998 Lee, 2009 Yannaki, 2010 Muchamuel, 2009
Lupus	Bortezomib	Decrease of plasma cells Decrease of anti-dsDNA Abrogation of lupus nephritis Survival prolongation	Neubert, 2008 Lee, 2010
ANCA-induced glomerulonephritis	Bortezomib	Depletion of plasma cell Decrease of anti-MPO antibody Decrease of proteinuria and hematuria Decrease glomerular necrosis and crescent	Bontscho, 2011
Experimental autoimmune encephalitis (EAE)	Bortezomib	Amelioration of clinical score Decrease T cell response to the peptide antigen	Fissolo, 2008
Myasthenia gravis	Bortezomib	Decrease of anti-AchR antibody Decrease of histologic lesions Decrease clinical symptoms	Gomez, 2011
Experimental colitis	PR-957 Bortezomib	Reduction of inflammation and tissue destruction Decrease of inflammatory mediators	Basler, 2010 Schmidt, 2010
Contact hypersensitivity	Epoxomicin Bortezomib	Decrease inflammatory reaction Decrease inflammatory cytokines	Meng, 1999 Yanaba, 2010

6. Proteasome inhibition in transplantation:

6.1. Experimental models of bone marrow transplantation:

As proteasome inhibitors have been developed mainly for the treatment hematologic malignancies, they have also been tested for the prevention and treatment of graft-versus-host disease (GVHD) in bone marrow transplantation (BMT). The clinical use of allogenic BMT for hematologic cancer treatment is seriously hampered by the occurrence of GVHD. However, treatments that reduce the incidence GVHD usually diminish graft-versus-tumor responses with increased tumor relapse. Using a mouse model of allogenic BMT, Sun et al have demonstrated that a brief course of treatment with bortezomib from day 0 to day 3 post-BMT protected mice from acute lethal GVHD as evidenced by a prolonged survival. Moreover, this treatment preserved graft-versus tumor responses after allogenic BMT in tumor-bearing mice. The protection from acute lethal GVHD was associated with an initial reduction of donor T cell engraftment, increased alloreactive T cell apoptosis, and reduction in systemic TNF-α (Sun et al, 2004). In marked contrast, delayed administration of bortezomib, when GVHD was ongoing (5-7

days post-BMT), resulted in accelerated mortality from GVHD (Sun et al, 2005). Pathologic evaluation revealed marked increase in lesions in the small intestine and colon, associated with a local upregulation of TNFR1, the principal proinflammatory and proapoptotic receptor for TNF-α. Accordingly, serum levels of TNF-α as well as other proinflammatory cytokines such as IL-1β and IL-6 were significantly increased. Because GVHD is a complex and multistage disease process, it is not surprising that bortezomib exhibits differential effects depending on the timing of administration. The induction phase of GVHD appears to be the most susceptible phase for successful bortezomib treatment. On the contrary, this drug should be avoided later on when GVHD is already progressive.

Interestingly, another study compared bortezomib and PS-1145, an IκB kinase (IKK) inhibitor that selectively inhibits NF-κB in the same mouse BMT model (Vodanovic et al, 2006). Similar findings were obtained for bortezomib. Early brief treatment with bortezomib protected mice from fatal GVHD, whereas delayed or prolonged treatment with bortezomib exacerbated GVHD-dependent mortality due to gut toxicity. Early brief treatment with PS-1145 also protected mice from lethal GVHD, but unlike bortezomib, prolonged treatment with PS-1145 was not associated with gut toxicity and resulted in more complete protection than that observed with an abbreviated treatment schedule. These results confirm a critical role of NF-κB in the pathophysiology of GVHD and indicate that targeted inhibition of NF-κB may have a superior therapeutic index.

6.2. Clinical bone marrow transplantation:

As discussed in the previous section, bortezomib was initially approved for used in relapsed/refractory multiple myeloma (MM) (Bross et al, 2004). Given the exciting efficacy in relapsed/refractory MM, bortezomib has rapidly moved forward to frontline chemotherapy. As high-dose chemotherapy followed by autologous stem cell transplant (ASCT) is the treatment of choice for MM patients younger than 65 years of age, bortezomib has subsequently been used in pretransplant induction therapy and then in maintenance therapy after ASCT. In a phase II, open-labeled trial, Harousseau et al have shown that bortezomib plus dexamethasone as induction treatment prior to ASCT was effective and well-tolerated in newly diagnosed MM patients (Harousseau et al, 2006). Subsequently, several randomized studies have demonstrated the efficacy of bortezomib-based regimens used in both the induction and maintenance therapy in ASCT. For example, the results from a recent French randomized trial have shown that bortezomib plus dexamethasone was superior to vincristine plus doxorubicin plus dexamethasone (VAD) as induction therapy prior to ASCT in newly diagnosed multiple myeloma (Harousseau et al, 2010). Bortezomib plus dexamethasone significantly improved post-

induction and post-transplantation complete response/nearly complete response and at least very good partial response rates compared with VAD and resulted in a trend for longer progression-free survival. Similarly, an Italian randomized study comparing bortezomib plus thalidomide plus dexamethasone (VTD) to thalidomide plus dexamethasone (TD) as induction therapy before and consolidation therapy after double ASCT in newly diagnosed multiple myeloma also demonstrated the superiority of the VTD regimen as evidenced by a higher rate of complete/near complete response (Cavo et al, 2010). As a result, bortezomib-based regimens have been increasingly recommended for induction therapy before and maintenance therapy after ASCT (Rajkumar, 2011).

6.3. Experimental models of organ transplantation:

Using in-house synthesized dipeptide boronic acid (DPBA), which is the chemical name of bortezomib, Luo et al in Montreal have demonstrated that DPBA prolonged graft survival in a mouse heart allograft model (Luo et al, 2001). A subsequent study by the same group has shown that DPBA also prolonged graft survival in a mouse islet allograft model (Wu et al, 2004). However, in these two studies, the mechanisms of action of DPBA were not analyzed in detail. In addition, the effect of treatment on post-transplant DSA levels was not studied, whereas we now know that the most important reason to use proteasome inhibitors in transplantation is to take advantage of its proapoptotic effect on plasma cells in order to abrogate alloantibody formation. In our present study (article 1), we will demonstrate that proteasome inhibition by itself can decrease DSA formation in rat heart transplant models of acute and chronic rejection.

6.4. Clinical organ transplantation:

In 2008, Everly et al reported the use of bortezomib, plasmapheresis, IVIg with or without rituximab in 6 kidney transplant patients with mixed acute T-cell mediated and antibody mediated rejection (Everly et al, 2008). The treatment was associated with a rapid reversal of rejection and marked reductions in DSA levels. However, two of the six patients finally lost their graft in less than 1 year post-transplant. Similarly, Perry et al reported that bortezomib combined with plasmapheresis and IVIg rapidly reduced DSA levels in two renal transplant patients undergoing acute AMR and this treatment was associated with a transient decrease in bone marrow plasma cell number (Perry et al, 2008). In another study, Trivedi et al were able to decrease DSA levels in 9 of 11 kidney transplant patients with bortezomib combined with plasmapheresis and pulse methylprednisolone (Trivedi et al, 2009). Because in these studies, all patients also received other treatments for AMR, it is not clear whether bortezomib per se can reduce DSA levels. Contrary to these three reports, Sberro-Soussan et al treated four renal

transplant patients with persistent DSA and subacute AMR with one cycle of bortezomib without concomitant plasmapheresis, IVIg, or rituximab and found no significant reduction in DSA levels in the 150 days post-treatment (Sberro-Soussan et al, 2009).

Because bortezomib has only been used in a few transplant centers for the treatment of AMR in the past two years, more case-series reports are necessary to accumulate experience on this new treatment modality. We will report our experience with three kidney transplant patients who received bortezomib or either acute or chronic AMR (article 2).

ARTICLE 1

original article

http://www.kidney-international.org

Immunoproteasome beta subunit 10 is increased in chronic antibody-mediated rejection

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Chronic active antibody-mediated rejection is a form of late rejection with a poor prognosis. To identify specific markers of this, we analyzed several microarray studies in the literature and performed mRNA profiling of 65 biopsies and 165 blood samples of a large cohort of renal transplant patients with precisely characterized pathologies. Immunoproteasome beta subunit 10 was found to be specifically increased in the graft and blood samples during chronic active antibody-mediated rejection and was also significantly increased in rat cardiac allografts undergoing acute rejection as well as chronic active antibody-mediated rejection. This syndrome is characterized by chronic transplant vasculopathy associated with diffuse C4d staining and circulating donor-specific antibodies. Using this animal model, we found that administration of the proteasome inhibitor, Bortezomib, delayed acute rejection and attenuated the humoral response in both the acute phase and established state of this syndrome in a dose-dependent manner. Following treatment with this reagent, donor-specific antibodies and C4d deposition were reduced. These studies highlight the role of the proteasome in chronic rejection and identify this molecule as a marker of this syndrome.

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KEYWORDS: chronic allograft rejection; gene transcription; kidney transplantation

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Long-term graft loss remains the bane of kidney transplantation. More recently, much attention has been paid to the 'humoral' theory of chronic allograft rejection.[1] Evidence for the involvement of a humoral arm of the immune response to allografts has come from studies analyzing the impact of anti-human leukocyte antigen (HLA) antibodies on graft outcome,[2,3] and evidence of complement cascade activation within kidney grafts diagnosed by intragraft deposition of the complement split product C4d.[4,5] These data were recently reinforced when the definition of *chronic active antibody-mediated rejection* (CAMR) was introduced into the Banff classification of kidney graft injury as an association of specific histological lesions associated with diffuse C4d deposition in peritubular capillaries and circulating donor-specific anti-HLA (DSA) antibodies.[6]

Current therapies to combat the acute form of antibody-mediated rejection aim to reduce antibody titers through the use of intravenous immunoglobulin,[7] plasmapheresis,[8] or B-cell-targeting antibodies such as Rituximab,[9] but these have not gained notoriety in the chronic form. Moreover, these strategies do not seem to have an effect on plasma cells, the source of antibodies. Thus, the development of more effective strategies would benefit patients suffering from this type of late graft rejection with such a poor prognosis.[10] The identification of molecular markers associated with CAMR would not only facilitate its diagnosis, but could also help to understand the pathophysiology of CAMR and thus aid in the design of new therapeutic strategies. Currently, CAMR diagnosis depends on the triad of graft lesions together with intragraft C4d and DSA mentioned above. Neither one of these alone can diagnose CAMR; DSA are predictive of graft loss but have not yet been shown to tightly correlate with intragraft lesions, and C4d has recently been shown to be absent in up to 50% of CAMR cases.[11] Here, using a gene-set comparison approach, we describe the identification of a well-known molecule, the immunoproteasome-β subunit 10 (PSMB10, also known as MECL-1) as a potential marker of CAMR in humans.

Moreover, we obtained concordant results in a rat heart allograft model that we have recently described as a pertinent model of CAMR (histological lesions of chronic vascular damage, persisting antidonor antibodies and diffuse C4d deposits on the graft vasculature).[12] Here we show that, in this model, inhibition of the proteasome significantly prolongs allograft survival by preventing acute rejection, and also attenuates established humoral immune responses (decreases DSA and C4d deposition) in both short- and long-term surviving recipients. Our data suggest the implication of the immunoproteasome in CAMR with PSMB10 as a potentially useful marker and point toward proteasome inhibition as a means of treating both acute rejection and CAMR.

RESULTS

Identification of the inducible member of the immunoproteasome, PSMB10, as a potential intragraft biomarker of chronic graft injury in human kidney transplantation

In order to identify potential diagnostic markers of chronic graft injury in humans, we compared several microarray data sets published in the literature in the context of human kidney transplant biopsies with chronic lesions (referred to as CAN or chronic rejection in the studies in question; Table 1). In total, less than 20 molecules were found to be common between at least two data sets (not shown). Among them, PSMB10 was found to be upregulated in chronic rejection in a study by Donauer *et al.*[13] and in Banff grade 3 versus Banff grade 0 in a study by Flechner *et al.*[14] PSMB10 was chosen from among the others because of its function as an instrumental member of the immunoproteasome and the availability of reagents to test it as a potential therapeutic target. In fact, the immunoproteasome was shown to be involved in antigen processing for presentation,[15] and proteasome inhibition was shown to blunt antibody responses in mice.[16] In healthy individuals, PSMB10 is expressed constitutively by the immune system (the spleen, peripheral blood, and lymph nodes; Supplementary Figure S1A) and by lymphocytes and monocytes, where its expression is regulated by cell activation (Supplementary Figure S1A and B). It can also be induced through exposure to interferon-γ.[17]

PSMB10 is upregulated in CAMR in human kidney transplant recipients

We analyzed PSMB10 in graft biopsies with different histological diagnoses (Table 2). As shown in Figure 1a, PSMB10 mRNA was specifically upregulated in biopsies with CAMR ($P<0.001$). Of note, PSMB10 expression was not correlated with proteinuria ($r=-0.16$; $P=0.63$). Receiver operator characteristic (ROC) curve analysis revealed that PSMB10 mRNA had an excellent capacity to discriminate CAMR from the other histological diagnoses, with an area under the curve of 0.92 ($P<0.0001$, 95% confidence interval of 0.84–0.97). At a cut-off of 1.95, there was a sensitivity of 0.85 and a specificity of 0.83 (Figure 1b). Thus, PSMB10 shows potential as an intragraft marker of CAMR in humans.

Given that ACR is becoming a rare phenomenon, we next focused on CAMR, to determine whether the specific regulation of PSMB10 was reflected in the peripheral blood. PSMB10 was analyzed in 150 kidney transplant recipients with stable graft function and 15 patients with CAMR (Table 3). As shown in Figure 2, patients with CAMR had significantly higher levels of PSMB10 than those with stable graft function ($P<0.01$). Again, PSMB10 expression was not correlated with proteinuria ($r=-0.01$; $P=0.98$).

To assess whether PSMB10 could potentially serve as a minimally invasive clinical decision-making tool, ROC curve analysis was performed. The results (Figure 2b) showed that this molecule analyzed in recipient blood could still discriminate CAMR from the other groups of patients well, albeit less than in the biopsies, with an area under the curve of 0.72 ($P<0.01$; 95% confidence interval of 0.57–0.84). At a cut-off of 0.96, there was a sensitivity of 0.67 and a specificity of 0.64.

Potential confounding factors influencing the expression of PSMB10

We next performed a multivariate analysis on the 150 stable patients to evaluate the potential impact of clinical and demographic factors on the expression of PSMB10 in the peripheral blood (Table 3). Of all the parameters tested (legend to Figure 2), the statistically significant parameters identified as being associated with PSMB10 mRNA expression were recipient gender ($P<0.05$) and time post-transplant ($P<0.01$). Thus, creatinine clearance, proteinuria,

Table 1 | Studies used to identify common biomarkers of late graft injury

First author, journal/year	Subject	Sample	Microarray platform	Gene list
Hotchkiss, *Transplantation*/2006	'Chronic allograft nephropathy' (CAN)	Biopsies	Affymetrix	Selected upregulated genes in CAN (gene list published)
Flechner, *Am J Transplant*/2004	Banff grade 3 vs Banff 1	Biopsies	Affymetrix	Genes upregulated in Banff 3 vs Banff 01 (gene list available on the authors' website)
Scherer, *Transplantation*/2003	'Chronic Rejection' (CR)	Biopsies	Affymetrix	Genes upregulated in biopsies at 6 months which went on to develop CR at 1-year vs biopsies at 6 months with no CR at 1-year (exhaustive gene list kindly provided by the authors)
Donauer, *Transplantation*/2003	'Chronic Rejection' (CR)	Biopsies	'Homemade' according to Stanford protocol	Upregulated in CR vs normal and polycystic kidneys (gene list published)

Table 2 | Patients included in analysis of biopsies (Nantes and Hanover biopsies pooled; see Materials and Methods section).

Group	N	IF/TA	CNI tox	CAMR[a]	ACR[b]
n	13	16	14	13	9
Recipient age, years: median (range)	40.0 (18–69)	49.5 (24–66)	51.5 (29–66)	48 (24–71)	45 (25–61)
Recipient gender ratio (M/F)	9:4	9:7	8:6	7:6	5:4
Donor age, years: median (range)	35.0 (12–69)	51.0 (23–77)	41.5 (16–56)	34 (14–75)	34 (5–72)
Time post-transplant years: median (range)[c]	1	2 (1–3)	5 (1–11)	11 (1–18)	0.67 (0.08–5)
Donor gender ratio (M/F)	8:5	10:6	8:6	5:6 (*n*=2 NA)	8:1
HLA incompatibilities: mean ± s.d.	2.5 ± 1.7	2.5 ± 1.2	2.4 ± 1.4	3.5 ± 1.4	3.1 ± 1.1
% First transplant	100	100	79	92	78
Cockroft creatinine clearance (ml/min): mean ± s.d.	75.9 ± 26.4	49.9 ± 17.0	52.4 ± 24.1	25.8 ± 16.7	32.6 ± 16.2
Proteinuria (g/24 h): median (range)	0.10 (0.03–0.43)	0.11 (0.01–6.37)	0.09 (0.03–4.47)	1.94 (0.80–4.00)	0.46 (0.21–0.49)
Banff c grade: mean ± s.d.	0.00 ± 0.00	1.25 ± 0.58	0.80 ± 1.03	2.27 ± 0.79	1.5 ± 0.53
Immunosuppression protocol (% of patients) at the time of biopsy	MMF: 69% Aza: 8% FK506: 62% CsA: 23% Rapa: 0% Steroids: 54%	MMF: 63% Aza: 13% FK506: 63% CsA: 13% Rapa: 13% Steroids: 69%	MMF: 64% Aza: 14% FK506: 29% CsA: 71% Rapa: 7% Steroids: 57%	MMF:46% Aza: 15% FK506:38% CsA: 54% Rapa: 0% Steroids: 23%	MMF: 78% Aza: 0% FK506: 67% CsA: 0% Rapa: 22% Steroids: 33%

Abbreviations: ACR, acute cellular rejection; Aza, azathioprine; CAMR, chronic antibody-mediated rejection; CNI tox, calcineurin inhibitor toxicity; CsA, cyclosporin A; FK 506, tacrolimus; DSA, donor-specific anti-HLA; HLA, human leukocyte antigen; IF/TA, interstitial fibrosis and tubular atrophy; MMF, mycophenolate mofetil; N, normal histology; TG, transplant glomerulopathy.
[a]The DSA were directed against class II (*n*=6), class I (*n*=2), or both (*n*=5).
[b]These were grade Ia (*n*=3), Ib (*n*=2), and grade IIa (*n*=4 with moderate intimal arteritis-vi) – all were C4d-negative and DSA-negative.
[c]Biopsies for cause only.

donor and recipient age, number of HLA incompatibilities (A + B + DR), and maintenance immunosuppression were not confounders, neither were presence or absence of anti-HLA. Thus, PSMB10 is not simply a reflection of presence of anti-HLA antibodies. These data are modeled in Figure 2c, where predicted values of PSMB10 are expressed according to time post-transplant and recipient gender. PSMB10 was thus significantly higher in the peripheral blood mononuclear cells (PBMCs) of male recipients compared with female recipients ($P<0.05$) and displayed a distinctive inverse bell-shaped relationship with time post-transplant. However, these potentially confounding factors could not explain the difference in PSMB10 between stable (STA) and CAMR, because there was no difference in time post-transplant or recipient gender between the STA and CAMR groups (P = not significant). Thus, overall these data show that PSMB10 may also be a peripheral blood biomarker of CAMR in humans, but some potential confounding factors may exist and need to be taken into consideration.

PSMB10 as an intragraft and peripheral blood marker of acute rejection as well as CAMR in a rat cardiac allograft model

To determine whether we could reproduce the above data in a rodent model, PSMB10 mRNA was analyzed in the grafts and PBMCs of rat recipients of cardiac allografts undergoing acute rejection or CAMR. We found that PSMB10 was significantly increased in the cardiac allografts during both acute rejection (at day (D)7 post-transplant) and CAMR (analyzed at D100 post-transplant) compared with syngeneic controls (Figure 3a; $P<0.01$ and $P<0.0001$, respectively). A different expression profile was observed in the PBMCs, where PSMB10 displayed no change during acute rejection, suggesting an effect of DST priming on PBMCs. Moreover, similar to in humans, a significant increase was observed during CAMR at D100 post-transplant (Figure 3b; $P<0.05$). Thus, PSMB10 shows potential as an intragraft and peripheral blood marker of CAMR in rats and humans.

The proteasome as a therapeutic target for AR: prolonged survival and attenuation of the humoral response during AR in a rat cardiac allograft model upon immunoproteasome inhibition with Bortezomib

Given this upregulation of PSMB10 in acute rejection and CAMR in this rodent model, we set out to determine whether proteasome inhibition could influence graft outcome. In the context of acute rejection, recipients of major histocompatibility complex (MHC)-mismatched cardiac allografts were treated with Bortezomib every other day from D0–D20. The data presented in Figure 4a show that Bortezomib dose-dependently prolonged cardiac allograft survival with an optimal effect at 0.1 mg/kg, giving a mean survival of 31.7 days ($n=6$) (versus 6.3 days in untreated controls ($n=4$); $P<0.01$). Moreover, at D7, there were significantly lower circulating anti-donor MHC class I and II antibodies for total immunoglobulin G (IgG) ($P<0.01$) as well as IgG1 ($P<0.01$), and IgG2c ($P<0.05$), with no reduction for IgG2a or IgG2b (Figure 4b).

The proteasome as a therapeutic target for CAMR: attenuation of the humoral response during CAMR in a rat cardiac allograft model upon immunoproteasome inhibition with Bortezomib

In the context of CAMR, Bortezomib was initially administered every other day from D80 to D100 at 0.1 mg/kg in rat recipients of cardiac allografts that had received DST before

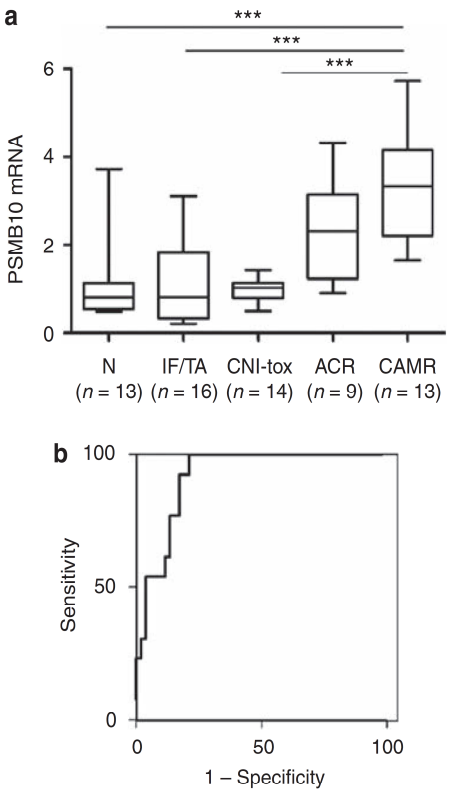


Figure 1 | Differential expression of mRNA for the immunoproteasome subunits PSMB10 and PSMB8 in renal transplant biopsies and their capacity to diagnose acute and chronic rejection. (**a**) Transcription of PSMB10 mRNA in renal transplant biopsies displaying normal histology, interstitial fibrosis and tubular atrophy (IF/TA) of unknown etiology, lesions evocative of calcineurin inhibitor toxicity (CNI tox), acute cellular rejection (ACR) and chronic antibody-mediated rejection (CAMR) (see Materials and Methods for precise definitions). Results represent pooled data for 6-month protocol biopsies and biopsies taken at ⩾1-year post-transplantation (similar results were found for the two cohorts). Statistical significance according to a Kruskal–Wallis test followed by a Dunn's Multiple Comparison test is indicated: ***$P<0.001$. PSMB10 mRNA was measured by quantitative PCR using a TaqMan probe set (Hs00160620_m1). Expression levels were calculated using the $2-\Delta\Delta C_t$ method where the reference represents onefold expression, as previously described.[44] Hypoxanthine phosphoribosyl transferase (Hs99999909_m1) was used as an endogenous control to normalize RNA starting quantity. (**b**) Receiver operator characteristic (ROC) curve analysis of PSMB10 in the combined 6-month and ⩾1-year biopsy cohorts; its capacity to discriminate CAMR from all the other biopsies is analyzed.

Table 3 | Patients included in analysis of PBMCs

Group	STA	CAMR[a]
n	150	15
Recipient age in years: median (range)	53 (20–85)	51 (29–76)
Recipient gender (% males)	62	47
Donor age in years: median (range)	36 (9–69)	41 (14–74)
Donor gender (% males)	75	60
Time post-transplant in years: median (range)	8 (5–21)	6 (1–25)
HLA incompatibilities: mean ± s.d.	3.4 ± 1.3	3.5 ± 1.7
% First transplantations	100	80
Cockroft creatinine clearance (ml/mn): mean ± s.d.	65.6 ± 14.2	34.3 ± 19.7
Proteinuria (g/24 h): median (range)	0.16 (0.04-0.16)	2.61 (1.95–11.52)
IS protocol at blood sampling	MMF: 51% Aza: 26% FK506: 26% CsA: 72% Steroids: 15%	MMF: 60% Aza: 7% FK506: 67% CsA: 20% Steroids: 27%

Abbreviations: Aza, azathioprine; CAMR, chronic antibody-mediated rejection; CNI, calcineurin inhibitor; CsA, cyclosporin A; DSA, donor-specific antibody; FK 506, Prograf; HLA, human leukocyte antigen; MMF, mycophenolate mofetil; NA, not applicable; PBMC, peripheral blood mononuclear cell.
[a]The DSAs were directed against class II (*n*=11), class I (*n*=1), or both (*n*=3).

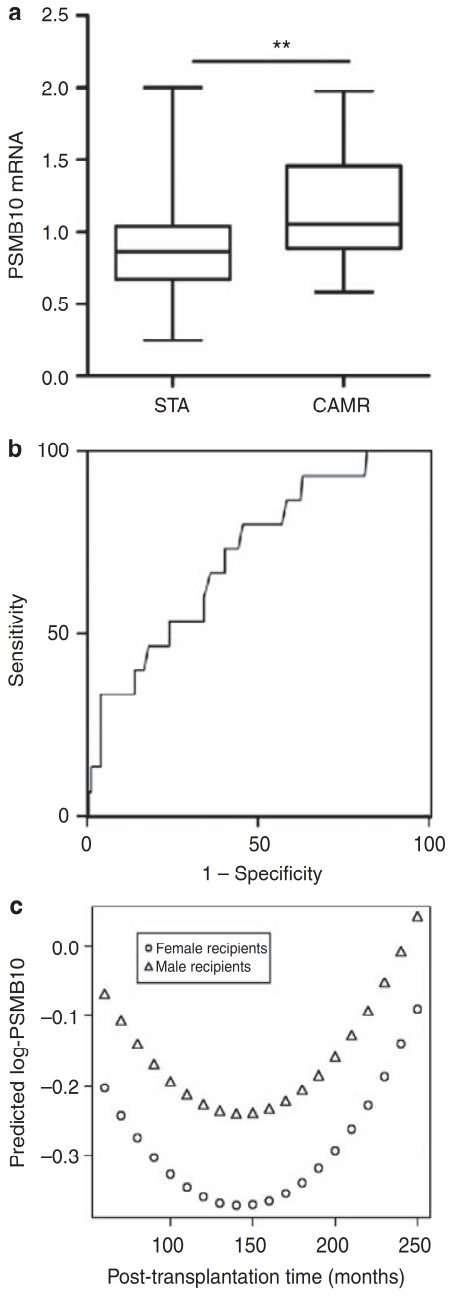
transplantation and were surviving long-term. The DST + Bortezomib recipients had significantly reduced levels of circulating anti-donor MHC class I and II antibodies for total IgG as well as IgG1, IgG2a, IgG2b, and IgG2c (Figure 5a). Bortezomib-treated recipients tended to display less intra-graft complement deposition (Table 4). In fact, although the grafts of DST-treated animals displayed diffuse linear expression of C4d (Figure 5b, left-hand panel and magnified insert), two Bortezomib-treated animals showed only vague background staining, whereas the others displayed minimal staining (Figure 5b, right-hand panel and magnified insert). There were no differences between the two groups in terms of infiltrate, fibrosis, vascular obstruction, vascular lesions, and number of affected arteries; however, myocyte necrosis was absent in four of the five animals in the Bortezomib-treated group (Table 4 and examples in Figure 5c, far left and middle panels). We thus performed further experiments in which treatment was initiated earlier at D60 and continued until D100. Again, a significant decrease in circulating anti-donor MHC class I and II antibodies for total IgG as well as for three of the four IgG subtypes tested was noted at D100 post-transplant in the Bortezomib-treated recipients (Figure 5d). This decrease was not simply a time-dependent effect. because recipients had significantly less DSA after the 40-day treatment versus before treatment, whereas DSA levels were unchanged in untreated animals over the same time period (Figure 5e). C4d staining at D100 in this group was heterogeneous (Table 4). A histological analysis at D100 showed that there was a tendency toward reduced vascular lesions and a significant reduction in fibrosis compared with the untreated DST group ($P<0.05$; Table 4 and an example Figure 5c, far right panel). Thus, in this model and at the dose and schedules studied, Bortezomib is able to significantly attenuate the humoral immune response in graft recipients undergoing CAMR with a reduction in fibrosis and a tendency to reduce the histological lesions of transplant vasculopathy.

Finally, as PSMB10 expression did not correlate with proteinuria in the blood or graft of humans, we wished to confirm this in a rat model. For this purpose, we analyzed PSMB10 in Buffalo/Mna rats, a well-known rat model of proteinuria due to spontaneous idiopathic nephritic syndrome of unknown origin (see Materials and Methods

section for details). As shown in the Figure 6, PSMB10 was not significantly differentially expressed between rats with or without proteinuria, despite radical differences in proteinuria.

DISCUSSION

Here we used a literature gene-set comparison approach and identified PSMB10 as a potential biomarker of chronic graft injury. Further profiling by quantitative PCR in biopsies and PBMCs of renal transplant recipients revealed this molecule to be specifically increased in the graft and blood in CAMR. PSMB10 was also significantly increased in rat cardiac allograft models with acute or chronic rejection. In the acute model, administration of the proteasome inhibitor



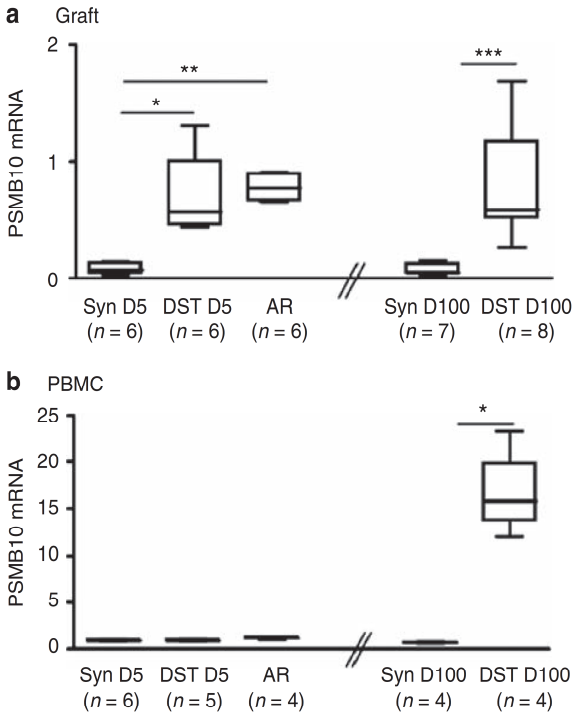


Figure 3 | PSMB10 mRNA profiles in the allografts and PBMCs of rat recipients of MHC-mismatched heart grafts displaying acute and chronic rejection. PSMB10 mRNA transcription in the grafts and PBMCs of rat recipients of MHC-mismatched and DST-treated or syngeneic cardiac transplants. Analyses were performed at D5 and D100 post-transplant as well as during acute rejection (mean 6.3 days post-transplant). PSMB10 was measured by quantitative PCR as described in the legend to Figure 1 using TaqMan probes for rat PSMB10 (Rn01432424_g1) and rat HPRT (Rn01527838_g1). Statistical significance according to a Kruskal–Wallis test followed by a Dunn's Multiple Comparison test is indicated: ***$P<0.001$; **$P<0.01$ and *$P<0.05$. In this model, DST treatment induces long-term survival but with histologic lesions of chronic transplant vasculopathy[40] associated with deposition of the complement split product C4d and circulating donor-specific antibodies.[12]

Figure 2 | Differential expression of PSMB10 mRNA in the peripheral blood mononuclear cells of renal transplant patients, its capacity to diagnose chronic rejection and its interaction with confounding factors. (**a**) PSMB10 mRNA expression in patients with stable graft function under standard immunosuppression (STA; $n=150$) and chronic antibody-mediated rejection (CAMR; $n=15$) (see Materials and Methods section for precise definitions). Statistical significance according to a Mann–Whitney test is indicated: **$P<0.01$. PSMB10 mRNA was measured by quantitative PCR as described in the legend to Figure 1. (**b**) Receiver operator characteristic (ROC) curve analysis of PSMB10 in the PBMCs of renal transplant patients. The capacity of PSBM10 to discriminate CAMR from the other transplant groups is analyzed. (**c**) Results of a multivariate linear regression analysis on log-transformed PSMB10 values. Statistical modeling of the relationship between PSMB10 mRNA values, time post-transplant and donor gender is illustrated. Parameters analyzed included donor and recipient age and gender, time post-transplant, creatinine clearance, proteinuria, number of HLA incompatibilities (A + B + DR), presence or absence of anti-HLA, and maintenance immunosuppression (presence/absence of steroids, high versus low trough level CsA (<125 versus ⩾125 ng/ml) versus high and low trough level FK-506 (<5 versus ⩾5 ng/ml).

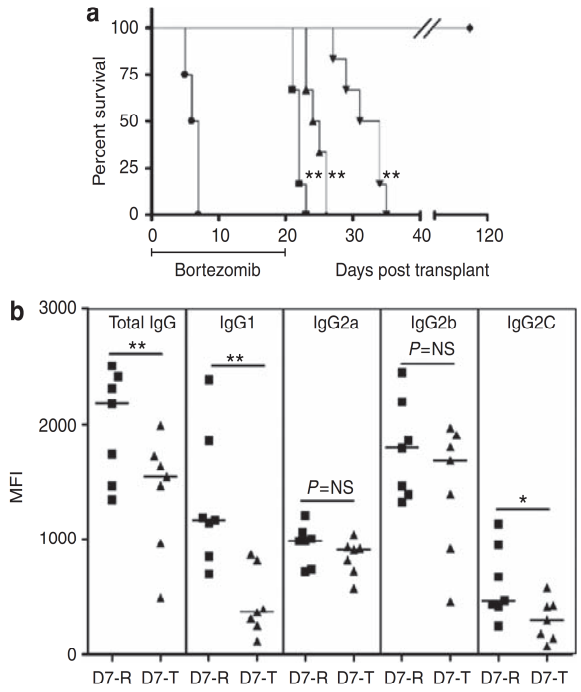


Figure 4 | Induction treatment in rat recipients of MHC-mismatched cardiac allografts with Bortezomib. (**a**) Kaplan–Maier survival analysis of cardiac allograft recipients untreated (●) or treated every other day for 20 days with Bortezomib at 0.025 (■), 0.05 (▲), or 0.1 (▼) mg/kg. Statistical significance is indicated $^{**}P<0.01$. (**b**) Measurement (as described in the Materials and Methods section) of anti-donor MHC class I and II IgG1, IgG2a, IgG2b, and IgG2c subtypes in the serum of MHC-mismatched cardiac allograft recipients at D7 post-transplant treated with Bortezomib (D7-T) or without Bortezomib treatment and thus undergoing rejection (D7-R). Results are expressed as mean fluorescence intensity (MFI). Statistical significance is indicated $^{*}P<0.05$, $^{**}P<0.01$.

Bortezomib not only dose-dependently delayed acute rejection but also attenuated the humoral response. In the chronic model with established CAMR, Bortezomib treatment decreased DSA and C4d deposition and improved some aspects of the chronic tissue injury. These data thus suggest PSMB10 as a potential marker and the proteasome as a therapeutic target for CAMR.

Proteasomes are large protease complexes located in cytoplasm and nuclei that degrade cellular proteins in a ubiquitin-dependent and adenosine triphosphate-dependent manner present in immune cells.[18] In response to interferon-γ, the three catalytic subunits are replaced by their homologous subunits, PSMB 8, 9, and 10, to form the so-called immunoproteasome, which is essential for processing antigenic peptides for presentation through the class I MHC complex.[15] Immunoproteasomes have additional effects that are independent to class I processing, for example, they inhibit T-cell proliferation, as T cells lacking immunoproteasome subunits hyperproliferate *in vitro* and *in vivo*, and KO mice have higher numbers of central memory CD8+ cells.[19] Experiments in KO mice have revealed that PSMB10 is involved in controlling homeostatic equilibrium between T-cell subsets,[20] thereby controlling the T-cell repertoire.[21]

Given the expression of PSMB10 by lymphocytes and monocytes, it is possible that the increase in PSMB10 in the graft in CAMR may be the result of specific infiltration of these cells. B cells in particular may be involved in this because patients with chronic rejection undergo lymphoid neogenesis with the development of intragraft B-cell germinal centers.[22] Likewise, increased PSMB10 expression may come from locally present activated monocytes or mature dendritic cells, as the immunoproteasome is known to be increased in these cells.[23] Given that the proteasome decreases during late phase plasma cell differentiation,[24] it is unlikely that the increase in PSMB10 in the peripheral blood stems from an increase in plasma cells in chronic rejection. ROC curve analysis suggested that the level of PSMB10 in the blood of graft recipients could be useful to diagnose CAMR, although it may not be sufficient as a stand-alone marker and could be combined with other markers to improve its diagnostic performance. Moreover, we found PSMB10 levels to be influenced by recipient gender and time post-transplant (but not presence of anti-HLA or renal function), highlighting the need to take confounding factors into account in biomarker analyses. Thus, PSMB10 is not simply a reflection of DSA or poor renal function, but rather of histological lesions together with C4d and DSA. Future analyses on simultaneous biopsy blood samples, which were not possible here because of lack of material, could be considered to directly compare intragraft and peripheral blood diagnostic performance.

The intragraft upregulation of PSMB10 we observed in CAMR in both humans and rats prompted us to test the proteasome inhibitor Bortezomib in the rat models of both acute rejection and CAMR. Currently, Bortezomib is used in the clinic for multiple myeloma and solid tumors.[25] However, a limited number of studies have analyzed the effect of this drug on acute rejection in murine models of transplantation, showing a preventative effect on both heart[26] and islet[27] allograft acute rejection. On the basis of blood biochemistry, Bortezomib was also associated with side effects, but these were reversible upon cessation of treatment.[27] Our data in a rat model of acute heart rejection are similar to those reported in the mouse, with a similar survival prolongation observed in both models. In the chronic model, diminution of some aspects of chronic injury was observed. The preventative effect on acute rejection is compatible with the hypothesis that proteasome inhibition reduces alloantigenicity by reducing class I presentation. Moreover, we observed a significant decrease in antibody production, suggesting an attenuating effect on the humoral response. Very recent data showed that proteasome inhibition specifically induces death of activated T cells,[28] and suppresses essential immune function of CD4+ T cells upon activation by allogeneic dendritic cells.[29] Moreover, proteasome inhibition has been shown to modulate toll-like receptor4-induced dendritic cell activation,[30] which could have a role here, as we recently

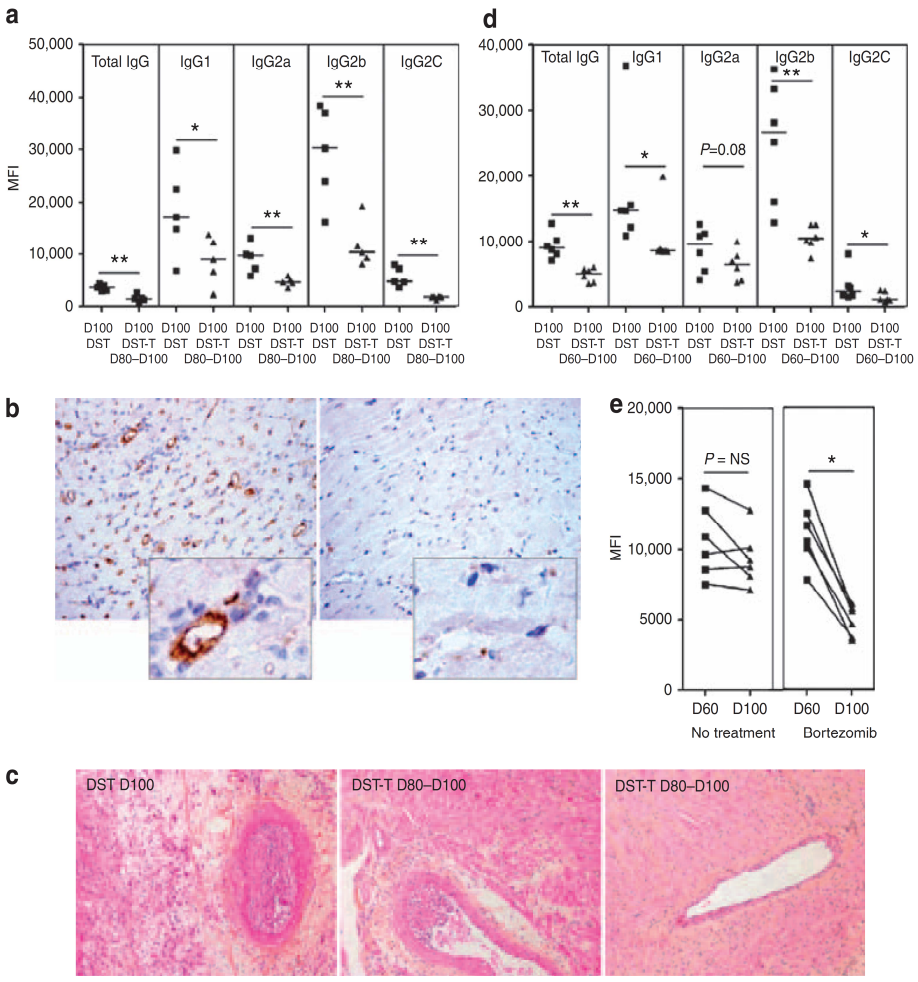


Figure 5 | Maintenance treatment in rat recipients of MHC-mismatched cardiac allografts with Bortezomib. (**a**) Measurement (as described in the Materials and Methods section) of anti-donor MHC class I and II total IgG and IgG1, IgG2a, IgG2b, and IgG2c subtypes in the serum of MHC-mismatched cardiac allograft recipients treated with DST or with DST followed by Bortezomib from D80–D800, at D100 post-transplantation. Results are expressed as mean fluorescence intensity (MFI). Statistical significance is indicated: $*P<0.05$. (**b**) Immunoperoxidase staining of C4d at D100 post-transplantation in recipients of MHC-mismatched cardiac allografts treated with DST alone (left-hand panel and insert) or with DST followed by Bortezomib from D80 to D100 (right-hand panel and insert) (see Materials and Methods section for details). (**c**) Histology (hematoxylin and eosin) at D100 post-transplantation in recipients of MHC-mismatched cardiac allografts treated with DST alone (far left panel) or with DST followed by Bortezomib from D80 to D100 (middle panel) or from D60 to D100 (far right panel) at $\times 100$ magnification. (**d**) Measurement (as described in the Materials and Methods section) of anti-donor MHC class I and II total IgG and IgG1, IgG2a, IgG2b, and IgG2c subtypes in the serum of MHC-mismatched cardiac allograft recipients treated with DST or with DST followed by Bortezomib from D60 to D100, at D100 post-transplantation. (**e**) Paired measurement of anti-donor MHC class I and II total IgG at D60 and D100 in untreated recipients and recipients treated with Bortezomib from D60 to D100.

found toll-like receptor 4 to be increased in the graft and blood of CAMR patients.[31]

We also found that in a rat model of CAMR, Bortezomib decreased the humoral immune response by significantly reducing DSA. These data are in accordance with a recent report that proteasome inhibition blunts Ab responses following *in vivo* B-cell activation in mice, by promoting apoptosis.[16] In the latter study, immunoproteasome subcomponents decreased during B-cell differentiation, in parallel with increased immunoglobulin synthesis. More recently, Bortezomib was shown to rapidly and efficiently deplete both short- and long-lived plasma cells in the spleens and bone marrow in a murine model of lupus-like disease, leading to reduced autoantibody development.[32] Moreover, Bortezomib has recently been shown to induce apoptosis of plasma cells, to block anti-HLA Ab secretion *in vitro*, and to transiently decrease plasma cells *in vivo* in renal transplant recipients.[33,34] However, in the latter studies in humans, Bortezomib was used in combination with other immunosuppressors and plasma exchange making it difficult to pinpoint the specific role of Bortezomib among the treatment arsenal. Moreover, its effect on graft histology has yet to be determined. In our model, we show a clear-cut effect of Bortezomib monotherapy on DSA and C4d, significantly

Table 4 | Histological analysis of DST-treated animals (D7 and D14 before transplantation) subsequently untreated or treated every other day from D80 to D100 or from D60 to D100 (CAMR lesions already established) with Velcade at 0.1 mg/kg

Rat	Group	Graft infiltrate	Fibrosis	Necrosis	Vascular lesions	Vascular obstruction	No. of affected arteries (art/section)	C4d
1	DST	2	3*	+	2	2	3 (28)	+++
2	DST	2	3	+	0	0	0	+++
3	DST	3	4*	+	2	4	13 (16)	+++
4	DST	2–3	2	+	2	1	1 (21)	+++
5	DST	2–3	3	+	1	1	3 (19)	+++
1	DST+Bortezomib D80–D100	2–3	3–4*	+	1–3	4	13 (33)	+
2	DST+Bortezomib D80–D100	2	3	–	2	4	8 (48)	–
3	DST+Bortezomib D80–D100	1–2	3–4	–	0	0	0	–
4	DST+Bortezomib D80–D100	1–2	3	–	2	4	7 (38)	++
5	DST+Bortezomib D80–D100	1–2	3*	–	1	1	2 (16)	++
1	DST+Bortezomib D60–D100	2	1*	+	0	0	0	+++
2	DST+Bortezomib D60–D100	2–3	2*	+	0	0	0	++
3	DST+Bortezomib D60–D100	1–2	3	Focal	3	3	7 (31)	+++
4	DST+Bortezomib D60–D100	2	1	Focal	1	1	3 (8)	+++
5	DST+Bortezomib D60–D100	1–2	2	–	0	0	0	–
6	DST+Bortezomib D60–D100	2	2	Focal	0	0	0.	–
		P=NS	*P*<0.05	*P*=NS	*P*=NS	*P*=NS		

Abbreviations: CAMR, chronic antibody-mediated rejection; DST, donor-specific antibody; NS, not significant.
Infiltrate was graded from 0 to 4 (0, absence; 1, minimum; 2, discrete; 3, moderate; 4, abundant). Fibrosis was graded from 0 to 4 (0, absence; 1, focal; 2, diffuse-minimal; 3, diffuse-moderate; 4, diffuse-abundant; *, edema). Vascular obstruction was graded from 0 to 4 (0, 0; 1, <20%; 2, 20–50%; 3, 50–80%; 4, >80%). Vascular lesions were graded from 0 to 3 (0, normal; 1, leuco-intimal adhesion; 2, inflammatory endarteritis; 3, fibrous endarteritis). C4d was graded from – to +++ (–, <20%; +, 20–50%; ++, 50–75%, and +++, >75%). Necrosis was graded as presence (+) or absence (–) of myocyte damage. Statistical significance was measured using a non-parametric Kruskal–Wallis test with Dunn's Multiple Comparison test.

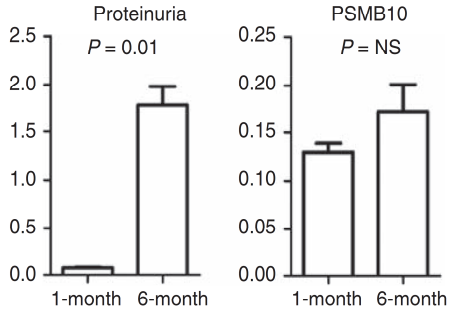


Figure 6 | PSMB10 and proteinuria in a rat model of idiopathic nephritic syndrome. Proteinuria (left-hand graph) and PSMB10 expression (right-hand graph) in Buffalo/Mna rats at 1-month of age and 6 months of age (see Materials and Methods section for details).

reduced allograft fibrosis and a tendency toward reduced vascular lesions. Therefore, early treatment with Bortezomib may be pivotal to prevent irreversible chronic lesions. Further analyses in a rodent model of kidney transplantation would also be interesting, although for the moment there are no true models of CAMR in the kidney. Indeed, in the Fischer to Lewis kidney transplant model of chronic allograft nephropathy (reviewed by Marco[35]), our own experience revealed chronic pyelonephritis with no vascular lesions (data not shown).

On the whole, our data point toward PSMB10 as a potentially useful intragraft and peripheral blood marker for CAMR, with immunoproteasome inhibition as a valuable treatment strategy for antibody-mediated rejection.

MATERIALS AND METHODS

Gene-set comparison

With the aim of identifying relevant biomarkers of late graft injury, we compared the gene sets from four microarray studies of late kidney graft injury published in the literature[13,14,36,37] (see Table 1). To overcome the problem of a single gene having numerous denominations, accession numbers, and so on, all genes were converted into a single common identifier, the 'gene ID,' using the gene ID conversion tool available on the DAVID Bioinformatic Resources 2006 website (http://www.david.abcc.ncifcrf.gov/home.jsp). Gene IDs from the different studies representing were sorted in Microsoft Excel and repeats of the same ID identified.

Human kidney transplant recipients

Study groups. All patients who participated in this study gave informed consent, and the study was approved by the University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks. The study was performed on 65 biopsies and 165 blood samples. Samples were included prospectively on the basis of histological or clinical criteria.

(a) *Biopsies* were collected at two centers, Nantes, France ($n=45$) and Hanover, Germany ($n=20$). Of the total

65 biopsies, 29 were protocol biopsies at 6 months or 1 year and the remaining 36 were biopsies for cause. All were classified according to the updated Banff classification criteria,[6] as displaying normal histology (N- C4d-negative and DSA-negative; $n=13$) or lesions of interstitial fibrosis and tubular atrophy of unknown etiology (IF/TA-C4d-negative and DSA-negative; $n=16$) or lesions of calcineurin inhibitor toxicity (tox-C4d-negative and DSA-negative; $n=14$), or lesions of acute cellular rejection (ACR; $n=9$) or CAMR (defined by the presence of circulating DSA associated with transplant glomerulopathy and deposition of C4d in peritubular capillaries ($n=13$). C4d staining was performed on frozen sections with >50% staining being considered as positive. The demographic and clinical data for all of these patients are provided in Table 2.

(b) *Peripheral blood mononuclear cells* (*PBMCs*) were prepared from the blood of 165 kidney transplant patients (largely independent from those included in the biopsy analysis) whose statuses were defined on a histological and/or clinical basis: Stable graft function (patients under standard immunosuppression with stable graft function (creatinemia <150 μmol/l and proteinuria <1 g/24 h for at least 3 years including over the two previous 6-month follow-up appointments with <20% change between the two time points: STA; $n=150$), or CAMR as defined above ($n=15$). The demographic and clinical data for these patients are provided in Table 3.

Rodent transplant models

Study groups. Inbred male adult rats (200–250 g) of the LEW.1A (RT1a) and LEW.1W (RT1u) MHC-incompatible congeneic strains were purchased from Janvier (Le Genest-Saint-Isle, France) and maintained in an animal facility under standard conditions according to the European and Institutional Guidelines.

(a) *Acute rejection of heart allografts.* LEW.1A rats received heterotopic heart allografts from LEW.1W donors as previously described[38] and received no immunosuppressive treatment. Rejection (cessation of heart beating assessed by daily abdominal palpation) occurs at a mean of 6.3 days post-transplant.

(b) *Chronic antibody-mediated rejection of heart allografts.* LEW.1A rat recipients were transfused intravenously with 1 ml of blood from a LEW.1W donor 14 and 7 days before heterotopic LEW.1W cardiac transplantation as described.[39] Graft function was evaluated as above. In this strain combination, donor-specific transfusion induces long-term graft survival but does not inhibit the indirect pathway of allorecognition, resulting in histological lesions of chronic transplant vasculopathy[40] associated with deposition of the complement split product C4d and circulating DSAs.[12]

(c) *Bortezomib treatment for the prevention of acute rejection.* Heart allograft recipients received Bortezomib (Velcade, Millennium Pharmaceuticals, Cambridge, MA, USA) intraperitoneally from the day of transplantation and every other day until day (D) 20. Three different doses were tested: 0.025, 0.05, and 0.1 mg/kg.

(d) *Bortezomib treatment of CAMR in the context of DST-induced long-term allograft survival.* Heart allograft recipients were treated with DST as described above and received Bortezomib intraperitoneally at 0.1 mg/kg every other day either from D80 until D100 or from D60 until D100. At this time, all allografts present vascular lesions with diffuse C4d deposits and circulating anti-DSA.

Rodent proteinuria model

The Buffalo/Mna rat strain maintained in our laboratory was provided by Dr Saito (Central Experimental Institute, Nokawa, Kawasaki, Japan). These rats have spontaneous idiopathic nephritic syndrome of unknown origin as previously described.[41] They do not have proteinuria at 1 month of age but develop significant proteinuria by 6 months of age. Animal care was in accordance with our institutional guidelines. The rats were placed in metabolic cages for 24 h and total urinary protein concentration (g/l) was measured by a colorimetric method using a Hitachi autoanalyzer (Boehringer, Reims, France). Proteinuria was expressed according to the formula proteinuria (g/mmol) = urinary proteins (g/l)/urinary creatinine (mmol/l), and it was considered to be abnormal at values >0.2 g/mmol.

Sample preparation

Human kidney transplant biopsies were processed as described.[42] Rat heart transplant or kidney tissue was processed as described.[40] Human and rat peripheral blood was collected in EDTA vacutainers and PBMCs separated by density centrifugation using Lymphosep, lymphocyte separation media (Bio West, Nuaille, France). PBMCs were stored in TRIzol (Invitrogen, Cergy Pontoise, France) at −80°C until use.

RNA extraction and reverse transcription. Human biopsies were processed as described in detail.[42] Rat heart allografts and rat and human PBMCs were processed using the TRIzol method (Invitrogen) according to the manufacturer's instructions. RNA quality and quantity was determined using an Agilent 2100 BioAnalyzer (Palo Alto, CA, USA). Genomic DNA was removed by DNase treatment (Roche, Indianapolis, IN, USA). RNA was reverse transcribed into cDNA using polydT oligonucleotide and Maloney leukemia virus reverse transcription (Invitrogen).

Real-time quantitative PCR. Real-time quantitative PCR was performed in an Applied Biosystems GenAmp 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) as described in detail,[42] using commercially available primer and probe sets (Applied Biosystems).

Rat cardiac allograft histological analyses and C4d immunostaining. Heart allografts were prepared as described by

Ballet *et al.*[12] and analyzed by a pathologist (KR) (see also Table 4 and legend). C4d deposition was localized as described by Ballet *et al.*[12] using an affinity-purified polyclonal rabbit antibody to C4d kindly provided by Baldwin and group[43] (department of pathology, John Hopkins Medical Institutions, Baltimore, MD, USA).

Measurement of circulating anti-donor antibodies in rat recipients of cardiac allografts. Anti-donor class I and II total IgG and subtypes in the sera of untreated or Bortezomib-treated recipients at D7 post-transplantation, and in DST-treated and DST + Bortezomib-treated animals at D60 and D100 post-transplantation were measured as described by Ballet *et al.*[12]

Statistical analyses

The non-parametric Mann–Whitney test was used for comparison between two groups. Note that there was an imbalance in sample size between the CAMR and STA patients ($n = 15$ versus 150); this was because we preferred to use all available STA samples to better reflect the relatively low prevalence of CAMR and to use all samples that were used for the other analyses (multivariate analyses and ROC). Such an imbalance is authorized within the context of the non-parametric Mann–Whitney test. The non-parametric Kruskal–Wallis test was performed for comparison of three or more groups, followed by Dunn's multiple comparisons test. ROC curve analysis was performed to determine the cut-off point of PSMB10 in biopsies and/or blood that yielded the highest combined sensitivity and specificity in diagnosing CAMR (see Figure 1 legend for explanation). Note that the $n = 15$ CAMR were compared with a large number of STA patients ($n = 150$) in order to better reflect the relatively low prevalence of CAMR in this diagnostic test. The effect of the different clinical and demographic parameters on the expression of PSMB10 in patients with stable graft function, a multivariate linear regression analysis, was performed following log transformation of the data taking into account the various parameters (see legend to Figure 2). The statistical software used was GraphPad prism 5.

DISCLOSURE

Several authors hold a patent for PSMB10 expression as a biomarker of CAMR. JA-C and MG are currently employees of TcLand Expression, Nantes France, a company developing blood biomarkers in transplantation.

ACKNOWLEDGMENTS

This research was funded by a grant from the 'Foundation Progreffe' (Nantes, France). The 164 stable patients were recruited in Nantes in the context of the Genhomme PHRC. This work is also part of a French Transplantation Research Network (RTRS) supported by the 'Fondation de Coopération Scientifique' CENTAURE. We thank Dr W Baldwin (Cleveland Clinic, OH, USA) for kindly providing the anti-rat C4d antibody.

SUPPLEMENTARY MATERIAL

Figure S1. PSMB10 mRNA expression within the immune system and various cell subtypes of healthy individuals.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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DISCUSSION OF ARTICLE 1

I will not discuss the identification of PSMB10 as a potential biomarker of chronic rejection because this work had been done by our colleagues, mainly Ashton-Chess and Jovanovic before I joined the lab. I will limit my discussion on the second part of the study where my contributions were essential to demonstrate the role of proteasome inhibition in rat heart allograft models.

Although several years before our study, the proteasome inhibitor dipeptidyl boronic acid (bortezomib) had been shown to prolong heart and islet allograft survival in mice (Luo et al, 2001, Wu et al, 2004), the mechanisms underlying its effects had not been studied in detail. We have demonstrated for the first time that bortezomib by itself can decrease donor-specific antibody formation in both models of acute and chronic heart allograft rejection in the rat. In addition, it also decreased capillary C4d deposition, a hallmark of chronic antibody-mediated rejection (AMR). Following our study, bortezomib has also been shown to be effective in a rat kidney allograft model of chronic AMR, especially when combined with sirolimus (Vogelbacher et al, 2010).

Since second generation PIs are being developed and may be more potent than bortezomib in inducing plasma cell apoptosis and decreasing antibody formation, we are currently contacting some pharmaceutical companies to test these new products in our animal models of transplantation (see perspective).

ARTICLE 2

Bortezomib in the Treatment of Antibody-Mediated Rejection – A Report of 3 Cases

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INTRODUCTION

Antibody-mediated rejection (AMR), either in the acute or chronic form, is increasingly recognized as an important subset of renal transplant rejection in the past decade (1,2). It will relentlessly lead to graft loss if not effectively treated. Unfortunately, the current therapies for AMR including plasma exchange, intravenous immunoglobulin (IVIg), and rituximab are still far from satisfactory. In fact, none of our current immunosuppressants affects plasma cells (PC), which are the antibody-secreting cells.

Bortezomib is the first proteasome inhibitor approved by FDA for the treatment of multiple myeloma (3). But it can also induce non-malignant PC apoptosis and decrease alloantibody production (4). In addition, it has been shown to inhibit essential functions of T cells (5) and dendritic cells (6) in vitro. In vivo, it prolongs graft survival in murine transplant models (7). Furthermore, we have also shown that bortezomib decreased alloantibody production and capillary C4d deposition in a rat heart transplant model of chronic rejection (8).

In human renal transplantation, three reports have shown encouraging effects of bortezomib either in patients with acute AMR (4,9) or in patients with high donor-specific antibodies (DSA) following a tolerance induction protocol (10). In most of these patients, other anti-rejection treatments such as pulse corticosteroids, rabbit antithymocyte globulin (rATG), rituximab, or plasmapheresis were given either concomitantly or shortly before bortezomib. On the contrary, bortezomib as single agent failed to decrease DSA levels in a recent report on 4 patients with subclinical AMR (11). Therefore, further studies are needed to determine the efficacy of bortezomib. We herein report three renal transplant patients including two with acute and one with chronic active AMR who received bortezomib either following other anti-rejection treatments or alone. Bortezomib helped decrease DSA levels in one patient, whereas in the other two, it was ineffective and may possibly have important side effects.

METHODS

Treatment

The study was approved by the drug advisory committee at our institution. All patients received IV bortezomib (Velcade®) 1.3 mg/m^2 on day 1, 4, 8, 11. A second cycle of bortezomib can be considered after at least ten days from the last injection. Valganciclovir (Rovalcyte®) and trimethoprim-sulfamethoxazole (Bactrim®) were given for cytomegalovirus and Pneumocystis prophylaxis, respectively.

Anti-HLA Antibody Testing

Anti-HLA antibodies were tested by the Luminex technique using test kits purchased from One Lambda, Inc., Canoga Park, CA. Patients on our waiting list for renal transplantation were first screened for the presence of anti-HLA antibodies with the LABScreen® Mixed Class I & II, followed by the determination of panel reactive antibody (PRA) percentage with the LABScreen® PRA Class I & II if the screening was positive. After transplantation, patients with suspected AMR were tested for DSA using the LABScreen® Single Antigen Class I & II. DSA levels were reported as mean fluorescence intensity (MFI) with the positive threshold set at 2000. Crossmatches were performed just prior to transplantation with the standard complement-dependent cytotoxicity test (CDC-CXM). Renal transplants were performed only when CDC CXMs were negative.

CASE REPORTS

The main characteristics of the three patients were summarized in Table 1.

Patient 1

A 32-year old highly-sensitized man with end-stage renal disease (ESRD) secondary to IgA nephropathy received a second cadaveric, three HLA-mismatched renal transplant in May 2009. His first cadaveric, three-mismatched kidney transplant at the age of 24 was complicated by a chronic allograft nephropathy probably linked to a non-compliance leading to the return to hemodialysis at 2 year post-transplant and a transplantectomy 1 year later. His peak PRA pretransplant were 80 percent for HLA class I and 100 percent for HLA class II.

Immunosuppression included rATG induction, corticosteroid (CS), mycophenolate mophetil (MMF), and tacrolimus (Tac). An early renal biopsy

Table 1. Patient characteristics.

	Patient 1	Patient 2	Patient 3
Recipient characteristics			
Sex	M	F	M
Age (years)	32	55	50
Nephropathy	IgAN	VUR	Undetermined
Previous renal transplants	1	0	0
Transplant characteristics			
HLA mismatches	3	4	5
Pretransplant class I/II PRA (%)	80/100	53/49	0/0
Induction	rATG	rATG	none
Immunosuppression	Tac/MMF/CS	Tac/MMF/CS	CyA/MMF/CS
Renal biopsy diagnosis	Acute AMR	Acute AMR	Chronic active AMR
BANFF scores			
Acute	g0, i0, t1, v0, ptc2	g0, i0, t0, v0, ptc0	g3, i0, t0, v0, ptc2
Chronic	cg0, ci0, ct0, cv0, mm0, ah0	cg0, ci0, ct0, cv0, mm0, ah0	cg3, ci0, ct1, cv0, mm0, ah1
C4d	+	+	+
Anti-rejection treatment prior to bortezomib	PE, IVIg, Ritux, pulse CS	PE, IVIg, pulse CS	Increase CyA, MMF
At the first bortezomib dose			
Posttransplant day	61	47	2145
Serum creatinine (µmol/l)	239	97	159
Proteinuria (g/d)	0.74	0.11	2.43

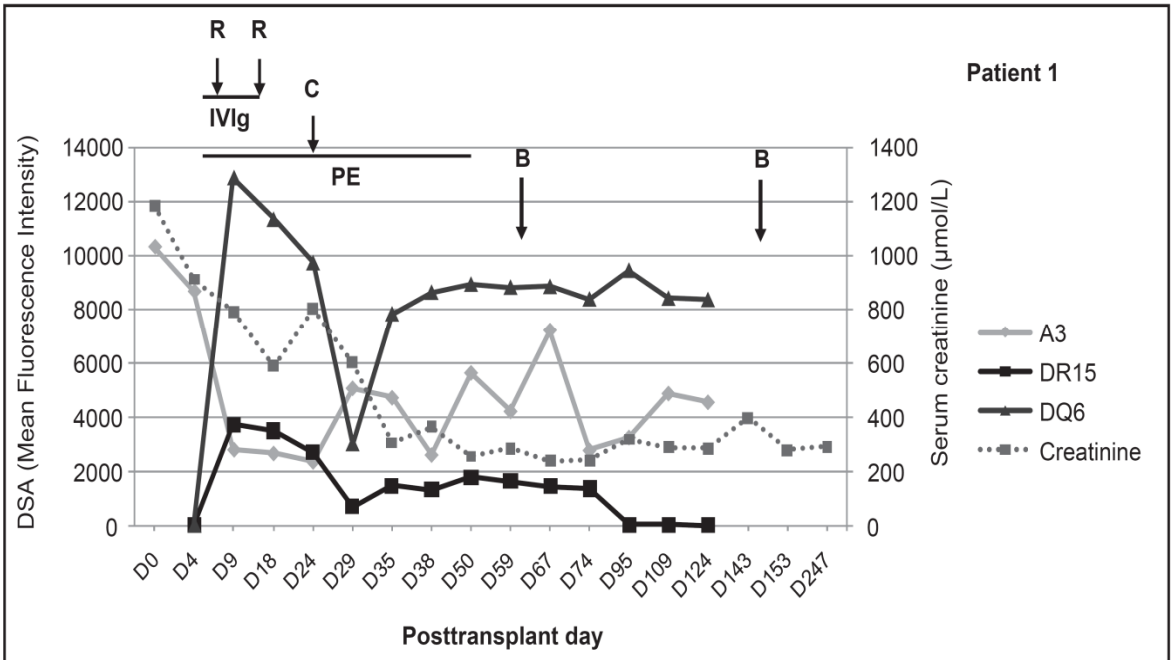
M: male; F: female; IgAN: IgA nephropathy; VUR: vesicoureteral reflux; PRA: panel-reactive antibodies; rATG: rabbit antithymocyte globulin; Tac: tacrolimus; MMF: mycophenolate mophetil; CS: corticosteroid; AMR: antibody-mediated rejection; PE: plasma exchange; IVIg: intravenous immunoglobulin; Ritux: rituximab.

on post-transplant day (PTD) 4 prompted by the detection of high levels of DSA anti-HLA A3 (MFI at 10291 and 8644 with serum of PTD 0 and 4, respectively) revealed diffuse acute tubular necrosis (ATN) and mild glomerulitis with polymorphonuclear neutrophils (PMNs) but the frozen fragment contained no renal tissue for C4d staining. A diagnosis of suspicious acute AMR was made and the patient was treated with rituximab, IVIg, and daily plasma exchange (PE). On PTD 12, he had a hemorrhagic shock secondary to a large perigraft hematoma necessitating a surgical drainage. A graft biopsy during operation confirmed an acute AMR with peritubular capillary (PTC) PMNs and C4d staining, associated with lesions of thrombotic microangiopathy (TMA) in the glomerular capillaries. A second dose of rituximab was given, PE continued 3 times per week, and Tac withdrawn. The patient was still on hemodialysis but his urine output began to increase from PTD 22. A third renal biopsy on PTD 25 disclosed an acute cellular rejection (ACR) grade IIa in addition to acute AMR, but no lesions of TMA. Three pulses of CS were given and Tac was reintroduced. His renal function improved and dialysis was stopped. A fourth renal biopsy on PTD 38 revealed a resolution of ACR but not of AMR as there were always diffuse C4d staining and mononuclear cells in PTC.

DSA followup as shown in Figure 1 revealed: 1) diminution of anti-HLA A3 to a nadir of 2347 on PTD 24 followed by an increase to the range of 4000 to 5000, 2) appearance of two de novo anti-HLA DR15 and anti-HLA DQ6 from PTD 9, the former was moderate and disappeared at PTD 29, but the latter was high and persistent around 8000. His renal function did not improve further with the serum creatinine (sCr) around 240 to 270 µmol/L (2.7 to 3.1 mg/dL).

Having failed to control AMR with all conventional anti-rejection therapies, including 23 sessions of plasma exchange from PTD 4 to PTD 52, we decided to start of bortezomib on PTD 61. At that moment, his DSA levels were 4200 and 8812 for anti-HLA A3 and DQ6, respectively. Anti-HLA A3 decreased to 2767 at the end of the treatment on PTD 74, but rose shortly thereafter to attain the pretreatment levels on PTD 109 and PTD 124. But anti-HLA DQ6 and sCr were essentially unchanged. A renal biopsy on PTD 95 failed to obtain renal tissue.

On PTD 143, we tried a second cycle of bortezomib. Both cycles were initially well tolerated



Abbreviations : B: bortezomib; C: pulse corticosteroid; IVIg: intravenous immunoglobulin; PE: plasma exchange; R: rituximab

Figure 1. Followup of DSA and serum creatinine of patient 1.

besides a transient thrombocytopenia which had already happened before bortezomib. However, he developed a transient leucopenia on PTD 160 at $2.7x10^9$/L, followed by a prolonged one persisting from PTD 189 ($2.9x10^9$/L) up to the last followup on PTD 247 ($2.1x10^9$/L), despite the discontinuation of valganciclovir and MMF on PTD 209 and PTD 227, respectively. More importantly, on PTD 227, he developed a complete atrioventricular block necessitating pacemaker implantation on PTD 244. He also had an acute renal failure secondary to dehydration caused by diarrhea, but his last sCr level on PTD 247 has returned to 290 µmol/L (3.3 mg/dL). The patient relocated just after the end of the second bortezomib cycle and DSA followup has not yet been done.

Patient 2

A 55-year old woman with ESRD secondary to a vesicoureteral reflux received a first four HLA-mismatched cadaveric renal transplantation in October 2009. The patient had six children and had previously received one blood transfusion. The peak pretransplant PRA were 53 and 49 percent for HLA class I and class II, respectively.

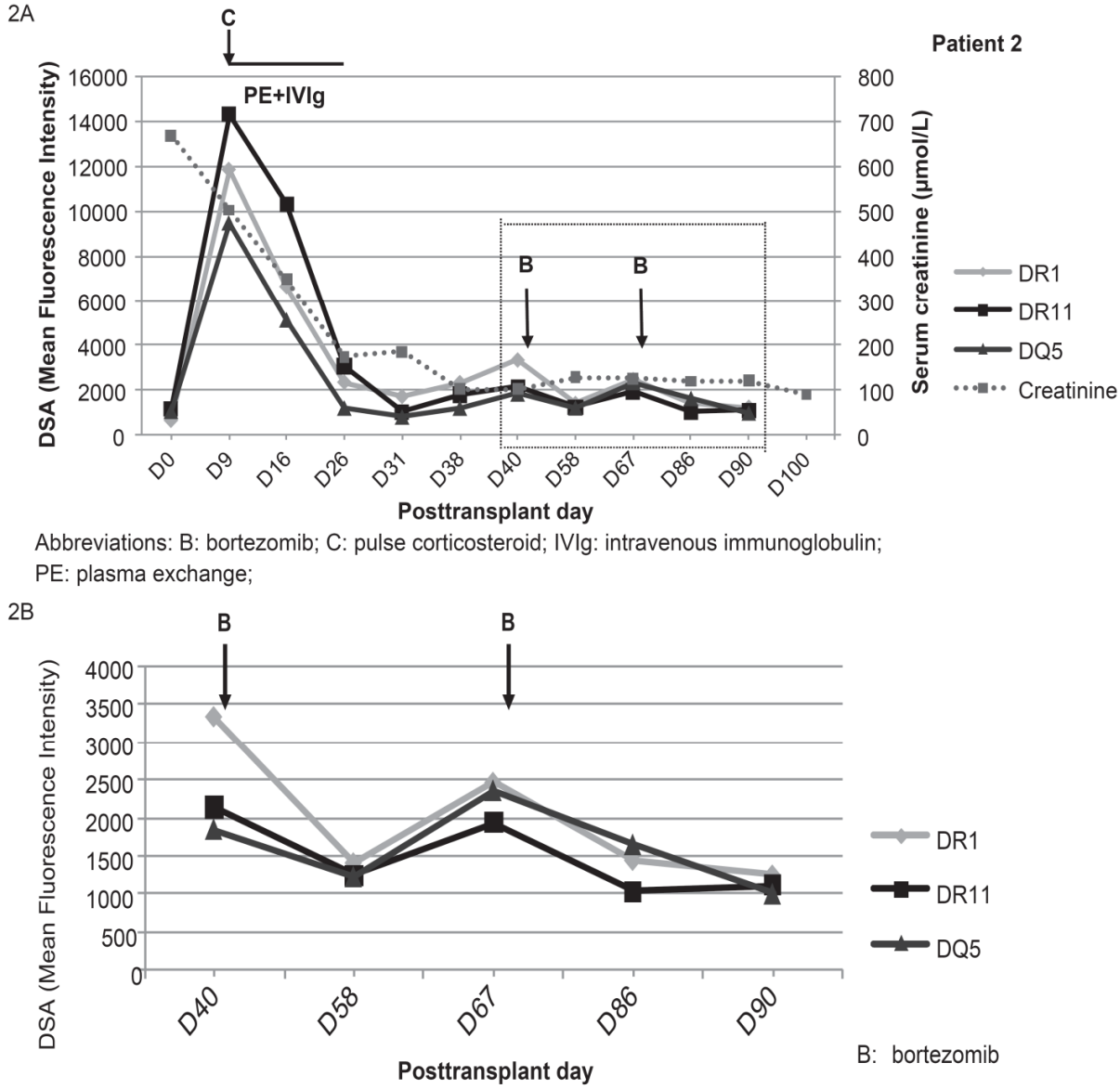


Figure 2. (A) Followup of DSA and serum creatinine of patient 2; (B) Followup of DSA of patient 2 after bortezomib.

Her immunosuppression included rATG induction, Tac, MMF, and CS. On PTD 9, three de novo DSAs were detected: anti-HLA DR1, DR11, and DQ5 with MFI of 11850, 14343, and 9505, respectively. A renal biopsy on the same day revealed an acute AMR grade I with ATN and diffuse PTC C4d deposition. The patient was treated with pulse IV corticosteroids, 10 sessions of plasma exchange from PTD 9 to PTD 25, and IVIg but not rituximab because she developed a Quincke's edema immediately after a few drops of IV rituximab. Her renal function improved progressively but she still needed occasional hemodialyses for fluid removal. On PTD 26, she developed a deep vein thrombosis of the right external iliac vein approaching the kidney graft anastomosis, necessitating a treatment.

DSA followup (Fig. 2A) showed that all three types of DSA decreased to below our positivity threshold of 2000 on PTD 31, but then increased on PTD 40 with anti-HLA DR1 and DR11 at 3335 and 2136, respectively. Because the patient now had stable sCr levels around 100 µmol/L (1.1 mg/dL) and was fully anticoagulated, we did not perform a second renal biopsy. We decided to start of bortezomib on PTD 47 which reduced all DSAs to one-haft of the pre-treatment values on PTD 58 (Fig. 2B). Nevertheless, the effect was transient as DSAs increased again on PTD 67. Because the first cycle was well tolerated, we tried a second cycle of bortezomib on PTD 76, which was associated with a moderate thrombocytopenia and leucopenia necessitating a delay of the third and fourth injections. Again this treatment decreased all DSAs on PTD 90, and sCr level on PTD 100 was 90 µmol/L (1.0 mg/dL).

Patient 3

A 45-year old man with ESRD of unknown etiology received a first cadaveric renal transplant with five HLA mismatches in January 2004. The pretransplant PRA were negative. He received cyclosporine A (CyA), MMF, and CS. The post-transplant course was uneventful, his sCr levels decreased gradually to 156 µmol/L (1.8 mg/dL) on PTD 31 and 121 µmol/L (1.4 mg/dL) on PTD 235. On PTD 786, two types of DSA were detected: anti-HLA DQ7 and anti-HLA DQ9, with MFI of 12362 and 12474, respectively (Fig. 3). However, his renal function remained stable during the two following year with sCr levels around 120-130 µmol/L (1.4-1.6 mg/dL). On PTD 1549, a renal biopsy prompted by the appearance of a moderate proteinuria disclosed a chronic active AMR with diffuse PTC C4d deposition. An attempt to switch from CyA to Tac was unsuccessful because of Tac intolerance.

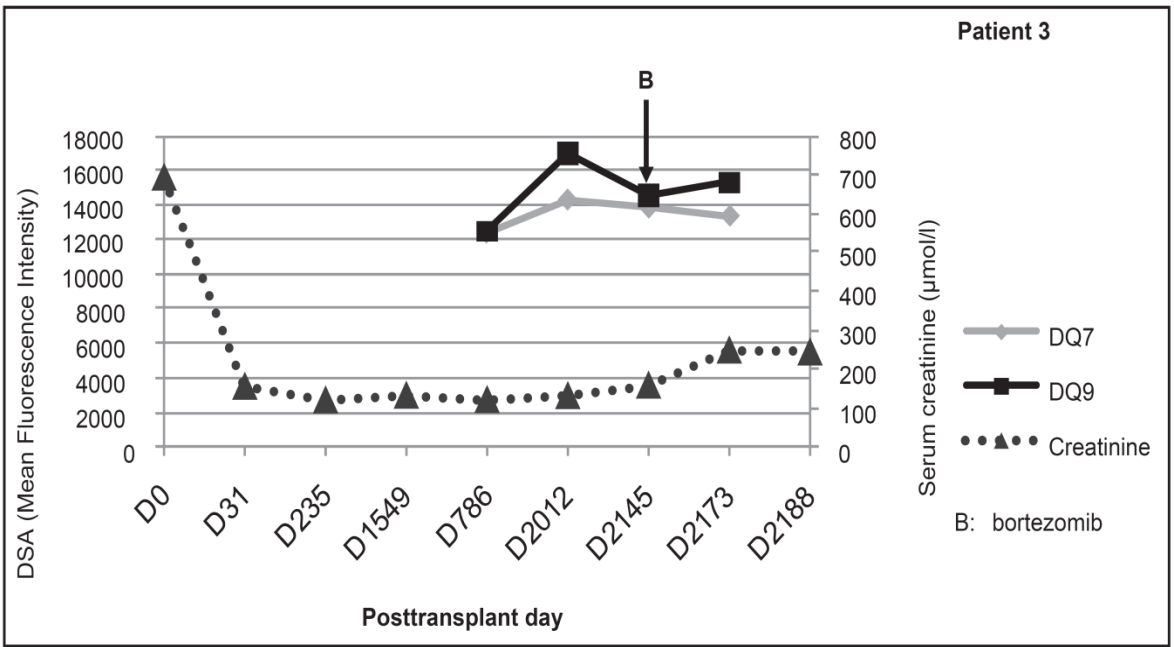


Figure 3. Followup of DSA and serum creatinine of patient 3.

Therefore, immunosuppression adjustment relied on an increase in the dose of both CyA and MMF. Nevertheless, DSA testing on PTD 2012 showed an increase of both anti-DQ7 and DQ9 with MFI of 14279 and 17013, respectively. In addition, his sCr levels began to increase gradually to 140-150 µmol/L (1.6-1.7 mg/dL).

Bortezomib was started on PTD 2145 when sCr was 159 µmol/L (1.8 mg/dL), and anti-HLA DQ7 and DQ9 levels were 13841 and 14548, respectively. It was associated with a nausea, a transient leucopenia, and an increase in C_0 (trough level) of CyA to 643 ng/mL at PTD 2149, which decreased rapidly and stabilized around the baseline of 100 ng/mL after dose adjustment. However, his renal function degraded rapidly with sCr increased to 210 µmol/L (2.4 mg/dL) on PTD 2149 and 249 µmol/L (2.8 mg/dL) on PTD 2173. Furthermore, DSA levels failed to decrease with anti-DQ7 and anti-DQ9 on PTD 2173 at 13378 and 15337, respectively (Fig. 3). A renal biopsy performed on PTD 2173 obtained only a small fragment for immunofluorescence, which showed no PTC C4d deposition.

DISCUSSION

With the advent of more sensitive techniques to detect anti-HLA antibodies and better pathological diagnostic criteria for renal allograft biopsies, antibody-mediated rejection has been more frequently diagnosed and become one the main concerns of the transplant community. The current therapy of acute AMR which usually includes rapid DSA removal by plasmapheresis, immunomodulation with IVIg, and B-cell depletion by rituximab has obtained significant success (12) but is still far from optimal with substantial graft loss rate (reviewed in (13)). Bortezomib with its ability to induce apoptosis of plasma cells – the antibody producing cells – emerged as a potential therapeutic agent for AMR. The first three reports on bortezomib treatment in combination with other conventional therapies were encouraging with rejection reversal in most of the patients associated with prolonged suppression of DSA (4,9,10). However, in the report by Trivedi et al (10), 4/11 patients had either persistent elevation or reappearance of DSAs after treatment, and in the report by Everly et al (9), 2/6 patients finally lost their grafts in less than one year posttransplant. Furthermore, in a recent report by Sberro-Soussan et al (11), all four patients with subclinical AMR (stable sCr but high DSA levels and histological lesions of AMR) showed no decrease in DSA levels after one course of bortezomib as a single agent. Therefore, more reports are needed to accumulate experience on this novel therapeutic modality.

The first patient in our report was highly sensitized and had mixed acute AMR and ACR. He received nearly all of our current anti-rejection armamentarium, with only partial resolution of rejections and partial diminution of DSAs. Bortezomib given 9 days after the last PE session caused only a transient reduction of one of the two DSA specificities. A second cycle of bortezomib was unlikely to be effective as there was no improvement in renal function (last sCr at 290 µmol/l), despite the lack of DSA follow-up. Moreover, the patient developed two severe adverse events including a leucopenia persisting for 2 months and a complete atrioventricular block (AVB) occurring 2 months after the last dose of bortezomib. Although it is difficult to establish a causal relationship in this case, cardiovascular side effects of bortezomib have been well-documented. For example, in a series of 69 cancer patients who received bortezomib treatment, there were 8 patients with cardiac complications, including 2 AVB requiring pacemaker implantation (14).

The second patient with acute AMR was apparently less severe than the first one because she had stable sCr and reduced levels of DSAs after conventional treatment. Because effective DSA reduction is associated with enhanced renal allograft survivals (15), we reinforced the treatment with two cycles of bortezomib, which effectively decreased all DSAs further to about one-half of the pretreatment levels. Noteworthy, DSA reduction in this patient can reasonably be attributed to bortezomib per se because there was no rituximab and the last session of PE and the last IVIg dose were given 22 days before, whereas in the study of Trivedi et al (10), all patients received 2 to 4 plasmapheresis

uring their bortezomib treatment course. However, the moment of this writing, the follow-up is too ıort to know if there will be a rebound of DSAs.

The third patient in our report with chronic :tive AMR received bortezomib as the sole erapy. However, it was initiated very late in the sease course after the failure of CyA and MMF ›se adjustment. This may explain the inefficacy bortezomib in reducing DSA. Although the time follow-up is short and only one DSA testing was erformed after 4 weeks, in our 2 previous cases ıd in other reports (4,9,10), the effect of bortezoıb if present can be seen in the first few weeks ter treatment. Therefore, it is unlikely that later SA testings will show a decrease in DSA levels. ı the other hand, the rapid increase in sCr after ›rtezomib treatment in this case needs special tention. It may initially be associated with CyA 'erdose. Since both bortezomib and CyA are etabolized via cytochrome P450, such interaction ın be predictable. But even when this problem as resolved rapidly with CyA dose reduction, sCr vels continued to increase. Unfortunately, the post-treatment allograft biopsy failed to obtain renal tissue. It is unclear whether bortezomib can aggravate renal insufficiency in the setting of chronic allograft rejection. On the contrary, bortezomib treatment in multiple myeloma patients with renal impairment is usually associated with improvement of renal function (16).

In summary, our case study with short follow-up does not permit to have a definite conclusion. Obviously, large, well-designed multicenter studies are needed to evaluate the efficacy and safety of bortezomib in renal transplantation. However, several important points can be drawn from this study: 1) bortezomib by itself is effective in reducing serum DSA levels in at least a proportion of patients with AMR; 2) bortezomib may be ineffective when given late in the course of the disease as a rescue therapy; 3) cautions must be taken concerning side effects, especially cardiotoxicity and renal impairment, since little is known about its interaction with other immunosuppressive drugs used in transplantation.

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DISCUSSION OF ARTICLE 2

Our article, together with those from other transplant centers at that time, helps to accumulate experiences in the use of this novel drug in treating AMR in kidney transplant patients. Because acute AMR is an urgent medical condition, it is very difficult to design randomized control studies to evaluate the efficacy of a new drug. Therefore, case-series reports are necessary to provide insights into the efficacy and safety profile of bortezomib-based protocols in the treatment of AMR. In a review article, Everly summarized experiences with bortezomib use in transplantation across 29 transplant centers with more than 70 cases and showed that bortezomib gave better results in the treatment of acute AMR than chronic AMR (Everly, 2009). Bortezomib has also shown a good safety profile in the setting of acute kidney allograft rejection, where patients concomitantly received other immunosuppressive therapies. However, side effects such as thrombocytopenia, gastrointestinal disturbances, and peripheral neuropathy have been reported.

Some transplant centers have also investigated the use of bortezomib as a desensitization therapy to decrease DSA levels in highly-sensitized patients allowing subsequent kidney transplantations. Although bortezomib alone failed to decrease DSA in these patients (Wahrmann et al, 2010), plasma exchanges following bortezomib treatment decreased DSA levels to a greater extent than plasma exchanges alone without bortezomib (Diwan et al, 2011).

At the present time, although bortezomib always remains an off-label use in organ transplantation, more and more transplant physicians have included this novel drug to their armamentarium to combat AMR, one of the major hurdles of organ transplantation. With the advent of the accumulation of experiences over the past three years, we now know that current bortezomib-based therapies are not always effective, they are usually more effective when given promptly to treat early acute AMR (diagnosed within the first 6 months post-transplant) than when given to treat late acute AMR or chronic AMR (Woodle et al, 2011). Although bortezomib monotherapy has been shown to be able decrease DSA formation in experimental allograft models as in our study, face to a complex and multifactorial disease like acute AMR, bortezomib must be combined with other antibody and B-cell directed therapies such as plasmapheresis and rituximab in order to provide maximal efficacy.

PERSPECTIVES

Our studies will be pursued in the quest for novel therapies for antibody-mediated rejection (AMR). We will continue our study with bortezomib and newer proteasome inhibitors. But we will also explore other therapeutic approaches targeting plasma cells and/or B cells. We are planning to carry out our studies both at the experimental and clinical levels.

1. Experimental animal studies:

1.1. Second generation proteasome inhibitors in experimental transplantation:

We are currently working with several pharmaceutical companies that produce proteasome inhibitors (PIs). We expect to obtain soon some of these second generation PIs to use in our transplant models.

The capacity of these PIs in inducing non-malignant plasma cell apoptosis and thereby decreasing alloantibody formation will be tested at first in a mouse skin graft model. The advantage of mouse models is that we can easily quantify plasma cells in mice with anti-CD138 whereas such antibody does not exist in rats. Balb/c mice receive tail skin graft from C57BL/6 mice. Without treatment, skin grafts are rejected within 10 days associated with high levels of DSA which persists over time. Orbital sinus blood will be collected at post-transplant day (PTD) 15 when all skin grafts have already been rejected. From PTD 15 to 45, treated mice will receive a PI at 2 or 3 different dosages, whereas control mice will receive vehicle. Mice will be sacrificed at PTD 45 for blood and organ harvesting. DSA levels will be essentially unchanged between PTD 15 and 45 in control mice, whereas treated mice are expected to have a significant decrease in DSA levels and plasma cell numbers.

These PIs will then be tested using the same rat heart allograft models of acute and chronic rejection as decribed in article 1 to see if they can prolong graft survival, abrogate DSA formation, and ameliorate histological signs of chronic rejection.

1.2. Radioimmunotherapy targeting plasma cells in experimental transplantation:

Radioimmunotherapy refers to the use of a radioactive isotope linked to a monoclonal antibody (mAb) in order to deliver cytotoxic radiation to a target cell and has been used mainly for the treatment of cancers. Two radiolabeled anti-CD20 mAbs, (^{90}Y)-ibritumomab tiuxetan (Zevalin®) and (^{131}I)-tositumomab (Bexxar®) have been approved by FDA for the treatment of follicular non-Hodgkin lymphoma (Ahmed et al, 2010). Other radiolabeled mAbs have been tested in various types of cancer.

Dr Cherel's team at the "Institut de Biologie" in Nantes has used an anti-human CD138 mAb labeled with radioactive bismuth-213 to kill myeloma cells in vitro (Supiot et al, 2002) and to cure multiple myeloma in a mouse xenograft model (article under review). He is currently characterizing an anti-mouse CD138 and will label it with bismuth-213 for further studies in mouse models of cancer. We will cooperate with Dr Cherel's team to test this ^{213}Bi-labeled anti-mouse CD138 mAb in our aforementioned mouse skin graft model to see if it can also decrease the number of non-malignant plasma cells and decrease alloantiboby formation. This study will be a preliminary step in the search for alternative approaches in the treatment of refractory AMR. Of course, the risk to benefit ratio of immunoradiotherapy should be considered to determine if it can be accepted as a potential treatment for a non-malignant disease like AMR.

2. Clinical studies:

2.1. Bortezomib combined with plasmapheresis, IVIg, and methylprednisolone in the treatment of chronic active antibody-mediated rejection in kidney transplant patients:

With the advent of the routine follow-up of DSA and C4d staining of kidney transplant biopsy, chronic AMR is more frequently diagnosed and emerges as an important cause of late graft dysfunction and graft loss. As mentioned above, chronic AMR appeared to be less responsive to bortezomib than acute AMR. In one case report from Necker Hospital, none of the 4 patients with chronic AMR underwent significant reduction in DSA levels after one cycle of bortezomib monotherapy (Sberro-Soussan et al, 2010). One of the explanations may be that in acute AMR, almost all patients are concomitantly treated with other therapies such as corticosteroid, rituximab, IVIg, or plasmapheresis, which may have synergistic effects with bortezomib. Therefore, we will collaborate with Dr Snanoudj et al at Necker Hospital in Paris together with other transplant physicians in Lyon (CENTAURE collaborative network) to design a randomized controlled trial to evaluate the efficacy of a combination therapy using plasmapheresis, IVIg, bortezomib, and methylprednisolone in the treatment of chronic active AMR. Both treated group and control group will undergo an adjustment of maintenance immunosuppression using a triple therapy with tacrolimus, mycophenolate acid (Cellcept or Myfortic), and prednisone at well-defined dosages during the whole period of the study. At the same time, treated group will receive the aforementioned bortezomib-based therapy, whereas control group will receive no further therapy. Primary endpoints will be a reduction in DSA levels of at least 50% and a stabilization of histological lesions of chronic active AMR at 1-year biopsy. 50 patients for each group will be recruited over 2 years. We expected that this study will give us a definite answer to

the question whether bortezomib-based therapy is effective in chronic active AMR.

2.2. Belimumab in the treatment of de novo donor-specific anti-HLA antibody formation in kidney transplant patients:

Belimumab (Benlysta®, Glaxo-SmithKline) is a fully human monoclonal antibody that binds to B-lymphocyte stimulator (BLyS), also known as B cell activation factor of the TNF family (BAFF), thereby inhibits the binding of BAFF to their receptors on B cells. Since BAFF signaling is required for the survival, proliferation, and differentiation of B cells, belimumab effectively decreases autoantibody levels and improves clinical symptoms in lupus patients and has been approved for the treatment of systemic lupus erythematosus. It is reasonable to expect that belimumab can also decrease alloantibody formation after kidney transplantation.

We are currently working with Glaxo-SmithKline to initiate a phase II study to evaluate the efficacy of belimumab in the treatment of de novo donor-specific anti-HLA antibody formation in kidney transplant patients. This will be a randomized control multicenter study involving 30 patients from three participating transplant centers in Nantes, Angers, and Tours and coordinated by Pr Dantal in Nantes. Patients having de novo DSA with MFI levels higher than 1000 by LABScreen® single antigen assays (One Lamda) will undergo an adjustment of maintenance immunosuppression using triple therapy with tacrolimus, mycophenolate acid (Cellcept or Myfortic), and prednisone at well-defined dosages. Then a treated group will receive belimumab for 6 months, whereas control group receive no further treatment. The primary endpoint of study will be a reduction of DSA levels of at least 50% after 6 months of treatment. Secondary endpoints will include histological changes on follow-up biopsies, renal function, and safety profile. If proven effective, belimumab will be a novel approach to the treatment AMR.

We hopefully believe that our extensive studies targeting various pathways leading to alloantibody formation will help to find out an effective therapy for antibody-mediated rejection, one of the major challenges in clinical transplantation.

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PART 2: INTERLEUKIN-7 RECEPTOR BLOCKADE IN ORGAN TRANSPLANTATION

The first part of my thesis dealt with an emerging problem in organ transplantation – antibody-mediated rejection. Although antibodies do have an important role, T cells are the principal driving force behind the pathologic process leading to allograft rejection. In the second part of my thesis, I will explore a novel therapy directed at T cells through inhibition of the interlekin-7 receptor (IL-7R) by a monoclonal antibody. Since interleukin-7 (IL-7) is the main survival factor for T cells, IL-7R blockade will profoundly affect T cell homeostasis in a way that may be potentially beneficial for allograft survival.

INTRODUCTION

1. Interleukin-7/interleukin-7 receptor axis is essential for normal lymphopoiesis:

1.1. Interleukin-7:

The discovery of interleukin-7 and interleukin-7 receptor in the early 1990s is largely attributed to researchers at Immunex Research and Development Corporation, Seattle, WA (now absorbed by Amgen). The murine interleukin-7 (IL-7) was first identified as a 25-kDa glycoprotein that stimulated the proliferation of B-cell precursors in vitro (Namen et al, 1988). Soon thereafter, IL-7 was also found to be involved in T cell differentiation (Conlon et al,1989). The human IL-7 was subsequently cloned and found to have 60% homology in amino acid sequence with murine IL-7 (Goodwin et al, 1989).

Expression of IL-7 mRNA and/or protein has been detected in major organs of the immune/hematopoietic system, most abundantly in the thymus, but also in the bone marrow, spleen, lymph node and fetal liver (Komschlies et al, 1995, Kim et al, 2011). Thymic epithelial cells around the corticomedullary junction of the adult thymus produce the most important amount of IL-7 (Zamisch et al, 2005, Alves et al, 2009). Other cell types that express IL-7 include bone marrow, lymph node and splenic stromal cells (Guimond et al, 2009). Interestingly, some cell types that do not belong to the hematopoietic system also produce IL-7 such as skin keratinocytes (Heufler et al, 1993) and intestinal epithelial cells (Watanabe et al, 1995). On the contrary, both T and B lineage cells, the main cellular targets of IL-7 do not produce IL-7 by themselves.

1.2. Interleukin-7 receptor (IL-7R):

The interleukin-7 receptor is composed of 2 subunits: the interleukin-7 receptor alpha (IL-7Rα) or CD127 and the cytokine receptor common gamma chain (γc), also known as interleukin-2 receptor subunit gamma (IL-2Rγ) or CD132. IL-2Rγ is shared by the receptors of the common gamma-chain cytokines including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (Figure 7), whereas IL-7Rα is a component of not only the IL-7 receptor but also of the thymic stromal lymphopoietin (TSLP) receptor. TSLP is not a member of the common gamma-chain cytokines but has overlapping function with IL-7 (Liu et al, 2007). The TSLP receptor is composed of 2 subunits, the IL-7Rα and the TSLPR, the latter is closely related to γc (Pandey et al, 2000, Park et al, 2000). The human and murine IL-7Rα was cloned in 1990 by the same group that had cloned IL-7 one year earlier (Goodwin et al, 1990). IL-7R is expressed on T cells, pre-B cells

and dendritic cells.

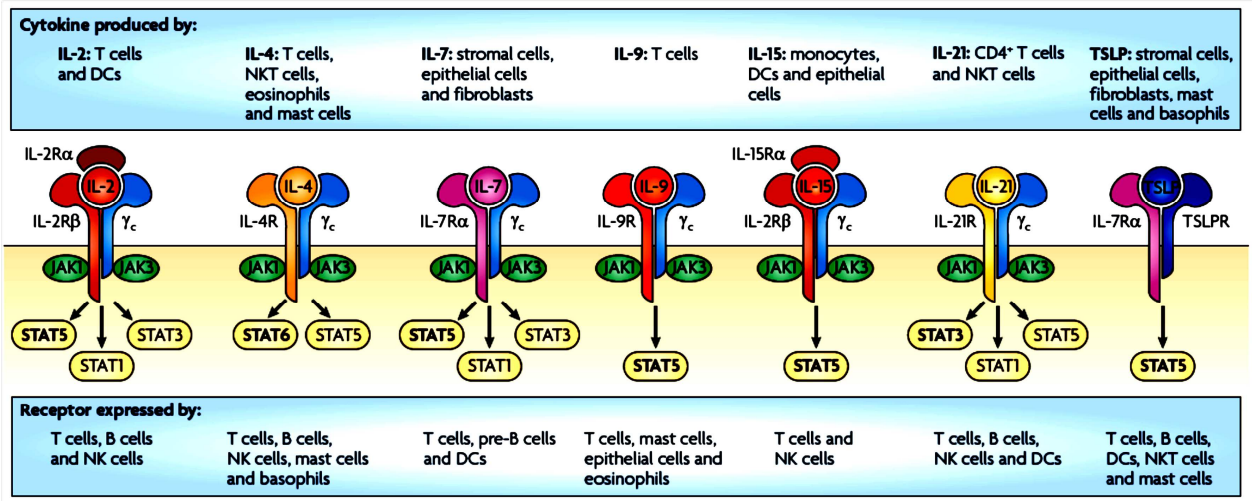


Figure 7: Receptors of the common gamma-chain cytokines and TSLP (*From Rochman et al, Nat Rev Immunol. 2009*). Shown are the receptors for interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15, IL-21 and thymic stromal lymphopoietin (TSLP). IL-2 and IL-15 are the only two of these cytokines to have three receptor chains. The receptors for these two cytokines share the common cytokine receptor γ-chain (γc; also known as IL-2Rγ) and IL-2Rβ, and the receptors for IL-7 and TSLP share IL-7Rα. Of the cytokines shown, only TSLP does not signal through a receptor containing γc. There are three classes of IL-2 receptor that bind IL-2 with low affinity (IL-2Rα alone), intermediate affinity (IL-2Rβ and γc) and high affinity (IL-2Rα, IL-2Rβ and γc); only the high-affinity IL-2 receptor is shown. The receptor for each γc family cytokine activates Janus kinase 1 (JAK1) and JAK3, whereas the receptor for TSLP has been reported to not activate any JAK. The main signal transducer and activator of transcription (STAT) proteins that are activated by these cytokine receptors are shown in bold. STAT5 refers to both STAT5A and STAT5B.

1.3. IL-7/IL-7R interaction leads to activation of the JAK-STAT signaling pathway:

The IL-7Rα and IL-2Rγ subunits do not have enzymatic activities in themselves but are bound to the Janus kinase JAK1 and JAK3, respectively. The binding of IL-7 to IL-7R leads to the transphosphorylation of these two kinases, which in turn phosphorylate signal transducers and activators of transcription (STATs). Phosphorylated STATs translocate into the nucleus where they regulate gene expression (Rochman et al, 2009).

1.4. IL-7 knockout (KO) mice and IL-7R KO mice have impaired lymphopoisis:

In order to study the function of the IL-7/IL-7R system, targeted disruption of the IL-7 or IL-7R genes were performed. IL-7R-deficient mice were first generated in 1994 also by researchers at Immunex (Peschon et al, 1994). IL-7-deficient mice were generated 1 year later by another group (von Freeden-Jeffry et al, 1995). The phenotypes of IL-7 KO mice and IL-7R KO mice were very similar. Gross pathological analyses of those mice did not reveal any nonlymphoid

anomalies and both sexes were fertile. However, they had profoundly reduced thymic, splenic, and lymph node cellularities which affect both T and B cell compartments.

Thymocyte development in IL-7- or IL-7R-deficient mice is blocked at an early development stage. During T cell development, T cell progenitors migrate from the bone marrow to the thymus to undergo expansion and T-cell receptor (TCR) rearrangement. These cells sequentially progress through the CD4-CD8- double-negative to the CD4+CD8+ double-positive stages to ultimately become CD4+CD8- or CD4-CD8+ single-positive mature T cells. In normal thymus, about 80 percent of thymocytes are double-positive, about 10 percent are single-positive, and less than 5 percent are double-negative cells. On the contrary, thymocytes from IL-7R-/- mice are composed primarily of CD4-CD8- cells, demonstrating that T-cell development in those mice is blocked at the double-negative stage.

Spleens from IL-7- or IL-7R-deficient mice also had greatly reduced numbers of total cells as well as CD4+ T cells, CD8+ T cells, and B cells compared to age-matched control mice. The total bone marrow cellularity was not different from control mice, but B lineage cells (B220+) were greatly reduced and mature B cells (B220+ IgM+) were virtually absent.

Interestingly γδ T cells was more severely reduced than αβ T cells, whereas natural killer (NK) cell number were not affected in either IL-7- or IL-7R-deficient mice (Moore et al, 1996, Maki et al, 1996, He et al, 1996).

Therefore, IL-7 or IL-7R knockout in mice leads to a T–B–NK+ severe combined immunodeficiency (SCID) phenotype. On the other hand, targeted deletion of the IL-2R common γ-chain in mice produced a T–B–NK– SCID phenotype in which T and B cell number were 10-fold reduced and NK cells were virtually absent (DiSanto et al, 1995).

1.5. Monoclonal antibody to IL-7 or IL-7R inhibits murine lymphopoiesis:

Another approach to study the function of the IL-7/IL-7R system is to produce blocking monoclonal antibodies (mAb) to IL-7 or IL-7R and inject these mAb to normal mice.

Grabstein et al at Immunex generated a mouse anti-human and mouse IL-7 mAb (clone M25) and injected that antibody to mouse for up to 12 weeks (Grabstein et al, 1993, Bhatia et al, 1995). They found a striking reduction in the number of B lineage cells in the bone marrow after 10 days of antibody injection, although the total bone marrow cells were not decreased. Thymic cellularity also markly decreased and the reduction in total cell number as well as in each thymic subset was proportionate to the duration of treatment, with an 80% and 99% reduction after 2 weeks and 12 weeks of antibody injection, respectively.

In parallel, Sudo et al in Japan generated and injected a rat anti-mouse IL-7Rα mAb (clone A7R34) to mouse for 2 weeks and also found a marked reduction in all thymic subsets in the thymus and B lineage cells in the bone marrow. The total cell number as well as the absolute number of CD4+ T cells, CD8+ T cells, B cells were also reduced in the spleen and lymph node of treated mice (Sudo et al, 1993).

In summary, injection of mAb to IL-7 or IL-7R to wild-type mice can induce lymphopenia similar to that seen with IL-7 or IL-7Rα gene knockout. As the IL-7Rα subunit is common to both the IL-7 receptor and TSLP receptor, we can speculate that IL-7Rα blockade or gene knockout produce a more severe phenotype than IL-7 blockade or gene knockout. The reason is that the former approach blocks both IL-7 and TSLP whereas the latter blocks only IL-7 but not TSLP, which may compensate for the absence of IL-7. However, this turned out not to be the case. In fact, targeting IL-7 or IL-7Rα gave essentially the same effect, which implies that TSLP cannot replace IL-7. Together, those experiments demonstrated that IL-7 in a non-redundant cytokine critical for normal lymphopoiesis.

1.6. Mutation of the IL-7R in human causes severe combined immunodeficiency (SCID):

Severe combined immunodeficiency (SCID) comprises a heterogeneous group of diseases characterized by a block in T cell development or function, variably associated with defects in B or NK cells (Fischer et al, 2005). At least 10 different genetic defects resulting in SCID have been fully characterized. The most common form of SCID is X-linked SCID (XSCID), accounting for at least 50% of the cases, and is caused by mutations in the gene encoding for the IL-2R common γ chain (IL-2Rγc). Patients with XSCID have a T–B+NK– immunological phenotype, which is characterized by a severely reduced number of T cells and NK cells, but a normal to increased number circulating B cells. Because Janus kinase 3 (JAK3) binds to the intracellular portion of IL-2Rγc and initiates the JAK/STAT signaling pathway, mutations of JAK3, though rarer than IL-2Rγc mutations, cause autosomal SCID with the same T–B+NK– phenotype as that of X-link SCID (Macchi et al, 1995).

Later on, it was discovered that mutation of the IL-7Rα also caused SCID, but these patients had only severe T cell lymphopenia, whereas NK cell and B cell number were unaffected (T–B+NK+ phenotype) (Puel et al, 1998, Giliani et al, 2005). Thus in human as well as in mouse, IL-7 is the cytokine responsible for T cell lymphopoiesis among the common gamma chain cytokines. But unlike in mouse, B cell lymphopoiesis in human is not dependent on IL-7 despite the fact that human bone marrow lymphoid progenitors do express IL-7R (Ryan et al, 1997). Perhaps other

hematopoietic growth factors can compensate for the absence of IL-7/IL-7R signaling in patients with IL-7Rα mutation.

2. IL-7/IL-7R system in T cell homeostasis:

The size of the peripheral T cell pool is constant despite the production of new T cells by the thymus and the expansion of existing cells after their stimulation by cognate antigen during immune response. This is achieved through specific homeostatic mechanisms that regulate T cell proliferation and survival. IL-7 has been shown to be essential for the homeostasis of both naïve and memory CD4+ and CD8+ T cells.

Schluns et al transferred CFSE-labeled OT-I TCR transgenic CD8+ naïve T cells which recognize an ovalbumin (OVA) peptide in the context of H-2K^{b} to either wild-type or IL-7-/- mice, both of them having undergone sublethal irradiation. While most of the transferred cells proliferated in wild-type mice, only one-third of them divided in IL-7-/- mice. Similarly, OT-I cells as well as polyclonal naïve CD4+CD44^{lo} and CD8+CD44^{lo} T cells proliferated in RAG-/- mice, but not in IL-7-/-RAG-/- mice (Schluns et al, 2000). This study and another study (Tan et al, 2001) confirmed that IL-7 is necessary for the homeostatic proliferation of naïve CD4+ and CD8+ T cells.

In order to study the generation of memory CD8+ T cells, a mixture containing equal numbers of OT-I cells and OT-I IL-7R-/- cells (from OT-I mice lacking IL-7R) was transferred to normal mice which were then infected with VSV-OVA in order to stimulate the immune response. Although both OT-I and OT-I IL-7R-/- cells underwent substantial early expansion, only normal OT-I cells were able to survive in the long-term. Thus IL-7 is required for the survival of memory CD8+ T cells (Schluns et al, 2000). The same is true for memory CD4+ T cells. CD4+CD44^{hi} T cells transferred to RAG-/- mice underwent extensive proliferation during the first week, regardless of whether hosts had IL-7 or not. However, after 2 weeks, donor cell recovery was profoundly reduced in the absence of IL-7 or in the presence of IL-7R blocking antibodies (Tan et al, 2002, Seddon et al, 2003, Kondrack et al, 2003). Therefore, IL-7 is also necessary for the survival of CD4+ memory cells.

3. Regulatory T cells express low level of IL-7Rα (CD127):

The best-characterized regulatory T cells in human and in mouse are CD3+CD4+CD25+ FOXP3+. Although FOXP3 is the most important marker for regulatory T cells, it is a transcription factor located in the nucleus, and the staining for FOXP3 requires cell membrane permeabilization which renders subsequent functional studies and in vitro expansion of these

cells impossible. As a consequence, efforts have been made to look for a cell surface marker which can be a substitute for FOXP3. Liu et al at Bluestone's lab in San Franscisco performed gene expression microarray, flow cytometry, and functional assays and observed that IL-7R or CD127 was down-regulated on all human T cells after activation. Whereas CD127 was reexpressed on the majority of effector and memory cells, FOXP3+ T cells remained CD127 low or negative (Liu et al, 2006). Similar results were described in the study of another group published in the same issue of Journal of Experimental Medicine (Seddiki et al, 2006). FOXP3 expression and $CD127^{lo}$ phenotype were highly correlated within the CD4+CD25+ T cell population and $CD4+CD25+CD127^{lo}$ T cells had suppressive activity in vitro whereas $CD4+CD25+CD127^{hi}$ cells did not (Michel et al, 2008). Therefore, staining for CD127 is now routinely used to isolate $CD3+CD4+CD25+CD127^{lo}$ regulatory T cells for functional studies and ex vivo expansion for therapeutic purposes (Putnam et al, 2009).

4. IL-7/IL-7R axis as a therapeutic target:

4.1. Recombinant human IL-7 is being developed for treating lymphopenic patients:

Given the critical role of IL-7 in lymphopoiesis, IL-7 appears to be an ideal drug to increase the lymphocyte count in immunodeficient patients secondary to severe lymphopenia, including HIV-AIDS patients, patients undergoing chemotherapy for cancer, and patients with idiopathic CD4+ lymphocytopenia. A recombinant human IL-7 (rhIL-7) is currently developed by Cytheris, a French biotech and pharmaceutical company.

Two phase I clinical trials in patients with refractory or metastatic cancers showed that 2 weeks of rhIL-7 therapy increased the absolute count of CD4+ and CD8+ T cells up to 2 and 4 folds, respectively (Sportès et al, 2008, Rosenberg et al, 2006). CD8+ increased more strongly than CD4+ T cells and naïve T cells increased more than memory T cells. Preferential expansion of naïve subsets leaded to increased TCR repertoire diversity. On the other hand, the relative proportion of CD4+FOXP3+ regulatory T cells to total CD4+ cells decreased with rhIL-7 treatment. Finally, peripheral B cell numbers were unchanged after rhIL-7 therapy, confirming the limited role of IL-7 in human B lymphopoiesis.

A phase I/IIa clinical trial was carried out on HIV-infected patients with CD4+ T cell count from 100 to 400/µl and HIV RNA levels less than 50 copies/ml, all of them were on combined antiretroviral therapy. Patients were given subcutaneous rhIL-7 every other day for a total of 8 doses over 16 days. This treatment leaded to a peak increase in the CD4+ T cell counts to 3 folds the pretreatment levels at day 21. CD4+ T cells counts then decreased progressively over time, but were still higher than the baseline levels at 48 weeks after treatment (Levy et al, 2009). The

result was encouraging and suggested that intermittent rhIL-7 could be used together with combined antiretroviral therapy to facilitate immune restoration in patients with HIV infection. A phase II clinical trial in HIV-patients is underway.

4.2. Inhibition of IL-7/IL-7R by monoclonal antibodies has been tested in some murine models of autoimmune diseases or transplantation:

Although the concept of using IL-7 to stimulate lymphopoiesis has been widely recognized and clinical trials are being carried out, the potential of using antibodies to IL-7 or IL-7R as an immunosuppressive therapy for autoimmune diseases and transplantation has received much less attention. However, a few recent studies using IL-7/IL-7R blockade in murine models gave promising results.

4.2.1. *Autoimmune hemolytic anemia:*

Hoyer et al used IL-2 knockout mice as a model autoimmune hemolytic anemia (AIHA) (Hoyer et al, 2007). AIHA is a group of diseases in which red blood cells are attacked by autoantibodies leading to hemolysis. Patients usually present with anemia, jaundice, and splenomegaly. AIHA can be primary, affecting only the red blood cells, or secondary, associated with other diseases such as lupus, infection, or malignancy. In IL-2 KO mice, the absence of IL-2 causes a lack of regulatory T cells which in turn leads to uncontrolled activation of CD4+ T cells, formation of autoantibodies, and breakdown of self-tolerance. IL-2 KO mice develop AIHA and lymphoproliferative diseases at 3-4 weeks of age and die a few weeks later if left untreated. IL-7R blockade by an anti-IL-7R monoclonal antibody (clone A7R34) in these mice prolonged survival, reduced anti-erythrocyte autoantibody titers, and ameliorated anemia.

4.2.2. *Chronic colitis:*

As mentioned in the previous section, IL-7 is also produced by intestinal epithelial cells. Moreover, cryptopatches, small aggregates of lymphoid cells found in the intestinal lamina propria contain clusters of IL-7R+c-kit+ lympho-hemopoietic progenitors which are IL-7 dependent (Kanamori et al, 1996). The selective expression of IL-7 in enterocytes can restore the development of TCR-γδ intraepithelial lymphocytes as well as cryptopatches and Peyer's patches in the intestine of IL-7 deficient mice (Laky et al, 2000). Thus, the IL-7/IL-7R system plays a crucial role in the organization of mucosal lymphoid tissues and in the regulation of the normal immune response in the gut. Overexpression of IL-7 in IL-7 transgenic mice caused chronic colitis with remittent intestinal bleeding, histological examination of the colon in these mice revealed pathologic lesions resembling those of human ulcerative colitis (Watanabe et al, 1998). On the contrary, the use of a toxin-conjugated anti-IL-7R monoclonal antibody to

eliminate IL-7R^{high} T cells ameliorated colitis in a model of chronic colitis in TCRα-/- mice (Yamazaki et al, 2003).

4.2.3. *Experimental autoimmune encephalomyelitis (EAE):*

Experimental autoimmune encephalomyelitis is an animal model for human multiple sclerosis. Liu et al demonstrated that monoclonal antibody blocking IL-7 or IL-7R given at the onset of EAE markedly reduced disease severity accompanied by decreased CNS inflammation and demyelination. On the contrary, IL-7 administration at the onset of EAE exacerbated disease severity associated with an increase in the number of effector T_H17 cells in the lymph nodes and in the spinal cord (Liu et al, 2010). They also showed that IL-7 was crucial for the survival and expansion of murine and human T_H17 cells and IL-7R antagonism induced apoptosis of T_H17 cells. The study suggests that IL-7/IL-7R blockade may be beneficial for the treatment of multiple sclerosis, an autoimmune disease in which T_H17 cells play an important role.

4.2.4. *Graft-versus-host disease (GVHD) in experimental bone marrow transplantation (BMT):*

The IL-7/IL-7R signaling is a double-edge sword in bone marrow transplantation (BMT). BMT is follow by a period of profound immune deficiency, during which new T lymphocytes are generated from either hematopoietic stem cells or immature thymic progenitors, this process is IL-7-dependent. Bolotin et al gave recombinant IL-7 to irradiated C57BL/6 mice that had received syngeneic BMT. They found that transplanted mice treated with IL-7 had normalization of thymic cellularity, normal proportion of thymic subsets and TCR diversity, normal numbers of peripheral CD4+ T cells, and improved T and B cell functions. On the contrary, transplanted mice that had not received IL-7 had profound thymic hypoplasia, increased proportion of immature thymocytes, decreased numbers of peripheral T cells, and impaired T and B cell functions. They concluded that IL-7 promoted thymic reconstitution in BMT and may be useful in preventing post-BMT immunodeficiency (Bolotin et al, 1996).

However another study by the same group showed that IL-7KO mice which received IL-7 following allogeneic BMT had significantly increased rate of GVHD-related mortality and morbidity compared to IL-7KO BMT recipients which did not received IL-7. Therefore IL-7 is an important factor in the development of GVHD, presumably by supporting the survival and proliferation of alloreactive donor-derived T cells in the recipients (Chung et al, 2008). In other to modulate the IL-7/IL-7R signaling, they gave small doses of an anti-IL-7Rα monoclonal antibody for 4 weeks to irradiated C57BL/6 mice which had received allogeneic BMT and found that this treatment significantly reduced the rate of GVHD-related mortality and morbidity

(Chung et al, 2007). Although anti-IL-7R-treated mice had fewer donor CD4+ and CD8+ T cells at day 30 post-transplantation, they had better long-term thymic and immune function. The authors concluded that IL-7R blockade resulted in elimination of alloreactive T cells, prevention of GVHD, improvement of donor T-cell reconstitution, and might be useful in clinical BMT.

4.2.5. *Anti-IL-7 antibody in murine cardiac transplantation:*

After a comprehensive review of the literature (Racape et al, 2009), we found only one study in which IL-7 antagonism was tested in the setting of organ transplantation (Wang et al, 2006). Using a murine cardiac allograft model, Wang et al found that anti-IL-7 antibody alone did not prolong heart graft survival. However, anti-IL-7 antibody combined with CD40/CD40L costimulation blockade significantly prolonged graft survival compared to CD40/CD40L costimulation blockade alone. The authors showed that the combination of anti-IL-7 and CD40/CD40L blockade suppressed the generation of allospecific memory CD8+ T cells but did not induce CD4+CD25+FOXP3+ regulatory T cells.

In our study, we demonstrated that IL-7R blockade by an anti-mouse IL-7Rα mAb not only decreased the number of almost all T cell subsets but also increased regulatory T cell frequency. Using a model of murine islet transplantation, we showed that anti-IL-7Rα alone could not prolong graft survival when given at the time of transplantation, but could induce long-term graft survival if started 3 weeks before graft, giving the time necessary for the reduction of T cell number. We then demonstrated that the use of IL-7R blockade following a T cell depletion therapy by a combination of anti-CD4 and anti-CD8 mAbs delayed T cell reconstitution and decreased memory T cell numbers, and as a consequence, prolonged graft survival in a stringent skin allograft model in mice. Together, we showed that IL-7R blockade, especially when combined with a T cell depletion therapy is a clinically relevant approach that can be used to reinforce immunosuppression and inhibit the increase in memory T cells.

ARTICLE IN PREPARATION

Interleukin-7 receptor blockade decreases memory T cell numbers and prolong mouse skin graft survival after T cell depletion therapy

INTRODUCTION

T cell depletion is one of the most potent immunosuppressive therapies; it is used in organ transplantation either for the treatment of severe acute rejection or as induction therapy. The most widely used T-cell depleting antibodies in clinical transplantation are polyclonal anti-thymocyte globulin (ATG), anti-CD52 monoclonal antibody (alemtuzumab), and to a lesser extent, anti-CD3 murine monoclonal antibody (mAb). The use of induction therapies are increasing from 30 to 75 percent of kidney transplantation over a decade (1), with depletional therapy being the most commonly used. Initially, the two most common purposes of depletional induction therapy are: 1) to reinforce immunosuppression in the presence of high-risk factors for rejection such as pre-transplant high panel reactive antibodies (PRA) with or without donor-specific antibodies, prolonged cold ischemia time, or kidneys from extended criteria donors; 2) to facilitate the reduction of maintenance immunosuppression with calcineurin inhibitor (CNI)-free regimens, corticosteroid-free regimens, or CNI or sirolimus monotherapy. Nowadays, depletion therapy is used more widely in kidney transplantation at the discretion of transplant physicians, especially in the United States.

Depletion of T cells at the time of organ transplantation was once believed to be a treatment that could induce tolerance, since a short course of T cell depleting antibodies were able to induce long-term graft survival in experimental models of islet or heart transplantation in mice (2,3). However, it was soon recognized that T cell reconstitution after depletion therapy leads to the predominance of memory T cells, which occurs in rodent and non-human primate models (4,5) as well as in human organ transplantation (6). Memory T cells are more potent than naïve T cell in mediating graft rejection. Mice undergoing T cell homeostatic proliferation following depletion therapy rejected cardiac allograft despite costimulatory blockade by CTLA-4Ig, a treatment capable of inducing tolerance in non-depleted mice (7). In human, kidney transplant recipients who had received T-cell depletion therapy by high-dose alemtuzumab but no maintenance immunosuppression uniformly developed acute rejection within the first month after transplantation, a period during which there was a severe T-cell lymphopenia but a predominance of effector-memory T cells accounting for more than 80 percent of T cells (8).

T cell reconstitution after depletion therapy comprises both de novo thymopoiesis and homeostatic proliferation of remaining T cells and both processes are interleukin-7 (IL-7)

dependent. IL-7 signals through the IL-7 receptor (IL-7R) which is composed of two chains, the gamma chain is common for the receptor of interleukin 2, 4, 7, 9, 15, and 21, whereas the alpha chain (IL-7Rα, or CD127) is used by IL-7R and thymic stromal lymphopoietin receptor (TSLP-R) (reviewed in 9). IL-7 plays an essential, non-redundant role in lymphopoiesis since IL-7 or IL-7 receptor (IL-7R) knock-out mice have severe T and B cell lymphopenia (10,11) and infants with IL-7R mutations have severe T cell lymphopenia necessitating bone marrow transplantation for survival (12). IL-7 has also been shown to be necessary for the homeostatic proliferation of both naïve and memory CD4+ and CD8+ T cells in lymphopenic conditions as seen in recombinant activation gene deficient (RAG-/-) mice or after irradiation (13,14). Therefore, in the setting of organ transplantation, blockade of the IL-7/IL-7R signaling is expected to prolong the effects of T cell depletion therapy and inhibit the increase in memory T cell numbers which is a potential cause of graft rejection.

In this study, we demonstrated that IL-7R blockade by an anti-mouse IL-7Rα mAb not only decreased the number of almost all T cell subsets but also increased regulatory T cell frequency. Using a model of murine islet transplantation, we showed that anti-IL-7Rα alone could not prolong graft survival when given at the time of transplantation, but could induce long-term graft survival if started 3 weeks before graft, giving the time necessary for the reduction of T cell number. We then demonstrated that the use of IL-7R blockade following a T cell depletion therapy by a combination of anti-CD4 and anti-CD8 mAbs delayed T cell reconstitution and decreased memory T cell numbers, and as a consequence, prolonged graft survival in a stringent skin allograft model in mice. Together, we showed that IL-7R blockade, especially when combined with a T cell depletion therapy is a clinically relevant approach that can be used to reinforce immunosuppression and inhibit the increase in memory T cells.

MATERIALS AND METHODS:

Mice and allograft models:

Experiments were performed using seven to nine-week old male C57BL/6 (H-2b) and BALB/c (H-2d) mice, which were purchased from Centre d'Elevage Janvier (Le Genest-Saint-Isle, France). Islet transplantation was performed as previously described (15). Briefly, Balb/c recipients received one intra-peritoneal injection of 250 mg/kg of streptozotocin to induce diabetes which is defined as non-fasting blood glucose greater than 16.6 mmol/l (300 mg/dl). Pancreases were harvested from C57BL/6 donor mice after injection of collagenase solution into the common bile duct and islets purified by Ficoll gradient centrifugation. Five to ten days after streptozotocin injection, each diabetic Balb/c mouse received about 500 islets under the renal

capsule. Graft was functioning when blood glucose decreased to less than 10 mmol/l (180 mg/dl) and graft rejection was defined as blood glucose increase again to greater than 16.6 mmol/l. Concerning skin allograft model, tail skin from C57BL/6 donors was grafted to the dorsal trunk of BALB/c recipients. Rejection was defined as graft necrosis of more than 70 percent of the surface.

Monoclonal antibody (mAb) preparation:

Rat anti-mouse IL-7Rα (clone A7R34) hybridoma cell-line was a generous gift from Dr Shin-Ichi Nishikawa at Riken Center for Developmental Biology, Kobe, Japan. Rat anti-mouse CD4 (clone GK1.5) and CD8 (clone 2.43) hybridoma cell-lines were purchase from American Type Culture Collection (ATCC) via LGC Standards (Molsheim, France). Hybridomas were cultured in our lab following the providers' instructions, using ultralow-IgG fetal bovine serum (Gibco Invitrogen). Hybridoma supernatants were concentrated either by ammonium sulfate precipitation or by ultrafiltration and purified using HiTrap^{TM} Protein G HP columns (GE Healthcare). A rat anti-dinitrophenol (DNP) mAb (clone LO-DNP-61) was purchased from the Laboratory of Experimental Surgery, Universite Catholique de Louvain (Brussels, Belgium) and used as rat IgG2a isotype control for the anti-IL-7Rα mAb.

Lymphocyte phenotyping by flow cytometry:

Mice were euthanasized under general anesthesia, blood was drawn from cardiac puncture, spleen, thymus, mesenteric lymph nodes and axillary lymph nodes ipsilateral to the skin graft were harvested. Whole blood lymphocyte phenotyping was performed using BD TruCOUNT tubes (BD Biosciences) according to the manufacturer's instruction. Cells from spleen, thymus, and lymph nodes were isolated using BD Falcon Cell Strainers (BD Biosciences). The following anti-mouse antibodies were used for flow cytometry: anti-CD3, CD4, CD8, CD19, CD44, CD45-2, CD45R (B220), CD62L, CD127, Gr-1 (Ly-6G and Ly-6C), and FOXP3, all were purchased from BD Biosciences except anti-FOXP3 from eBiosciences and anti-CD127 (clone A7R34) prepared in our lab. Flow cytometry was performed using a BD LSRII flow cytometer.

Measurement of donor-specific antibodies (DSA):

Blood of Balb/c recipients were obtained either by retroorbital sinus bleeding or by cardiac puncture in the case of sacrifice and centrifuged for sera, which were stored at -20°C for later use. Sera of recipients were diluted at 1/20 and incubated for 20 minutes with cells freshly isolated from mesenteric lymph nodes of a donor-type C57BL/6 mouse. Cells were washed twice, incubated for 15 minutes with an FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Europe Ltd) and hamster anti-mouse CD3 (BD Biosciences), washed twice,

and analysed by flow cytometry. DSA levels were reported as mean fluorescence intensity (MFI).

Statistics:

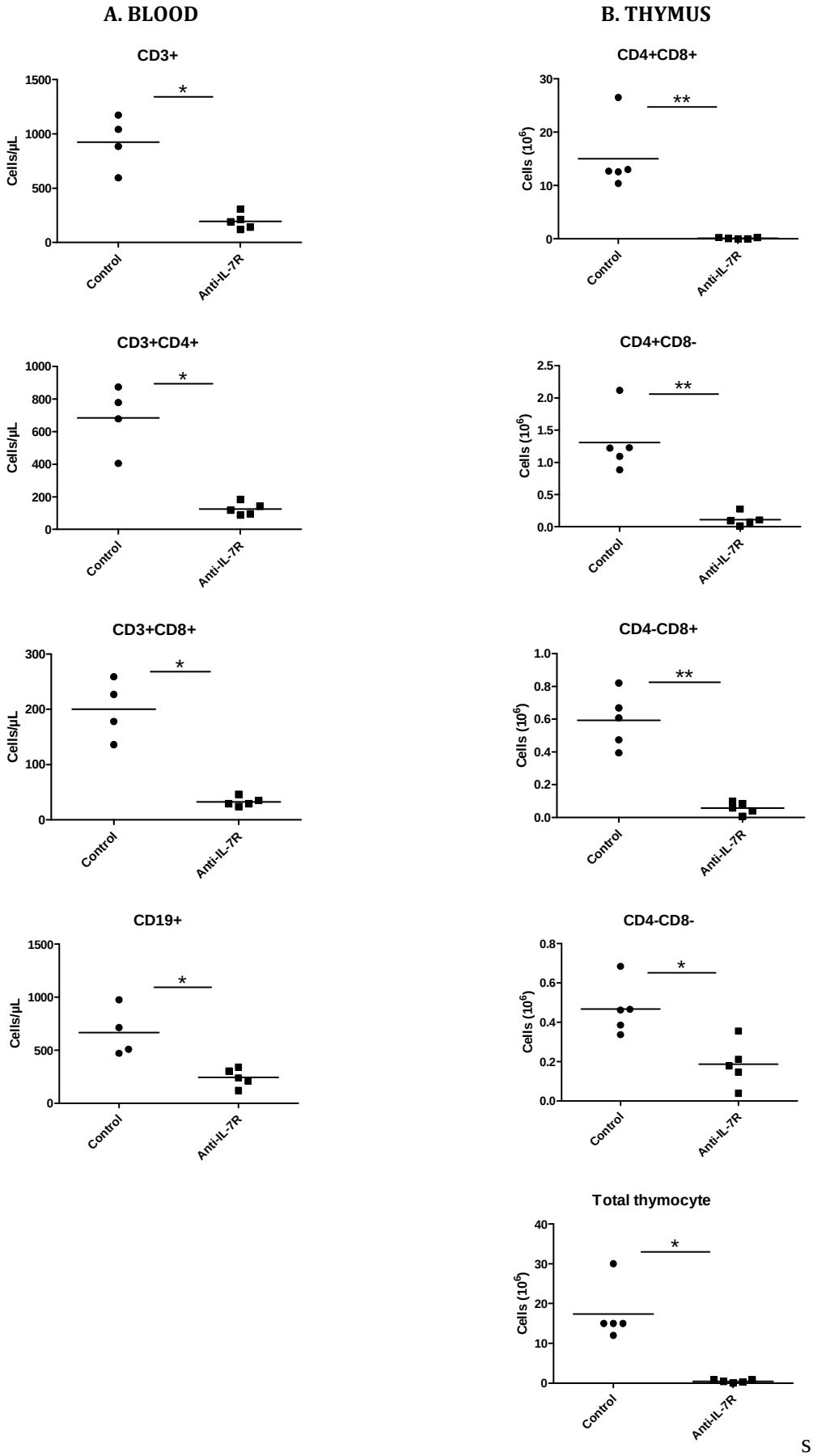
Statistics were done using the GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). Data were presented as mean ± SD. Survivals were compared using the log-rank test and other values were compared using the Mann Whitney test.

RESULTS

Anti-IL-7Rα antibody reduced lymphocyte number and increased regulatory T cell frequency:

Eight-week old male Balb/c mice received either anti-IL-7Rα mAb or isotype control 400 µg IP every other day for 3 weeks and were sacrificed (n = 5 for each group). Anti-IL-7R-treated mice had significantly lower number of T cells, CD4+ T cells, CD8+ T cells, and B cells in the blood, lymph nodes, and spleen than those of control mice (Figure 1A, C, and D and Table 1 A, C, D). In the thymus, total thymocytes as well as CD4+CD8+ double positive, CD4+CD8- or CD4-CD8+ single positive, and CD4-CD8- double negative cell numbers were all profoundly decreased in anti-IL-7R-treated mice (Figure 1B and Table 1B). Interestingly, the percentages of regulatory T cells (defined as CD3+CD4+CD25+FOXP3+) among CD4+ T cells in the spleen and in the lymph nodes of treated mice were increased compared to those of control mice (20.3 ± 3.6 vs 11.6 ± 1.3 and 21.4 ± 5.5 vs 12.5 ± 1.8 percent, respectively, all $p<0.01$), although the absolute number of Treg were also reduced due to the decrease in total cell numbers in these lymphoid organs (Figure 1E).

Figure 1: Anti-IL-7R antibody reduced all lymphocyte subset numbers and increased regulatory T cell frequencies.



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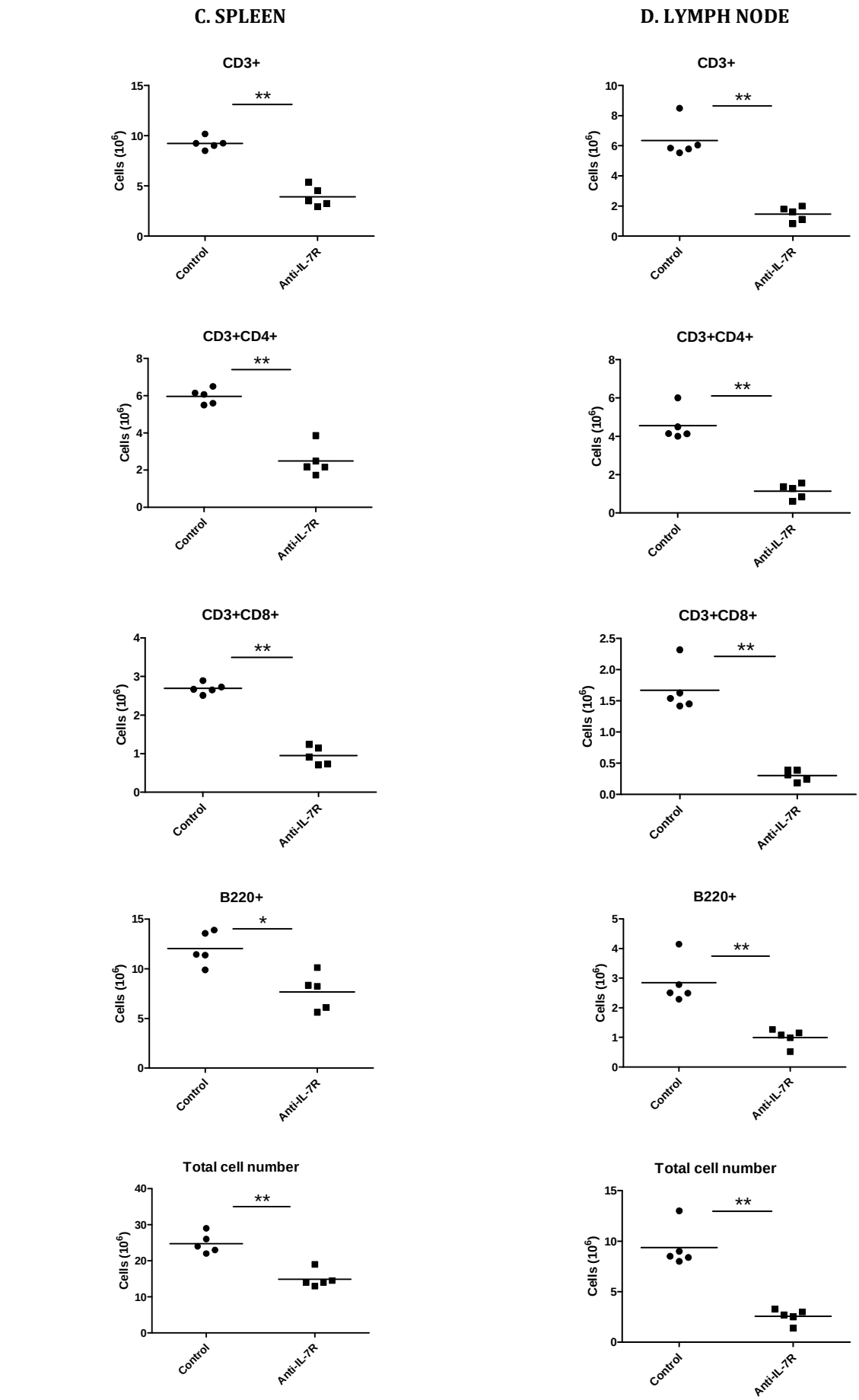

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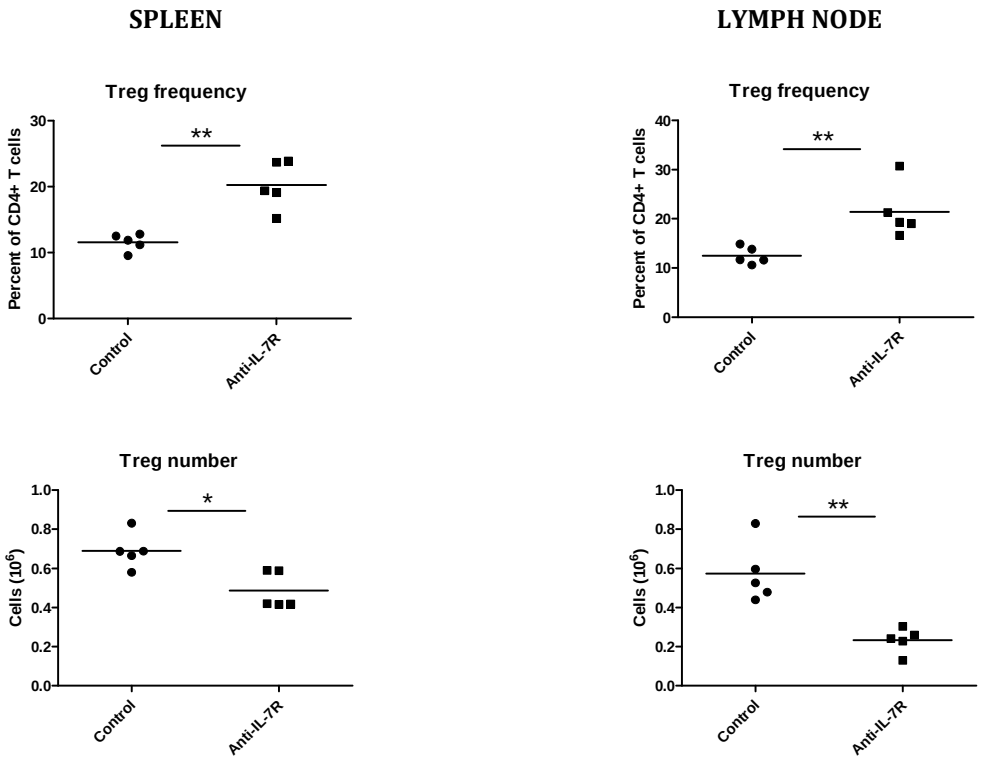
E. Regulatory T cells:



Legend to figure 1 and table 1: Eight-week old male Balb/c mice received either anti-IL-7R mAb or isotype control 400 µg IP every other day for 3 weeks (n = 5 for each group). Anti-IL-7R-treated mice had significantly lower number of T cells, CD4+ T cells, CD8+ T cells, and B cells in the blood (A), lymph nodes (C), and spleen (D) than those of control mice. In the thymus, total thymocytes as well as CD4+CD8+ double positive, CD4+CD8- or CD4-CD8+ single positive, and CD4-CD8- double negative cell numbers were all profoundly decreased in anti-IL-7R-treated mice (B). The percentages of CD3+CD4+CD25+FOXP3+ regulatory T cells in the spleen and in the lymph nodes of treated mice were increased compared to those of control mice, although the absolute number of Treg were also reduced due to the decrease in total cell numbers in these lymphoid organs (E). (*: $p<0.05$; **: $p<0.01$)

Table 1: Anti-IL-7Rα antibody decreased lymphocyte numbers.

A. Blood:

Leukocyte subsets (cells/µl)	Control group (n=4)	Treated group (n=5)	P value
CD3+	924 ± 248.3	194.6 ± 73.8	<0.05
CD3+ CD4+	684.3 ± 202.5	125.2 ± 39.3	<0.05
CD3+ CD8+	21.7 ± 1.1	17.3 ± 2.7	<0.05
CD19+	667.3 ± 231.6	242.8 ± 86	<0.05

B. Thymus:

Cell subsets (10^6 cells)	Control group (n=5)	Treated group (n=5)	P value
Total thymocytes	17.40 ± 7.16	0.47 ± 0.33	<0.05
CD4+ CD8+ double positive	15.03 ± 6.50	0.12 ± 0.12	<0.01
CD4+ CD8- single positive	1.31 ± 0.47	0.11 ± 0.10	<0.01
CD4- CD8+ single positive	0.59 ± 0.17	0.06 ± 0.04	<0.01
CD4- CD8- double negative	0.47 ± 0.13	0.19 ± 0.11	<0.05

C. Spleen:

Leukocyte subsets (10^6 cells)	Control group (n=5)	Treated group (n=5)	P value
Total cells	24.80 ± 2.78	14.9 ± 2.36	<0.01
CD3+	9.24 ± 0.60	3.92 ± 1.01	<0.01
CD3+ CD4+	5.96 ± 0.41	2.49 ± 0.82	<0.01
CD3+ CD8+	2.69 ± 0.14	0.95 ± 0.24	<0.01
B220+	12.04 ± 1.67	7.68 ± 1.84	<0.05
CD3+CD62L^{lo}	1.72 ± 0.15	0.86 ± 0.25	<0.01
CD3+CD62L^{lo}/CD3+ (%)	19.96 ± 0.92	29.08 ± 4.01	<0.01
Treg (CD3+CD4+CD25+FOXP3+)	0.69 ± 0.09	0.49 ± 0.09	<0.05
Treg/CD3+CD4+ (%)	11.59 ± 1.29	20.26 ± 3.63	<0.01

D. Lymph node:

Leukocyte subsets (10^6 cells)	Control group (n=5)	Treated group (n=5)	P value
Total cells	9.38 ± 2.06	2.58 ± 0.73	<0.01
CD3+	6.34 ± 1.21	1.47 ± 0.49	<0.01
CD3+ CD4+	4.56 ± 0.83	1.14 ± 0.40	<0.01
CD3+ CD8+	1.67 ± 0.37	0.30 ± 0.09	<0.01
B220+	2.84 ± 0.75	1.0 ± 0.29	<0.01
CD3+CD62L^{lo}	1.37 ± 0.35	0.43 ± 0.16	<0.01
CD3+CD62L^{lo}/CD3+ (%)	22.62 ± 2.75	33.42 ± 2.14	<0.01
Treg (CD3+CD4+CD25+FOXP3+)	0.57 ± 0.15	0.23 ± 0.06	<0.01
Treg/CD3+CD4+ (%)	12.52 ± 1.77	21.38 ± 5.47	<0.01

Anti-IL-7Rα antibody induced long-term islet allograft survival when started 3 weeks before graft:

In order to test if the decrease in lymphocyte number and the increase in Treg percentage by IL-7R blockade could prolong graft survival, we used a murine islet transplantation model in which streptozotocin-induced diabetic Balb/c recipients received islet grafts from C57BL/6 donors. Untreated mice rejected their islet grafts in a median of 21 days (range: 14-34 days) (n=16). Anti-IL-7R-treated mice that received anti-IL-7Rα mAb 400 µg IP every other day from the day

of islet grafts until rejection (n=5) did not have significantly prolonged graft survival compared to untreated mice as they rejected their grafts in a median of 29 days (range: 21-35 days) (p=0.16). The anti-IL-7Rα mAb used in our study can effectively block the IL-7/IL-7R interaction (16) but is unlikely to deplete IL-7R+ cells. Consequently, as shown in this study as well as in previous studies (16), this antibody must be given for at least 2-3 weeks before a significant reduction in lymphocyte numbers is seen. Given this fact, we then gave recipient mice anti-IL-7Rα mAb 400 µg IP qod 3 weeks before islet grafts and continued the treatment until graft rejection or post-transplant day (PTD) 90. With this treatment, 5 of 6 mice had indefinite graft survival over 180 days (p=0.0002 compared to untreated mice) (Figure 2A). Donor-specific antibody (DSA) measurement at PTD160 in mice with long-term graft survival showed minimal antibody levels, whereas there was a strong DSA response in untreated mice after graft rejection (n=5 for each group, p<0.01) (Figure 2B). Subsequent left nephrectomy in long-survival mice to remove the islet grafts led to severe increase in blood glucose, confirming that the maintenance of normoglycemia was due to graft function (data not shown).

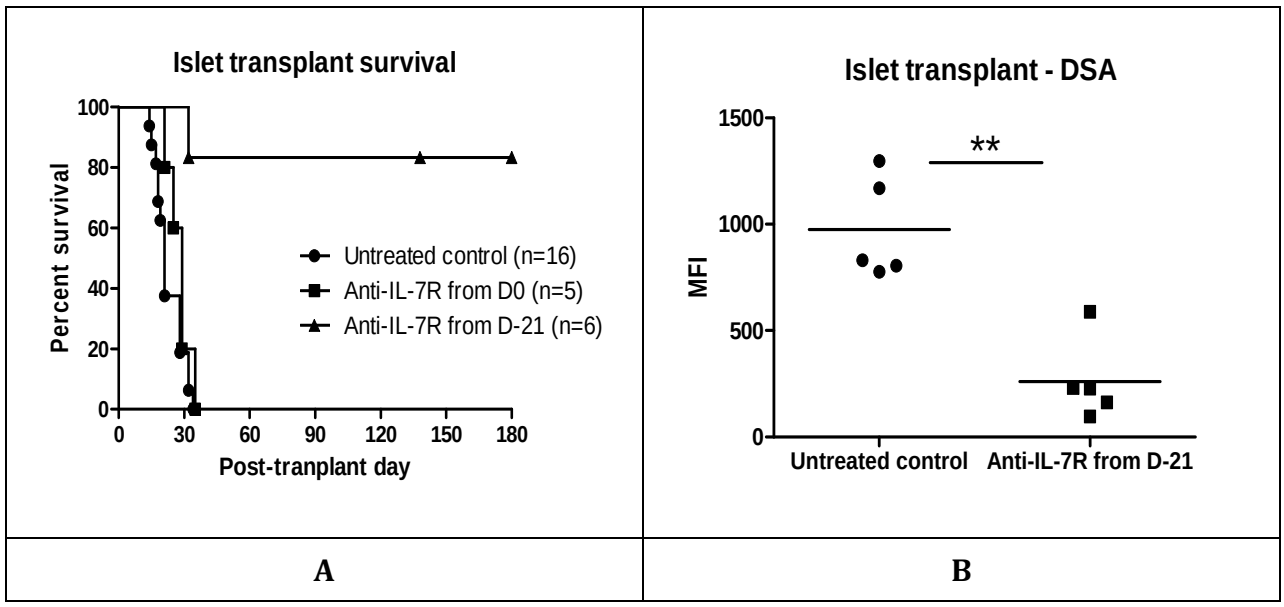


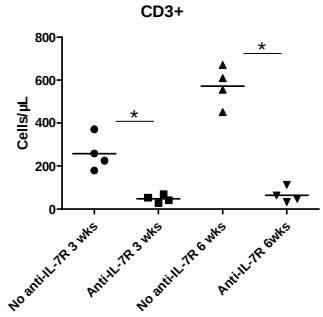
Figure 2: Anti-IL-7R antibody prolonged islet graft survival and abrogated humoral immune response. Streptozotocin-induced diabetic Balb/c recipients received islet grafts from C57BL/6 donors. A. Untreated mice rejected their islet grafts in a median of 21 days (range: 14-34 days) (n=16). Anti-IL-7R mAb-treated mice that received anti-IL-7R mAb 400 µg IP qod from the day of islet grafts until rejection (n=5) rejected their grafts in a median of 29 days (range: 21-35 days) (p=0.16 compared to untreated control). On the contrary, 5 of 6 anti-IL-7R mAb-treated mice that received 400 µg IP qod 3 weeks before islet grafts until graft rejection or post-transplant day 90 had indefinite graft survival over 180 days (p=0.0002 compared to untreated control). B. Donor-specific antibody (DSA) measurement at PTD160 in mice that received anti-IL-7R from 3 weeks before graft and had long-term graft survival showed minimal antibody levels, whereas there was a strong DSA response in untreated control mice after graft rejection (n=5 for each group, p<0.01).

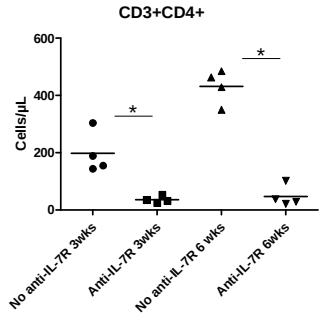
Anti-IL-7Rα antibody given after T cell depletion inhibited T cell reconstitution and decreased memory T cell number:

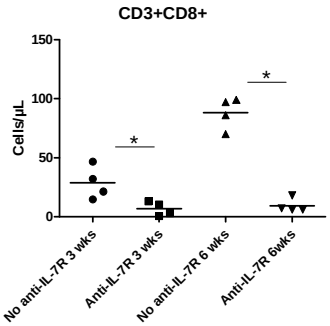
Although anti-IL-7Rα mAb alone can prolong islet graft survival, however the fact that it must be used 3 weeks before graft makes its clinical application unlikely. Therefore, we started the treatment of naïve mice with a combination of two known T cell depleting antibodies commonly used in experimental transplantation in mice: anti-CD4 mAb (clone GK1.5) and anti-CD8 mAb (clone 2.43) in order to rapidly reduce T cell numbers, followed by anti-IL-7Rα mAb to test if IL-7R blockade can prolong the T cell reduction and inhibit the post-depletion increase in memory T cell numbers. A total of 16 eight-week old male Balb/c mice were given GK1.5 100 µg plus 2.43 100 µg IP, followed 2 days later by either anti-IL-7Rα mAb (treated group, n=8) or isotype control (control group, n=8) 400 µg qod until sacrifice at 3 and 6 weeks (n=4 for each group at each time point). Treated group had significant lower numbers of almost all lymphocyte subsets, including CD3+, CD3+CD4+, CD3+CD8+, CD19+, and total lymphocytes in the blood, spleen, lymph nodes, and thymus than control group either at 3 weeks or 6 weeks. Importantly, $CD3+CD44^{hi}CD62L^{lo}$ memory T cell numbers were lower in treated group than in control group, although memory T cells accounted for a higher percentage of T cells in treated group. As in the case where IL-7R mAb was used alone, the treated group had increased frequency of regulatory T cells although their absolute numbers also decreased (Figure 3 and Table 2).

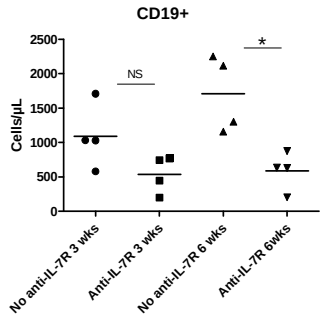
Figure 3: Anti-IL-7Rα antibody given after T cell depletion inhibited T cell reconstitution and decreased memory T cell number. A total of 16 eight-week old male Balb/c mice were given GK1.5 100 µg plus 2.43 100 µg IP, followed 2 days later by either anti-IL-7Rα mAb (n=8) or isotype control (n=8) 400 µg qod until sacrifice at 3 and 6 weeks (n=4 for each group at each time point). Treated group (anti-IL-7R) had significant lower numbers of almost all lymphocyte subsets, including CD3+, CD3+CD4+, CD3+CD8+, CD19+, and total lymphocytes in the blood (A), thymus (B), spleen (C), and lymph node (D) than control group (no anti-IL-7R) either at 3 weeks or 6 weeks. Importantly, $CD3+CD44^{hi}$, $CD62L^{lo}$ memory T cell numbers were lower in treated group than in control group, although memory T cells accounted for a higher percentage of T cells in treated group (E). Treated group had increased frequency of CD3+CD4+CD25+FOXP3+ regulatory T cells although their absolute numbers also decreased (F). (*: $p<0.05$; **: $p<0.01$; (1): $p=0.057$; NS: non-significant)

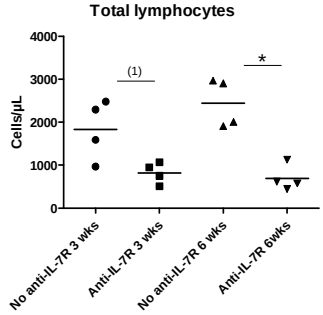
A. BLOOD



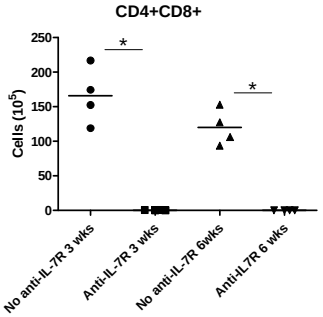


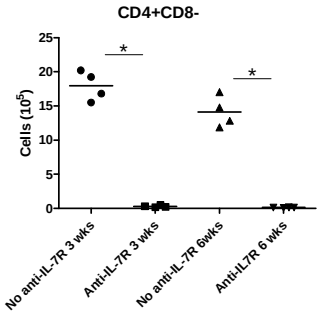


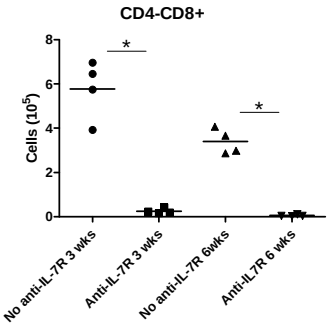


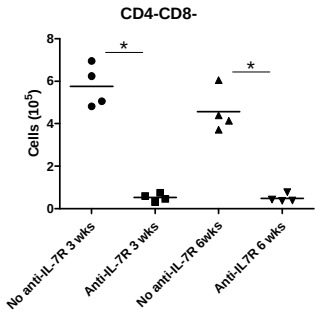


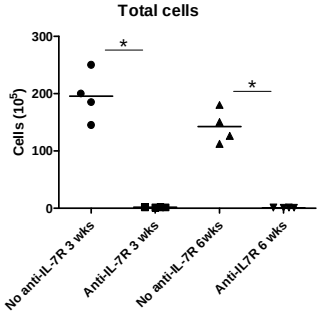
B. THYMUS











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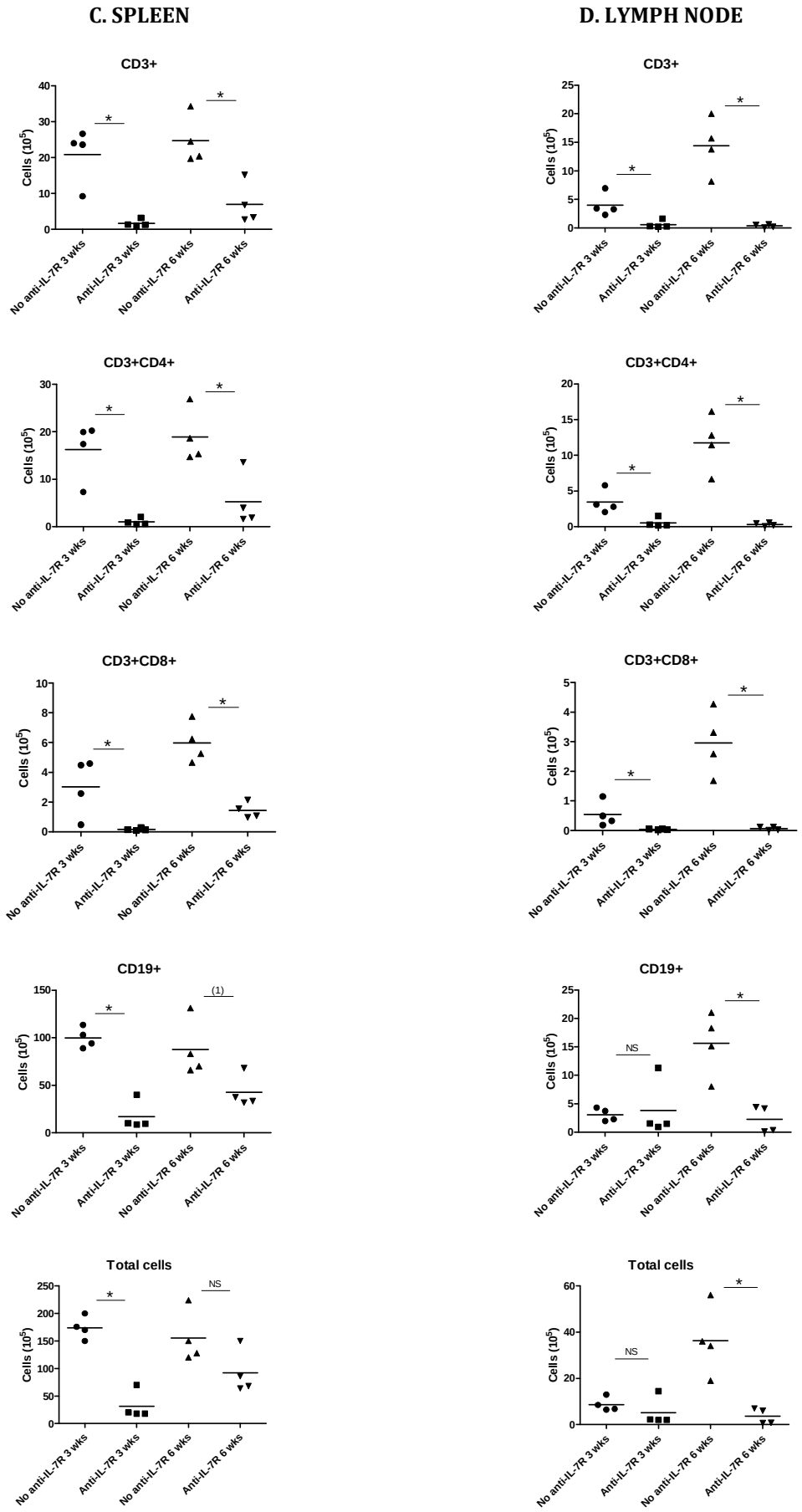

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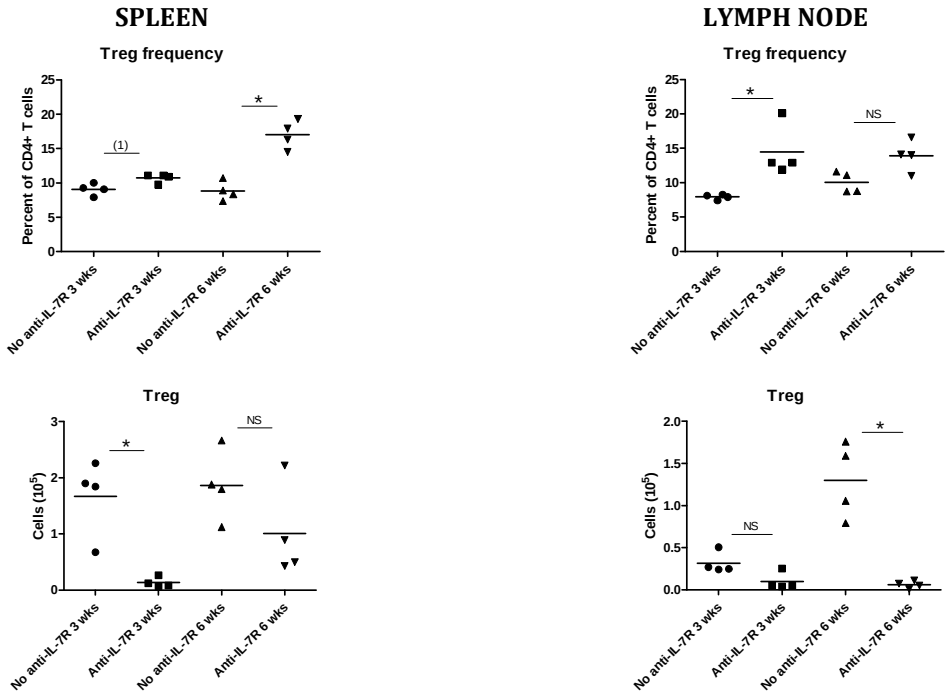
E. Regulatory T cells:



F. Memory T cells 6 weeks after depletion

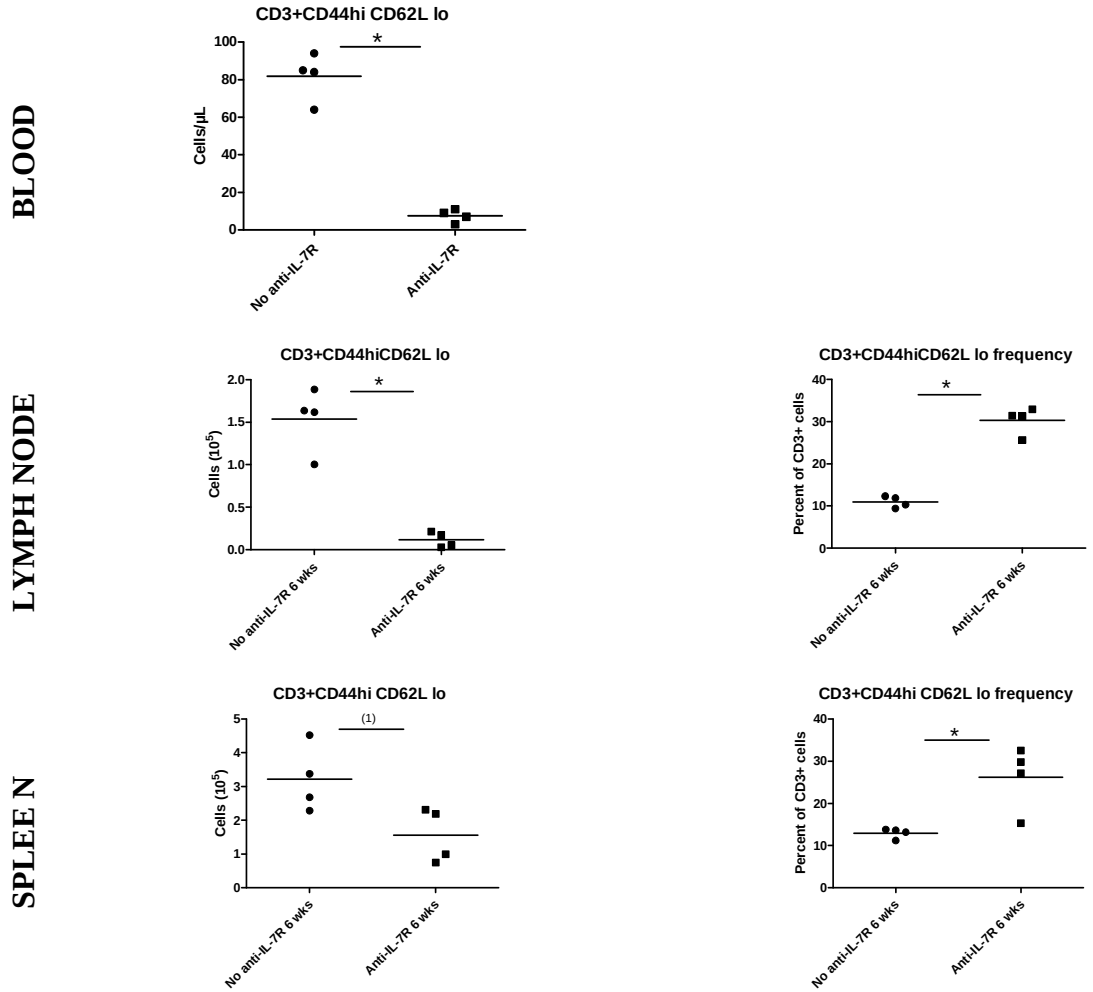


Table 2: Anti-IL-7Rα antibody given after T cell depletion inhibited T cell reconstitution and decreased memory T cell number.

A. Blood:

3 weeks after depletion

Leukocyte subsets (cells/µl)	Control group (n=4)	Treated group (n=4)	P value
Total lymphocytes	1831.0 ± 692.4	818.5 ± 244.9	0.057
CD3+	257.8 ± 81.5	47.0 ± 17.9	<0.05
CD3+ CD4+	197.0 ± 73.2	35.3 ± 11.9	<0.05
CD3+ CD8+	28.6 ± 13.9	6.7 ± 5.9	<0.05
CD19+	1086.0 ± 465.5	536.5 ± 270.3	0.11
Granulocytes	478.0 ± 168.7	483.3 ± 200.6	1

6 weeks after depletion

Leukocyte subsets (cells/µl)	Control group (n=4)	Treated group (n=4)	P value
Total lymphocytes	2446.0 ± 566.1	690.5 ± 299.9	<0.05
CD3+	571.0 ± 93.0	63.3 ± 34.9	<0.05
CD3+ CD4+	430.8 ± 59.2	46.8 ± 36.8	<0.05
CD3+ CD8+	88.0 ± 13.3	9.3 ± 5.9	<0.05
CD19+	1704.0 ± 556.9	584.5 ± 280.4	<0.05
Granulocytes	537.3 ± 85.0	578.0 ± 80.7	0.86
Memory T (CD3+CD44hiCD62Llo)	81.8 ± 12.7	7.5 ± 3.4	<0.05

B. Thymus:

3 weeks after depletion

Cell subsets (105 cells)	Control group (n=4)	Treated group (n=4)	P value
Total thymocytes	195.0 ± 43.4	1.4 ± 0.18	<0.05
CD4+ CD8+ double positive	165.5 ± 40.9	0.32 ± 0.17	<0.05
CD4+ CD8- single positive	17.95 ± 2.17	0.30 ± 0.14	<0.05
CD4- CD8+ single positive	5.77 ± 1.33	0.25 ± 0.12	<0.05
CD4- CD8- double negative	5.77 ± 1.0	0.53 ± 0.18	<0.05

6 weeks after depletion

Cell subsets (105 cells)	Control group (n=4)	Treated group (n=4)	P value
Total thymocytes	142.0 ± 29.8	0.74 ± 0.13	<0.05
CD4+ CD8+ double positive	119.9 ± 26.0	0.068 ± 0.060	<0.05
CD4+ CD8- single positive	14.13 ±2.28	0.130 ± 0.031	<0.05
CD4- CD8+ single positive	3.40 ± 0.57	0.054 ± 0.038	<0.05
CD4- CD8- double negative	4.57 ± 1.02	0.485 ± 0.192	<0.05

C. Spleen:

3 weeks after depletion

Leukocyte subsets (105 cells)	Control group (n=4)	Treated group (n=4)	P value
Total cells	174.0 ± 20.6	31.5 ± 25.7	<0.05
CD3+	20.8 ± 7.9	1.7 ± 1.0	<0.05
CD3+ CD4+	16.2 ± 6.1	1.0 ± 0.7	<0.05
CD3+ CD8+	3.03 ± 1.94	0.166 ± 0.082	<0.05
CD19+	99.9 ± 10.8	17.2 ± 15.2	<0.05
CD3+CD62Llo	6.17 ± 1.98	0.69 ± 0.48	<0.05
CD3+CD62Llo/CD3+ (%)	30.5 ± 3.3	40.5 ± 4.4	<0.05
Treg (CD3+CD4+CD25+FOXP3+)	1.67 ± 0.69	0.135 ± 0.090	<0.05
Treg/CD3+CD4+ (%)	9.06 ± 0.87	10.72 ± 0.64	0.057

6 weeks after depletion

Leukocyte subsets (105 cells)	Control group (n=4)	Treated group (n=4)	P value
Total cells	155.5 ± 47.4	92.0 ± 39.8	0.15
CD3+	24.7 ± 6.7	7.0 ± 5.7	<0.05
CD3+ CD4+	18.9 ± 5.6	5.3 ± 5.6	<0.05
CD3+ CD8+	6.0 ± 1.3	1.4 ± 0.5	<0.05
CD19+	87.7 ± 30.0	42.8 ± 17.0	0.057
Memory T (CD3+CD44hiCD62Llo)	3.22 ± 0.98	1.56 ± 0.81	0.057
Memory T/CD3+ (%)	12.95 ± 1.19	26.18 ± 7.58	<0.05
Treg (CD3+CD4+CD25+FOXP3+)	1.87 ± 0.63	1.00 ± 0.83	0.20
Treg/CD3+CD4+ (%)	8.83 ± 1.34	17.00 ± 2.07	<0.05

D. Lymph node:

3 weeks after depletion

Leukocyte subsets (105 cells)	Control group (n=4)	Treated group (n=4)	P value
Total cells	8.7 ± 3.0	5.2 ± 6.2	0.34
CD3+	3.99 ± 2.03	0.60 ± 0.68	<0.05
CD3+ CD4+	3.43 ± 1.63	0.55 ± 0.64	<0.05
CD3+ CD8+	0.538 ± 0.425	0.034 ± 0.011	<0.05
CD19+	3.07 ± 1.13	3.8 ± 5.0	0.34
CD3+CD62Llo	1.14 ± 0.53	0.26 ± 0.29	0.057
CD3+CD62Llo/CD3+ (%)	29.0 ± 2.9	45.0 ± 4.9	<0.05
Treg (CD3+CD4+CD25+FOXP3+)	0.316 ± 0.126	0.100 ± 0.102	0.11
Treg/CD3+CD4+ (%)	7.92 ± 0.38	14.45 ± 3.80	<0.05

6 weeks after depletion

Leukocyte subsets (105 cells)	Control group (n=4)	Treated group (n=4)	P value
Total cells	36.3 ± 15.2	3.63 ± 3.35	<0.05
CD3+	14.4 ± 4.9	0.37 ± 0.26	<0.05
CD3+ CD4+	11.8 ± 3.9	0.32 ± 0.23	<0.05
CD3+ CD8+	2.96 ± 1.10	0.066 ± 0.059	<0.05
CD19+	15.6 ± 5.6	2.26 ± 2.36	<0.05
Memory T (CD3+CD44hiCD62Llo)	1.54 ± 0.38	0.116 ± 0.089	<0.05
Memory T/CD3+ (%)	10.98 ± 1.35	30.30 ± 3.22	<0.05
Treg (CD3+CD4+CD25+FOXP3+)	1.30 ± 0.45	0.063 ± 0.042	<0.05
Treg/CD3+CD4+ (%)	10.05 ± 1.52	13.93 ± 2.29	0.11

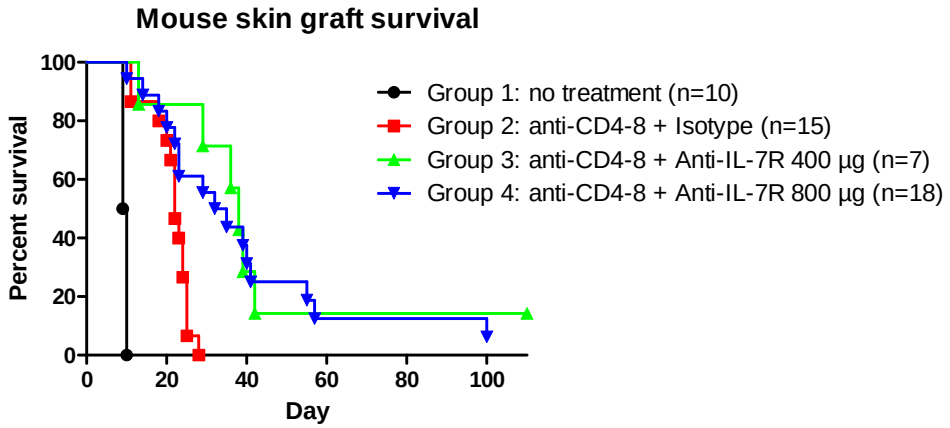
Legend to table 2: A total of 16 eight-week old male Balb/c mice were given GK1.5 100 µg plus 2.43 100 µg IP, followed 2 days later by either anti-IL-7Rα mAb (treated group, n=8) or isotype control (control group, n=8) 400 µg qod until sacrifice at 3 or 6 weeks (n=4 for each group at each time point). Treated group had significant lower numbers of almost all lymphocyte subsets, including CD3+, CD3+CD4+, CD3+CD8+, CD19+, and total lymphocytes in the blood (A), thymus (B), spleen (C), and lymph node (D) than control group either at 3 weeks or 6 weeks. Importantly, $CD3+CD44^{hi}$, $CD62L^{lo}$ memory T cell numbers were lower in treated group than in control group, although memory T cells accounted for a higher percentage of T cells in treated group. Treated group had increased frequency of CD3+CD4+CD25+FOXP3 regulatory T cells although their absolute numbers also decreased.

Anti-IL-7R antibody given after T cell depletion prolonged skin allograft survival and decrease humoral alloimmune response:

To determine whether the prolongation of T lymphopenia associated with the decrease in memory T cell number and the increase in regulatory T cell frequency by IL-7R blockade following T cell depletion is beneficial for graft survival, we used a stringent model of skin allograft in mice. This time, we did not use islet transplant to streptozotocin-induced diabetic mice because T cell depletion by anti-CD4 mAb could already induce long-term graft survival (2). Balb/c mice received tail skin grafts from C57BL/6 donors (Figure 4A). Untreated mice (group 1, n=10) rejected their grafts in a median of 9.5 days (range: 9-10 days). Mice which received a single dose of anti-CD4 100 µg and anti-CD8 100 µg IP 3 days before graft followed by isotype control 400 µg qod until rejection (group 2, n=15) had a median graft survival of 22 days (range: 11-28 days), which was significantly longer than that of untreated mice ($p<0.0001$). We then added IL-7R blockade to the treatment protocol to see whether it could further prolong graft survival. Mice were given the same dose of anti-CD4 and anti-CD8, followed by anti-IL-7Rα mAb 400 µg qod until rejection or PTD70 (group 3, n=7), this treatment resulted in a median graft survival of 38 days (range: 13 to >120 days), which was significantly longer than

that of group 2 (p=0.0006). The increase in the dose of anti-IL-7R mAb from 400 µg to 800 µg qod (group 4, n=18) did not further prolong graft survival, the median graft survival was 33.5 days (range: 10 to >100 days) (p = 0.71 and p=0.002 compared to group 2 and group 1, respectively). The prolonged graft survival with IL-7R blockade was associated with an abrogation of humoral immune response, as DSA levels at PTD30 were significantly lower in group 3 compared to group 2 (p<0.05) (Figure 4B).

A.



B.

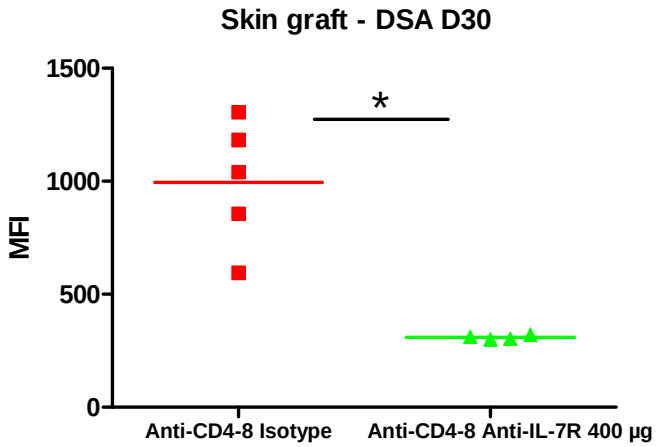


Figure 4: Anti-IL-7R blockade following T cell depletion prolonged mouse skin allograft survival and abrogated humoral immune response. (A) Balb/c mice received tail skin grafts from C57BL/6 donors. Group 1: no treatment (n=10); group 2 (n=15): single dose of anti-CD4 100 µg plus anti-CD8 100 µg IP 3 days before graft followed by isotype control 400 µg qod until rejection; group 3: single dose of anti-CD4 100 µg and anti-CD8 100 µg IP 3 days before graft followed by anti-IL-7Rα 400 µg qod until rejection or PTD70 (n=7); group 4: same as group 3, but with anti-IL-7Rα 800 µg qod (n=18). Median graft survival of group 1, 2, 3, and 4 were 9.5, 22, 38, and 33.5 days, respectively. Graft survival was significantly longer in group 2 than in group 1 (p<0.0001), it was further prolonged in group 3 and group 4 compared to group 2 (p=0.0006 and p=0.002, respectively), however it was not different between group 3 and group 4 (p=0.71). (B) Donor-specific antibodies (DSA) were significantly lower in group 3 compared to group 2 (p<0.05).

DISCUSSION

Since the discovery of the essential role of the IL-7/IL-7R axis in lymphopoiesis, this signaling pathway has become an interesting therapeutic target. Stimulation of this pathway by recombinant human IL-7 is currently tested in clinical trials to increase CD4+ T cell numbers in HIV patients (17). Conversely, inhibition of IL-7 or IL-7R has been tested in experimental models to treat graft-versus-host disease following bone marrow transplantation (18) or autoimmune diseases (19). Concerning organ transplantation, to the best of our knowledge, there is only one published study by Wang and al in which a neutralizing anti-IL7 antibody was used in a murine cardiac allograft model. In that study, anti-IL-7 antibody alone given from the day of transplantation was not effective, but anti-IL-7 antibody combined with CD40/CD40L costimulatory blockade significantly prolonged graft survival compared to costimulation blockade alone (20).

We began our study by giving anti-IL-7Rα mAb (clone A7R34) to naïve Balb/c mice to evaluate the levels of reduction in the numbers of different lymphocyte subsets. We found that 3 weeks of A7R34 400 µg IP qod significantly reduced the numbers of total T cells, B cells, CD4+ and CD8+ T cells in the blood, spleen, lymph nodes, and thymus; our results are similar to those previously shown by Sudo and al., who had created and characterized this mAb (16). However, we first show that anti-IL-7R also reduced the number of CD3+CD62L^{lo} memory T cells and increased the frequency of CD3+CD4+CD25+FOXP3+ regulatory T cells, this property is potentially beneficial in transplantation. We then tested anti-IL-7R treatment in a murine islet allograft model. Similar to Wang and al, we could not prolong graft survival by anti-IL-7Rα antibody monotherapy given from the day of transplantation. However, when we started anti-IL-7Rα antibody 3 weeks before islet graft and continued the treatment until 3 months post-transplant, we induced indefinite graft survival in 5 of 6 treated mice. The timing of treatment is important because this monoclonal antibody is not depleting and must be given for a few weeks before its IL-7R blocking effect translates into a reduction in lymphocyte numbers. Depleting antibodies that target IL-7Rα will be of great interest because IL-7Rα is expressed in almost all T cells except regulatory T cells, where the expression of IL-7Rα is low or absent (21, 22). In fact, the levels of expression of IL-7Rα or CD127 help to distinguish CD4+CD25+CD127^{lo} regulatory T cells from CD4+CD25+CD127^{hi} effector T cells (23). Therefore, depleting anti-IL-7Rα antibodies will deplete effector T cells while sparing regulatory T cells.

In the absence of depleting properties, a blocking anti-IL-7Rα mAb should be used in combination with other agents that have immediate immunosuppressive effects. Since IL-7 is required for T cell reconstitution after T cell depletion therapy, we treated mice with anti-IL-7Rα

mAb immediately after T cell depletion by a combination of two depleting mAbs: anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43). This combination of two depleting mAbs has been commonly used in murine allograft models and shown to deplete about 85 percent of T cells following by a T cell reconstitution with a predominance of memory T cells (7). As expected, mice that had received IL-7R blockade after T cell depletion had a profoundly suppressed T cell reconstitution as evidenced by a 2 to 15-fold reduction of peripheral total T cells, CD4+, and CD8+ T cells in the blood, spleen, and lymph nodes compared to mice that had received T cell depletion alone (Table 2 and Figure 2). The difference was even more pronounced in the thymus, where total thymocyte and thymocyte subset numbers were reduced up to 1750 folds in anti-IL-7R treated mice. Importantly, the absolute numbers of memory T cell, defined either as CD3+CD62Llo or CD3+CD62Llo CD44hi were reduced 2 to 13 folds in the blood, spleen, and lymph nodes of anti-IL-7R treated mice. Although IL-7 had previously been confirmed to be necessary for the proliferation and survival of naïve and memory T cells in lymphopenic host (reviewed in 24), almost all of these studies were carried out by transferring T cells into either constitutively lymphopenic mice such as rag-/- or severe combined immunodeficient (SCID) mice or sublethally irradiated wild-type mice. Our study, on the other hand, demonstrated that in normal mice, IL-7R blockade following a T cell depleting therapy commonly used in experimental transplantation prolonged T cell lymphopenia, inhibited the post-depletional increase in memory T cell numbers, and increased regulatory T cell frequency.

We subsequently confirmed these potentially beneficial effects of IL-7R blockade in a murine skin allograft model, which is simple to perform but more stringent than heart allograft or islet allograft in streptozotocin-induced diabetic mice. Just to remind that a brief course of a depleting anti-CD4 mAb alone could readily induce long-term survival of heart or islet allografts (2,3), whereas it has no effect on skin allograft survival (25). In our study, mice who received anti-IL-7R mAb 400 µg qod following a single dose of a combination of depleting anti-CD4 and anti-CD8 mAbs (referred to as anti-IL-7R-treated group) has significantly prolonged skin allograft to a median of 38 days compared to 22 days in mice who received only anti-CD4 and anti-CD8 (referred to as depletion-only group). A doubling of the dose of anti-IL-7R did not further improve graft survival, suggesting that IL-7 receptors are already saturated. When analyzing the survival curves (Figure 4), we recognized that some mice in both anti-IL-7R-treated groups and depletion-only group rejected their grafts in less than 15 days, which is earlier than expected. The explanation may be that the dose of depleting antibodies was not sufficient. Therefore, we recently increased the dose of anti-CD4 and anti-CD8 to 200 µg for each mAb, given at day -3 and -1, followed by either isotype control (n=6) or anti-IL-7R (n=6) 400 µg qod from day 1 post-

graft. Mice who received isotype control have a median graft survival of 29 days (range: 19 to 34 days), whereas all IL-7R-treated mice still maintained their graft at the time of this writing (PTD 47). These mice will be followed longer and more skin grafts will be performed using this dose schedule to determine graft survival.

Finally, IL-7R blockade is safe and appears to affect only the lymphoid system. Our anti-IL-7R-treated mice gained weight similar to control mice. No anemia, thrombocytopenia, or hepatic enzyme elevation was noted after 6 weeks of treatment. There was no death among more than 60 mice that we have treated with this anti-IL-7Rα mAb, even when combined with depleting anti-CD4 and anti-CD8 mAbs. However, our current study is not a comprehensive toxicological study, such a study will be necessary to confirm the safety of this therapy.

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PERSPECTIVES

1. Study of the mechanisms of action of IL-7R blockade in the prolongation of skin allograft survival:

As mentioned above, we will perform more skin grafts to confirm the effect of IL-7R blockade following a higher dose of T cell depletion in prolonging graft survival. We expect to obtain a substantial proportion of long-term graft survival with this protocol. Then further experiments will be performed to understand through what mechanisms IL-7R blockade can prolong graft survival and we will submit our article only after having obtained the results of these studies.

1.1. Reduction of alloreactive T cells:

Lymphocyte phenotyping has shown that the most evident effect of IL-7R blockade following T cell depletion is the prolongation of profound lymphopenia affecting almost all T cell subsets, and to a lesser extent, B cells. It is likely that an overall decrease in lymphocyte numbers also means a decrease in alloreactive T cells. To verify this hypothesis, we will use two different approaches.

In the first approach, we will use a one-way mixed lymphocyte culture (MLR), in which purified T cells from skin graft recipients are stimulated by irradiated donor APCs. T cells from anti-IL-7R treated mice are expected to proliferate less than those from depletion-only mice, demonstrating that IL-7R blockade reduces alloreactive T cells. T cell anergy will also need to be ruled out by demonstrating the failure of exogenous IL-2 at doses that only affect high-affinity receptors to restore the proliferation of T cells from anti-IL-7R-treated recipients in MLR.

In the second approach, we will perform direct interferon gamma (IFNγ)-Elispot using purified T cells from recipients and T cell-free APCs from donors. IFNγ-Elispot is commonly used in experimental as well as clinical transplantation to quantify allospecific T cells by counting the number of IFNγ secreting cells per 10^5 of total T cells. This method provides another way to identify alloreactive T cells through their cytokine secreting properties in response to allostimulation.

1.2. Role of regulatory T cells:

Since the frequency of regulatory T cells (Treg) is increased in anti-IL-7R-treated mice, these Treg will be purified and tested in MLR to see if they capable of suppressing T cell proliferation.

In order to answer the question whether the prolongation of graft survival was due mainly to T cell depletion or T cell regulation, we will perform adoptive transfer experiments using Balb/c SCID mice. Balb/c SCID mice do not reject skin allograft from C57BL/6 donors because they have severe immunodeficiency characterized by lymphopenia, absence of functional T cells and B cells, and hypogammaglobuminemia. Then we will perform adoptive transfer to several groups of Balb/c SCID mice followed by skin graft from C57BL/6 donors.

Group 1 will receive T cells from naive wild-type Balb/c mice, they will become immunocompetent and will reject their C57BL/6 skin graft.

Group 2 will receive T cells from wild-type Balb/c mice that have been treated with IL-7R blockade following T cell depletion by anti-CD4 and anti-CD8 and have accepted their C57BL/6 skin graft for more than 50 days (referred to as anti-IL-7R-treated mice). We expect that these Balb/c SCID mice will accept their C57BL/6 skin graft or at least will reject the graft in a much lower pace than group 1.

Group 3 will also receive T cells from anti-IL-7R-treated mice but this time CD3+CD4+CD25+CD127lo regulatory T cell (Treg) will have been removed by cell sorting prior to adoptive transfer. The essential role of Treg in prolonging graft survival will be confirmed if group 3 has significantly shorter graft survival than group 2. Conversely, if graft survival is not different between the two groups, it will signify that the prolongation of graft survival is due to the reduction of alloreactive T cells and not to the increase in Treg frequency.

1.3. Role of regulatory B cells?

Because B cell number is also reduced by IL-7R blockade, though to a lesser extent, we will explore if there are changes in the frequency of regulatory B cells defined as B220+CD1dhiCD5+ (Yanaba et al, 2008). Further experiments to clarify the function of these regulatory B cells will be planned when necessary.

2. IL-7R blockade following T cell depletion in other transplant models:

We are currently testing the same protocols of IL-7R blockade following T cell depletion in a model of islet transplantation in non-obese diabetic (NOD) mice. This model is very stringent and strong immunosuppression is usually needed to maintain long-term graft survival because NOD mice have both alloimmune and autoimmune responses to islet grafts.

We also intend to cooperate with another lab to study this protocol in a model of heart graft in mice. A short course of depleting anti-CD4 mAb with or without depleting anti-CD8 mAb can already induce long-term heart allograft survival. However, histologic studies of these heart

grafts at postransplant day 100 revealed transplant vasculopathy typical of chronic rejection. We will test if the addition of a blocking anti-IL-7R-mAb can reduce the histological signs of chronic rejection.

3. IL-7R blockade in a mouse model of type 1 diabetes:

In a preliminary study, we found that IL-7R blockade can effectively prevent the development of diabetes in NOD mice. NOD mice is the most commonly used animal model for the study of type I diabetes in human. Female NOD mice develop inflammation of pancreatic islets or insulitis from 7-8 weeks of age. Overt diabetes begins to be detected in these mice from 12 weeks of age as a large proportion of beta cells have been destroyed. By the age of 30 weeks, 80 percent of female NOD mice have become diabetic. As a pilot study, we treated 8 week-old female NOD mice with either anti-IL-7R mAb 400 µg IP qod (treated group, n=8) or PBS at the same volume and same schedule (control group, n=8) for 8 weeks (to 16 weeks of age). At 52 weeks of age, 5/8 (62.5%) of control mice develop diabetes, whereas only 1/8 (12.5%) of treated mice has diabetes (p = 0.028, log-rank test) (Figure 9). We are continuing our study by treating a series of NOD mice at 12 weeks of age with anti-IL-7R mAb for late diabetes prevention. At the same time, we also treated NOD mice at the time when diabetes was diagnosed for diabetic reversal.

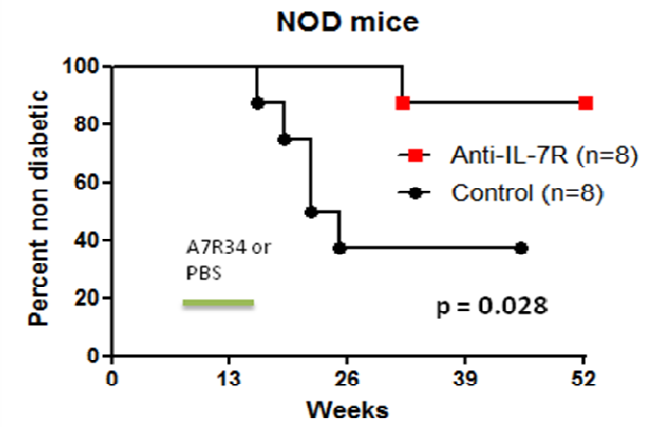

Figure 8 : Anti-IL-7Rα in the prevention of diabetes in NOD mice

4. IL-7R blockade following T cell depletion therapy – a strong immunosuppressive protocol with potential application in clinical transplantation:

Our results with the use of IL-7R blockade following a T cell depletion therapy may have important applications in clinical transplantation. Although CNI-based therapies remain the standard immunosuppression in organ transplantation, CNI nephrotoxicity is one the main

concerns of transplant physicians (Chapman, 2011). Therefore, new drugs are developed in order to replace CNIs. CNI-free regimens, as expected, usually resulted in better renal function at 1 year, but unfortunately, were associated with higher acute rejection rates compared to standard CNI-based regimens (Vincenti et al, 2010), sometimes leading to the early termination of clinical trials (Friman et al, 2011). More importantly, even CNI-free protocols that used a clinically approved drug such as sirolimus and reinforced by a depletion therapy by alemtuzumab or ATG still resulted in higher acute rejection rate or lower graft survival than conventional CNI-based treatments (Knechtle et al, 2003, Flechner et al, 2005, Glotz et al, 2010). Since the dose of sirolimus as well as of other non-CNI drugs can unlikely be increased further because of their side effects, we may need to add another drug to these CNI-free protocols to reinforce their efficacy. IL-7R blockade following a T cell depletion therapy presents as a novel and potent immunosuppressive protocol which can be combined with one of the non-CNI maintenance therapies in order to have an adequate immunosuppressive effect while keeping better renal function by avoiding CNI nephrotoxicity. A series of anti-human IL-7Rα is currently developed and will soon be tested in non-human primate transplant models.

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ANNEX

Others articles published during the PhD training period

ARTICLE 3

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JLB

Brief Conclusive Report

TNF blockade abrogates the induction of T cell-dependent humoral responses in an allotransplantation model

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ABSTRACT

TNF blockade modulates many aspects of the immune response and is commonly used in a wide array of immune-mediated inflammatory diseases. As anti-TNF induces anti-dsDNA IgM antibodies but not other antinuclear reactivities in human arthritis, we investigated here the effect of TNF blockade on the induction of TD humoral responses using cardiac allograft and xenograft models. A single injection of an anti-rat TNF antibody in LEW.1A recipients grafted with congenic LEW.1W hearts almost completely abrogated the induction of IgM and IgG alloantibodies. This was associated with decreased Ig deposition and leukocyte infiltration in the graft at Day 5. TNF blockade did not affect germinal-center formation in the spleen or expression of Th1/Th2 cytokines, costimulatory and regulatory molecules, and TLRs in spleen and graft of the recipient animals. Clinically, the abrogation of the induction of the alloantibodies was associated with a marked prolongation of graft survival. In contrast, anti-TNF did not alter acute xenograft rejection mediated by TI antibodies in a hamster-to-rat model. Taken together, these data indicate that TNF blockade abrogates the induction of TD humoral responses and accordingly, may have a beneficial effect in antibody-mediated inflammatory pathologies. *J. Leukoc. Biol.* 90: 000–000; 2011.

Introduction

Blocking TNF signaling has emerged as the first effective, targeted biological treatment in the early 1990s and has now become a treatment of choice for many chronic autoimmune diseases, including RA, SpA, Crohn's disease, and psoriasis. The clinical efficacy of anti-TNF antibodies as well as soluble TNFR fusion proteins has not only yielded a dramatic benefit in terms of patient care but also turned out to be a unique tool to study the modulation of the human immune system in vivo [1]. Translational studies in patients treated with TNF blockers have yielded important insights into the pathophysiology of these diseases as well as in the mechanism of action of the TNF-blocking agents [2]. Neutralizing TNF appears to target multiple inflammatory pathways downstream of TNF, including endothelial activation, leukocyte recruitment, proliferation, differentiation, and activation of hematopoietic and mesenchymal cells. Moreover, anti-TNF treatment can induce reverse signaling through transmembrane TNF and thereby, functionally modulate the TNF-producing cells, perhaps even leading to apoptosis. Finally, TNF blockers induce additional effects by interaction with FcRs, leading to activating or inhibitory signals in cells expressing these receptors.

A striking and unexpected observation in the early studies with infliximab, a chimerical mAb to TNF, in RA was the induction of ANA in a significant proportion of patients [3]. Confirming and extending these observations, we reported that this induction of novel autoantibody reactivities was not only observed in RA, a disease characterized by autoantibodies and defects in B cell tolerance, but also in SpA, a prevalent form of arthritis without known autoantibodies and hence, with a presumably normal B cell tolerance [4]. Clinically, the newly induced ANA were not associated with lupus-like disease and may even represent a physiological housekeeping mechanism to clear nuclear debris [5, 6]. From an immunological point of view, however, further characterization of the fine specificity of the antibodies yielded two striking observations [5]. First, all antibodies were of the IgM and IgA isotype, with an almost complete absence of IgG antibodies. These newly induced IgM ANA also disappeared upon interruption of TNF blockade in most of the patients. Second, the fine specificity

Abbreviations: ANA=antinuclear antibodie(s), AU=arbitrary unit(s), AUC=area under the curve, FoxP3=forkhead box P3, HPF=high-power field, HPRT=hypoxanthine guanine phosphoribosyl transferase, MARD/G/M=mAb-to-rat IgD/IgG/IgM, PALS=periarteriolar lymphoid sheath, qRT-PCR=quantitative RT PCR, RA=rheumatoid arthritis, SpA=spondyloarthritis, TD=T cell-dependent, TI=T cell-independent

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was almost exclusively restricted to dsDNA, which can induce a TI humoral response and did not spread to other TD nuclear antigens, even for the IgM isotype. Taken together, these observations—that the humoral autoimmune reaction toward nuclear antigens during TNF blockade was restricted to short-term TI responses—raised the hypothesis that TNF blockade may, in addition to the effects described previously, also affect the normal maturation of a humoral response and in particular, the TD response against protein antigens.

In the present study, we aimed to address this hypothesis experimentally by using a rat allograft model, in which congenic LEW.1W (haplotype $RT1^u$) heart grafts are heterotopically transplanted in LEW.1A (haplotype $RT1^a$) recipients. These congeneic animals have a complete mismatch for the class I, II, and I-like genes of the MHC region, leading to an acute rejection within 6.5 days. The rejection is mediated by Th1 cells as well as by alloantibodies, which appear as early as Days 6–8 [7–9]. Here, we assessed whether TNF blockade inhibits the induction of TD humoral responses by studying the induction of alloantibodies, the graft and spleen immunopathology, the global immune profile of graft and spleen, and the allograft survival in this model. As control, we studied a hamster-to-rat xenotransplantation model, where the acute rejection is mediated by TI antibodies [10, 11].

MATERIALS AND METHODS

Cardiac allograft transplantation model

LEW.1A ($RT1^a$), and LEW.1W ($RT1^u$) rats (Janvier, Savigny/Orge, France) were maintained under standard conditions, according to European and institutional guidelines. Heterotopic cardiac allografts were performed at Week 8 [7]. Animals were treated with an anti-rat TNF mAb (IgG2A κ; Centocor, Malvern, PA, USA) or the species-, isotype-, and concentration-matched control 3G8 antibody injected i.p. at 8 mg/kg. In specific experiments, anti-TNF was given at 15 mg/kg or was combined with a subtherapeutic dose of cyclosporine A (5 mg/kg) from Days 0 to 2. Orbital blood was obtained sequentially for alloantibody monitoring. For histology and mRNA analysis, animals were killed at Day 5 to recover cardiac allografts and recipient spleen. For experiments on graft survival, animals were monitored for heart beating up to Day 40. As control for this allotransplantation model, we additionally used cardiac hamster-to-rat xenografts as described previously [11]. Recipients were treated with cyclosporine A (10 mg/kg) daily in combination with anti-TNF (8 mg/kg) i.p. at Days 0, 3, and 6.

ELISA measurement of alloantibodies

Cellular proteins were extracted from LEW.1W splenocytes using the ReadyPrep sequential extraction kit (BioRad Laboratories, Veenendaal, The Netherlands) and coated on 96-well plates. Serum from LEW.1A-recipient animals (1:100 dilution) was incubated on the plates, and bound alloantibodies were detected with a HRP-conjugated rabbit anti-rat IgG antibody (Bethyl, Montgomery, TX, USA) and 3′,5,5′-tetramethyl benzidine substrate. Serial dilutions of high titer sera were used as a standard curve to assign AU for comparison between groups.

Immunohistology

Frozen sections of cardiac allografts were stained with mouse MARM-4 or MARG (both kindly provided by B.V., INSERM U643, Nantes, France) using a streptavidin-peroxidase technique and scored semiquantitatively by two independent observers blinded for the treatment protocol. Additionally, sections were immunostained using a three-step indirect immunoperoxidase technique using antibodies against the following cell-surface markers: CD45 for leukocytes (clone OX1-OX30); TCR for T lymphocytes (clone R7-3); CD4 (clone W3/25), CD8 (clone OX8), and CD45RA for B lymphocytes (clone OX33); CD68 for macrophages (clone ED1); and OX62 antigen for DCs (clone OX62; all clones from Bioatlantic, Nantes, France, except W3/25 clone from Serotec Laboratories, Oxford, UK). The number of positively stained cells/HPF was counted on each slide.

Frozen sections of the spleen were stained for B cells (CD45RA) and T cells (TCR). Some spleen sections were also stained for surface MARD-3 clone (Serotec Laboratories). B cell follicles were quantified as described previously [10]. Briefly, all B cell follicles present on one whole section were counted under the microscope, and the percentage of secondary follicles with a germinal-center formation was reported. To measure the follicular area, 10 consecutive pictures were captured for each spleen section at 100× original magnification. Image analysis was performed with the Metamorph software version 7.0 (Molecular Devices, Sunnyvale, CA, USA). Follicular areas, including primary and secondary follicles in each picture, were outlined manually and measured as number of pixels. All follicular areas present in these 10 pictures were summed and compared between treated and control groups.

qRT-PCR

Total RNA was isolated using TRIzol (Invitrogen, Cergy Pontoise, France), amplified using the SuperScript RNA amplification system (Invitrogen), and reverse-transcribed using a Moloney murine leukemia virus RT kit (Invitrogen). Real-time qPCR was performed with a GenAmp 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR core reagents (Applied Biosystems). Specific amplification products were checked by amplicon melting curves. The primers used for qRT-PCR and designed in the laboratory are shown in **Table 1**.The others primers (FoxP3, HO1, IDO, TLR1–10) were purchased from Applied Biosystems.

Statistics

Data were represented as mean ± SEM, and comparisons between treatment groups were performed using the parametric *t* test. For semiquantitative scores, the data were represented as median (interquartile range), and

TABLE 1. List of Primers Used for qPCR Analysis

	Forward primer	Reverse primer
IL-2	CCTTGTCAACAGCGCACCC	GCTTTGACAGATGGCTATCC
IL-4	CCACCTTGCTGTCACCCTGT	AGGATGCTTTTTAGGCTTTC
IL-10	TCAGCACTGCTATGTTGCC	CCTTGCTTTTATTCTCAGAGG
IL-13	AGCAACATCACACAAGACCAG	CACAACTGAGGTCCACAGCT
IFN-γ	TGGATGCTATGGAAGGAAAGA	GATTCTGGTGACAGCTGGTG
TNF-α	TGCTCCTCACCCACACCG	GGCTCTTGATGGCGGAGA
TGF-β	CTACTGCTTCAGCTCCACAG	TGCACTTGCAGGAGCGCAC
HPRT	CTGCTGGATTACATTAAAGCG	TCCCTGAAGTGCTCATTATAG
iNOS	ACTCAAGTTCAGCTTGGCGG	GGAGTGTCAGTGGCTTCCAG

comparisons between treatment groups were performed using the nonparametric Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

RESULTS

Anti-TNF blocks the induction of alloantibodies

To determine whether TNF blockade affects the induction of alloantibodies, we first measured by ELISA the appearance of serum alloantibodies over 20 days after LEW.1W-to-LEW.1A cardiac allograft in recipients treated with a single i.p. injection of the control 3G8 antibody (8 mg/kg; n=5) or the anti-TNF antibody (8 mg/kg; n=6) on Day 0 or the anti-TNF antibody (8 mg/kg) at Days 0, 3, and 6 (n=6). In line with previous observations [8], serum IgM alloantibodies appeared as early as Day 5, peaked at Day 10 at 4442 ± 1783 AU/ml, and dropped on Day 15 in the control animals (**Fig. 1A**). Single as well as triple injection of anti-TNF significantly suppressed the induction of IgM alloantibodies (P=0.030 and P=0.030, respectively, for AUC comparison with control animals) with peak titers of 227 ± 22 AU/ml (P=0.028 vs. controls) and 194 ± 48 AU/ml (P=0.027 vs. controls), respectively (Fig. 1A and B). IgG alloantibodies appeared from Day 5 on and peaked around Day 15 at 5698 ± 3067 AU/ml in control animals (Fig. 1C). As for IgM, single as well as triple administration of anti-TNF significantly suppressed the induction of IgG alloantibodies (P=0.017 and P=0.030, respectively, for AUC comparison with control animals) with peak titers of 277 ± 160 AU/ml (P=0.052 vs. controls) and 155 ± 56 AU/ml (P=0.030 vs. controls), respectively (Fig. 1C and D). These data demonstrate that even a single administration of anti-TNF antibody at the day of transplantation severely impairs the induction of IgM and IgG alloantibodies.

TNF blockade decreases IgG deposition and leukocyte infiltration in the graft

To assess whether the effect of TNF blockade on alloantibody levels in the serum was also reflected by altered deposition in the grafts, we performed histopathology on grafts obtained at Day 5 after transplantation in animals treated with a single injection of control 3G8 antibody (n=9) or anti-TNF antibody (n=9). Whereas there was a slight but not significant difference in IgM deposition (**Fig. 2A**), the degree of IgG deposition was significantly decreased in the graft of anti-TNF-treated animals versus animals treated with the control antibody (P=0.035; Fig. 2B). Immunohistochemical analysis of the inflammatory infiltration of the grafts additionally revealed a significant reduction of the total number of leukocytes in the anti-TNF-treated grafts (63±22 cells/HPF) versus the control-treated grafts (99±22 cells/HPF; P=0.003; Fig. 2C). Further characterization of the infiltrating leukocytes did not show significant differences in any of the analyzed leukocyte subsets, including $CD4^+$ and $CD8^+$ T cells, B cells, macrophages, and DCs (data not shown).

TNF blockade significantly prolongs allograft survival

We next assessed whether the observed inhibition of alloantibody induction and Ig deposition by TNF blockade affected

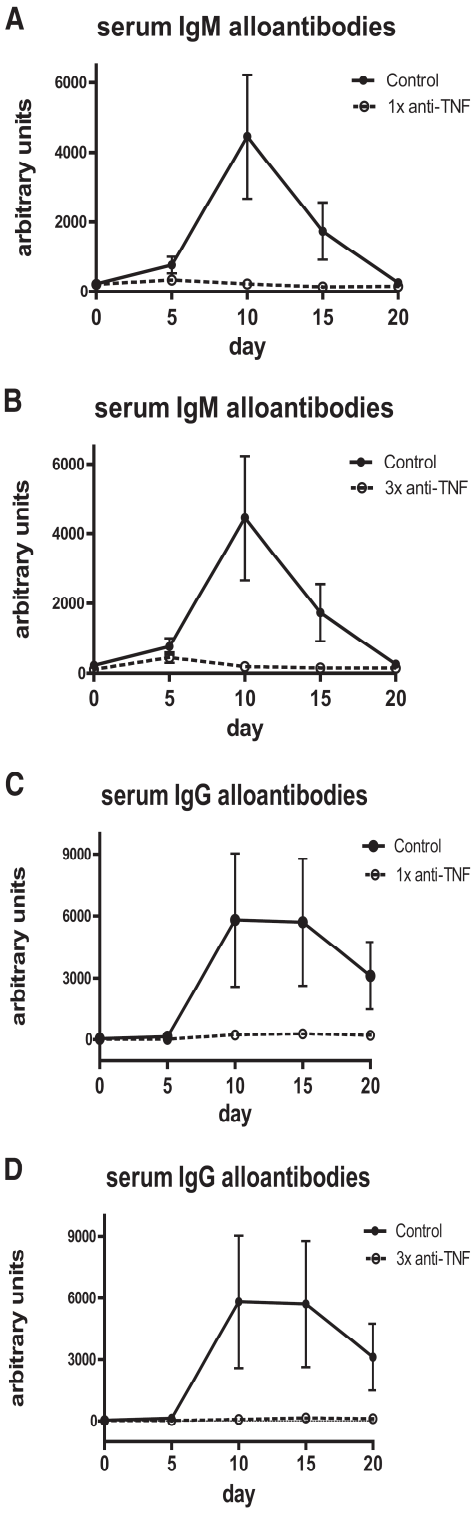

Figure 1. Time curve of the serum alloantibodies of the IgM (A and B) and IgG (C and D) isotype from Day 0 to Day 20 after cardiac allotransplantation. Control animals were treated with a single injection of a control antibody at Day 0. The treated groups received a single injection of anti-TNF at Day 0 (A and C) or three injections of anti-TNF at Days 0, 3, and 6 (B and D). Data are represented as mean ± SEM.

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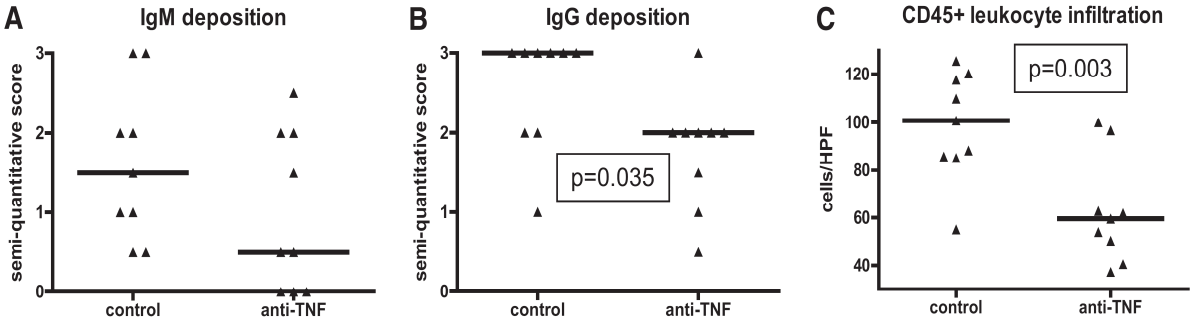

Figure 2. Histological and immunohistochemical analysis of cardiac allografts recovered at Day 5 after transplantation in animals treated with a single injection of control antibody or anti-TNF at Day 0. (A and B) Semiquantitive assessment of IgM and IgG deposition in the grafts, respectively. (C) $CD45^+$ leukocyte infiltration, as assessed by number of positive cells/HPF upon immunostaining.

the allograft survival. In the control group, the LEW.1W-to-LEW.1A cardiac allografts were consistently rejected around Day 7 (n=10). Treatment with a single injection of anti-TNF antibody at a dose of 8 mg/kg at Day 0 (n=10) significantly prolonged the survival to Day 20 (range 10–25; P<0.001; **Fig. 3A**). Triple injection with anti-TNF at Days 0, 3, and 6 (n=11) further prolonged the allograft survival to Day 25 (range 22–27; P<0.001 compared with control, as well as with single anti-TNF treatment; Fig. 3B). Modulation of the timing (Days −4, −1, and 2, instead of Days 0, 3, and 6) or the dose (15 mg/kg, instead of 8 mg/kg) of the triple anti-TNF administration did not further improve the graft survival (data not shown). These data indicate that even if TNF blockade does not induce long-term tolerance, the abrogation of alloantibody induction was associated with a significant prolongation of allograft survival.

TNF blockade selectively affects TD humoral responses

As the humoral alloresponses are strongly dependent on T cell help, in this model, we sought to provide additional evidence that TNF blockade was specifically affecting TD but not TI humoral responses steered by innate immune triggers [12]. First, we investigated whether anti-TNF had an additive effect to a T cell-directed therapy such as cyclosporine A. For this purpose, we used a suboptimal treatment with cyclosporine A (5 mg/kg from Days 0 to 2), which slightly prolonged graft survival (Day 14, range 6–19), compared with untreated animals (Day 8, range 5–11; P=0.007) but did not induce long-term tolerance to be able to evaluate an additional effect of TNF blockade (Fig. 3C). The combination of this subtherapeutic dose of cyclosporine A with a single injection of anti-TNF (median graft survival Day 18, range 5–20) was not superior to cyclosporine A alone or to anti-TNF alone (median 20, range 10–25), indicating that there was no additive effect (Fig. 3D). Next, we performed similar experiments in a hamster-to-rat cardiac xenograft model, where the acute rejection is not mediated by affinity-matured TD antibodies but by TI antibodies [10, 11]. In this model, xenografts were rejected at Day 3 in untreated animals. The median xenograft survival was 4 days [2–8] in treated animals (n=7), indicating that anti-TNF had no effect in this model. Finally, we assessed whether TNF blockade may affect alloantibody responses through modulation of TLRs, as we previously observed a strong effect of TNF blockade on TLR expression in humans [13] and as TLR-mediated innate immune responses contribute to allograft rejection [14]. As for T cell cytokines and regulatory molecules, however, there was no difference in TLR mRNA expression in the graft and the spleen of anti-TNF-treated versus control-recipient animals (**Fig. 4A** and **B**). Taken together, these data support the concept that TNF blockade selectively affects the TD humoral responses.

TNF blockade does not affect T lymphocyte profiles

As the alloantibodies in this model are TD [10, 15], we investigated whether the abrogation of alloantibody induction could be a result of an indirect effect of TNF blockade on T cell profiles. We analyzed the expression of genes of interest at Day 5 after transplantation in the graft and spleen of control 3G8-treated (n=9) versus anti-TNF-treated (n=9) recipients. As shown in Fig. 4C and D, the expression in the graft and in the spleen of IL-2, IL-4, IL-10, IL-13, IFN-γ, and TNF-α was not different between treated and control animals, indicating that TNF blockade did not induce a shift in the Th1/Th2 balance. There was also no alteration in the expression of CD40 and CD154, a pivotal ligand-receptor pair involved in the TD maturation of B cell responses (data not shown). As to regulatory pathways that may modulate T cell responses, the expression of TGF-β, HO1, IDO, and iNOS was similar in anti-TNF-treated and control grafts and spleen (Fig. 4E and F). The expression of FoxP3 was very low in all samples (data not shown). Taken together, this screening approach by gene expression analysis revealed no major impact of TNF blockade on intragraft or splcen T lymphocytc profilcs.

TNF blockage does not affect splenic germinal-center formation

The pivotal interaction of T and B cells in the maturation and amplification of the alloimmune responses occurs mainly in the germinal centers of secondary lymphoid follicles [10, 16, 17]. As previous studies in mice have indicated that blocking TNF could affect memory B cells and antibody responses by

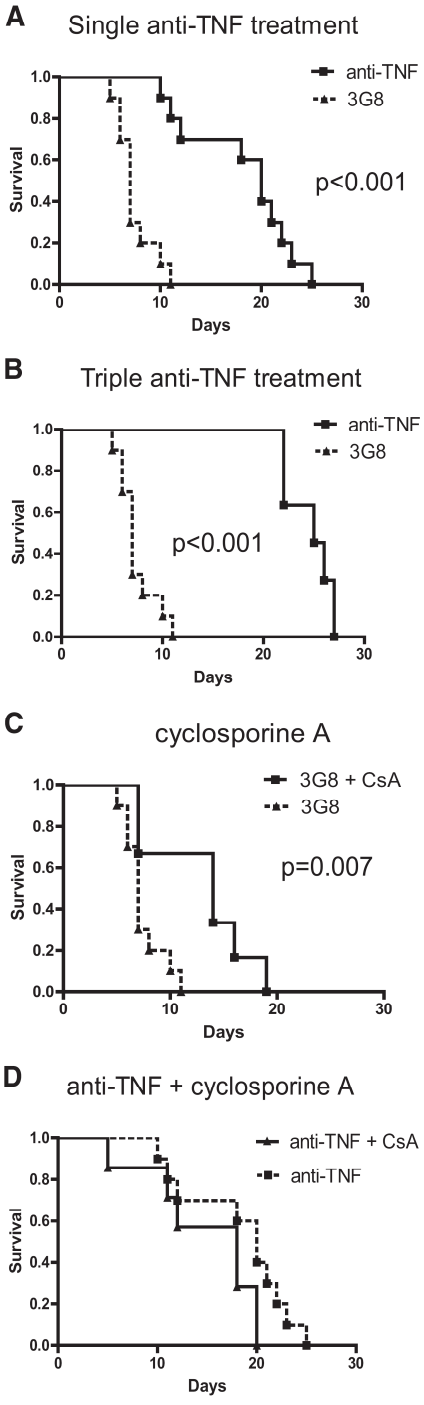

Figure 3. Survival of LEW.1W cardiac allografts in LEW.1A recipients. These were treated with a single injection of anti-TNF at Day 0 (A), a triple injection of anti-TNF at Days 0, 3, and 6 (B), a suboptimal dose of cyclosporine A at Days 0–2 (C), or the combination of a single anti-TNF injection with a suboptimal dose of cyclosporine A (D).

inhibition of germinal-center reactions [18, 19], we obtained spleens at Day 5 or Day 10 post-transplant (n=4 for each group) and immunostained T and B cells to quantify the percentage of secondary follicles and the total follicular areas as described previously [10] (**Fig. 5A** and **B**). We used CD45RA as the marker for rat B cells, but we also stained some sections for surface IgD, which gave the same staining pattern. At post-transplant Day 5, treated animals had 9.7 ± 2.4% of secondary follicles versus 13.6 ± 4.0% in the control group (P=0.48; Fig. 5C). At post-transplant Day 10, the percentages were 26.3 ± 3.1% and 27.1 ± 3.8% in the treated and control group, respectively (P=0.89; Fig. 5D). Similarly, the follicular area was comparable in the treated group versus the control group at post-transplant Days 5 and 10 (Fig. 5E and F). These data indicate that the splenic germinal-center reaction was not affected by TNF blockade in this model.

DISCUSSION

The main finding of this study in an experimental cardiac allotransplantation model is that TNF blockade abrogates the induction of antibodies directed toward a TD alloantigen. The first evidence for modulation of humoral responses by TNF blockade came from the observations that anti-TNF therapy induced ANA in RA and SpA patients [3–5]. Interestingly, this autoimmune response was not directed against a variety of protein and nonprotein nuclear antigens, as observed in systemic lupus erythematosus, but restricted to IgM and IgA antibodies toward the TI antigen dsDNA. We propose that this restriction in autoantibody response may relate to the fact that activation, maturation, and differentiation of B cells in the periphery can occur along two distinct pathways [12]. Upon relatively low-strength signaling through the BCR, naive B cells are preferentially directed toward the marginal zone of the spleen, where they can undergo some degree of somatic hypermutation but no class-switch recombination. This process mainly initiated by TI antigens, such as polysaccharides, is governed by neurogenic locus notch homolog protein 2 signaling. Marginal-zone B cells can then further differentiate toward IgM memory cells or IgM-producing plasma cells, which retain a global, low affinity for the antigens. In contrast, high-affinity B cell responses require the naive B cells to mature in a germinal-center reaction, a process that depends on high-strength signaling through the BCR, on Bruton's tyrosine kinase and runt-related transcription factor 1 (RUNX1) signaling, and on T cell help. Extensive somatic hypermutation and class-switch recombination in the germinal center allow the development toward high-affinity, isotype-switched memory B cells and plasma cells. This is not only the classical pathway for B cell responses against TD protein antigens but may also play a crucial role in the maturation of high-affinity responses to TI antigens. Our observations about ANA induction in arthritis patients treated with anti-TNF antagonists were therefore compatible with a selective induction of the marginal-zone pathway or a specific inhibition of the TD germinal-center reaction. The data obtained in this experimental study of allotransplantation strongly support the second hypothesis, as we observed a nearly complete abrogation of the antibody induction toward

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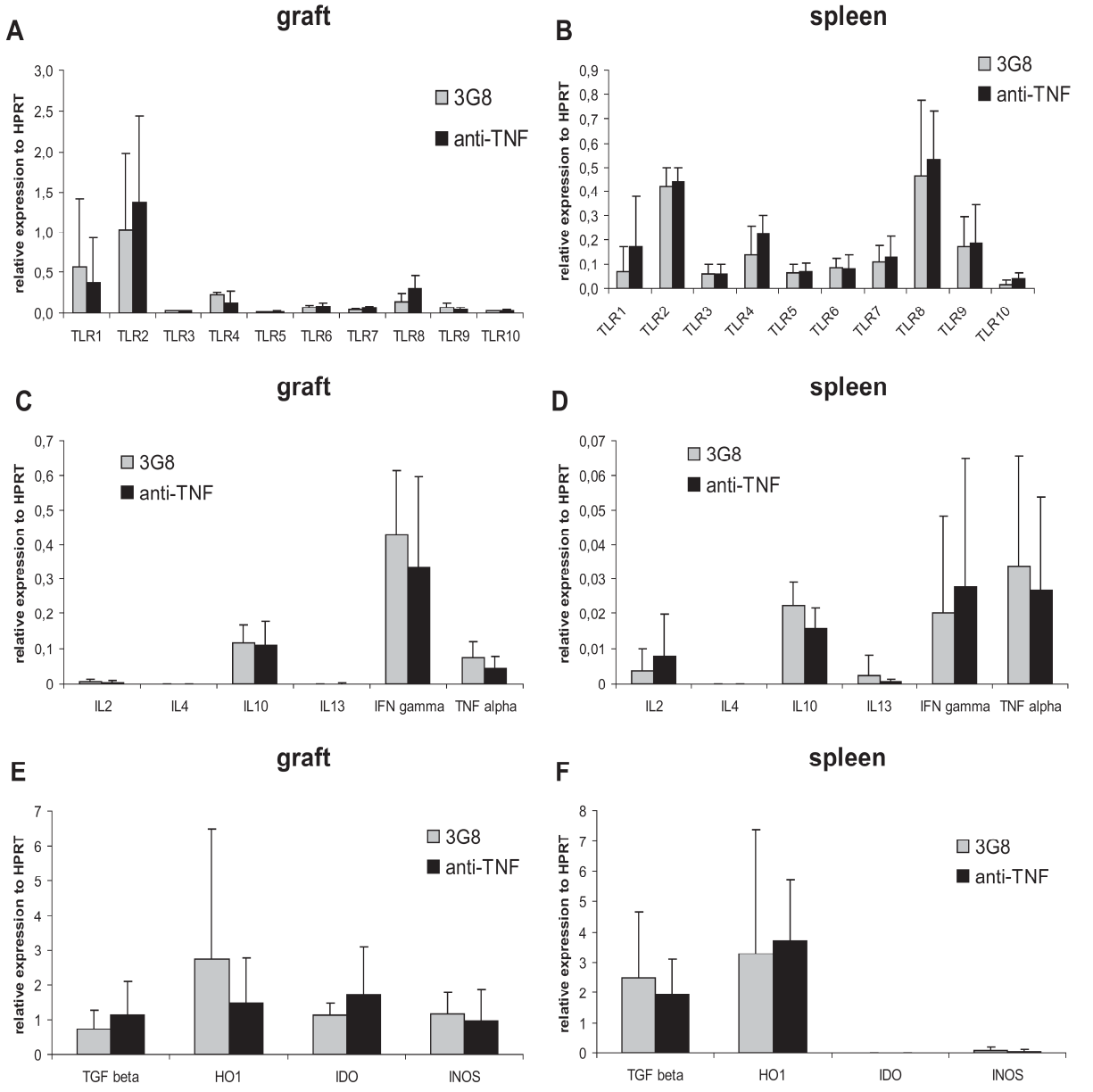

Figure 4. Expression of gene expression by qPCR in grafts (A, C, and E) and spleens (B, D, and F) of recipient animals at Day 5 after cardiac transplantation. The recipients were treated at the day of transplantation with a single injection of the 3G8 control antibody or the anti-TNF antibody. Data are represented as relative expression to the household gene HPRT. (A and B) TLRs. (C and D) T cell cytokines. (E and F) Regulatory molecules.

a TD alloantigen. Moreover, the absence of effect on TLR expression, the absence of additional effects of TNF blockade on cyclosporine A treatment, and the absence of effect in the xenograft model all corroborate to support that TNF blockade selectively affects TD humoral responses.

The second crucial finding of the study was that the abrogation of alloantibody induction by TNF blockade was associated with a decreased inflammatory infiltration of the graft and a prolonged graft survival. These findings are consistent with pioneering studies of TNF blockade in experimental transplantation models in the early 1990s, which showed in different models that TNF blockade doubles to triples the survival of allografts [20–23]. Several mechanisms have been suggested to contribute to this beneficial effect on graft survival, including decrease of the production of TNF itself by lymphocytes [24], modulation of ECM components important for the homing of lymphocytes to the graft [25], and modulation of cytokine and MHC expression [26]. Here, we propose that inhibition of the

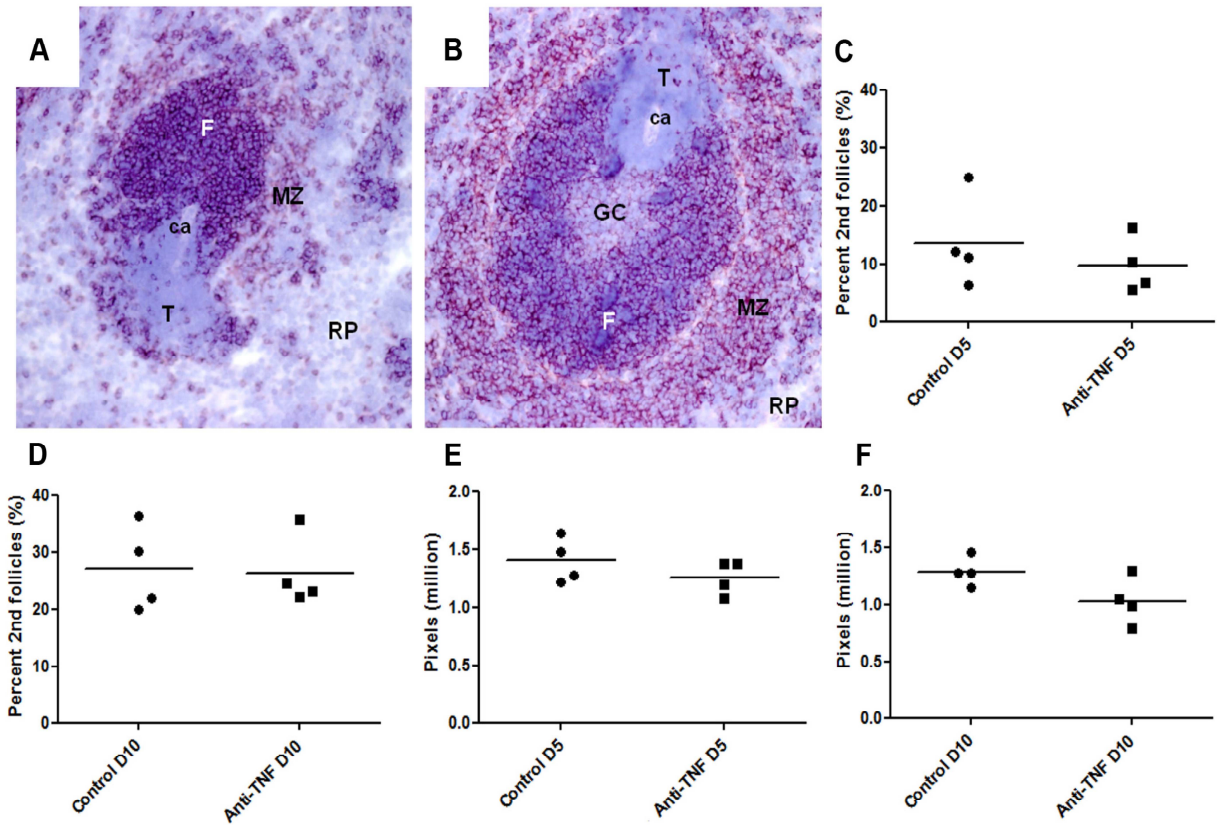

Figure 5. Analysis of germinal-center reactions in the spleen after transplantation. Immunostaining for B cells with CD45RA (clone OX-33) allowed identification of primary follicles (A) and secondary follicles (B) with germinal centers in the spleen of rats sacrified at Day 5 or at Day 10 after heart transplantation (original magnification, 200×). ca, Central arteriole; F, B cell follicle (dense concentration of $CD45RA^+$ B cells); GC, germical center (clear zone at the center of the follicle as a result of the absence of $CD45RA^+$ B cells; this zone is also devoid of T cells when anti-TCR staining is used); MZ, marginal zone (surrounding the follicle and the PALS with a less-dense concentration of $CD45RA^+$ cells); T, T cell-rich PALS (CD45RA-negative, but TCR+ in sections stained for TCR); RP, red pulp. Quantification of the percentage of secondary follicles to all follicles (C and D) or the total areas of all follicles (including primary and secondary follicles) measured in pixels (E and F) at Day 5 (C and E) and Day 10 (D and F) post-transplantation did not reveal differences between the control animals and the anti-TNF-treated animals.

alloantibody response is an additional mechanism contributing to the beneficial effects of TNF blockade in transplantation. The huge amounts of purified alloantibodies needed to perform transfer experiments in rats precluded us from formally proving this hypothesis, but the absence of effect on the other key factors in this model, including T cell cytokines and regulatory molecules, supports that the increased graft survival is related to effect of TNF blockade on the humoral response. The data obtained here with TNF blockade are also perfectly in line with our recent observation that attenuation of the humoral response by proteasome inhibition also delayed acute graft rejection in the same rat allotransplantation model [27].

These observations raise two important and related questions: what are the mechanisms of this effect, and to what extent can this effect be translated to other humoral responses in an experimental setting or more importantly, human disease? As to the mechanism, studies by Noorchashm et al. [16, 17], as well as other groups, have indicated that cognate T-B cell interactions are crucial in the maturation of the alloresponse and the subsequent rejection. In these interactions, which occur during germinal-center reactions, T cells will provide help to B cells for affinity maturation, but B cells can also play a crucial role as APCs in the activation of alloreactive T cells. Studies in knockout mice as well as in human RA treated with TNF blockers indicated that TNF signaling plays an important role in germinal-center reactions and thereby suggested that TNF blockade may impact the humoral alloresponse at this level. However, our experiments did not show alterations of the T cell cytokine profiles in spleen and graft or a decrease in the number or size of germinal-center reactions in the spleen. An additional observation in this context was that not only IgG but also IgM alloantibody formation was suppressed, suggesting that the effect of TNF blockade may occur at levels other than the germinal-center reaction as such. Dissection of the exact mechanism behind this abrogation of alloantibody induction will, however, require tools to track antigen-specific B cells to assess whether TNF blockade exerts its action at the level of naive B cell activation, the germinal-center reaction itself, or the postgerminal-center maturation toward fully differentiated plasma cells.

Our findings in this experimental model finally raise the question of whether the observed effect is more generally applicable to a TD humoral response and how TNF blockade could be used to modulate humoral rejection in human transplantation. We have preliminary evidence that TNF blockade in humans suppresses primary responses to TD vaccination (unpublished observations) but has only modest effects on pre-existing antibody titers [28], on recall responses against, for example, influenza [29, 30], and TI vaccinations against pneumococcal polysaccharides [31–34]. This supports the concept that anti-TNF not only abrogates the induction of alloantibodies but is more broadly suppressing primary TD B cell responses. As to the potential relevance for human applications, TNF blockade has barely been explored for clinical use in solid organ transplantation in humans despite ample evidence about the association of high levels of TNF with graft rejection in patients [35–37]. Pascher et al. [38, 39] have successfully used the chimerical anti-TNF mAb infliximab to treat steroid-resistant, acute cellular rejection of intestinal transplantation in four cases [38, 39]. The soluble TNFR construct etanercept was shown to have some modest effects on post-transplant cardiac allograft hypertrophy [40]. TNF blockade has, to the best of our knowledge, not been explored in other types of transplantation and/or graft rejection.

In conclusion, the present study provides experimental evidence that TNF blockade abrogates the induction of TD humoral responses. In this transplantation model, this effect was paralleled by a significant prolongation of graft survival. The exact immunological mechanisms as well as the relevance for human transplantation need to be explored in more detail.

AUTHORSHIP

S.B. and D.B. designed the study. S.B., V.J., B.V., H-L.M., F.B., C.U., F.M., and D.B. conducted in vivo experiments and G.F., V.J., and D.B., in vitro experiments. S.B., G.F., B.V., P.P.T., J-P.S., and D.B. interpreted the data. S.B., G.F., B.V., P.P.T., J-P.S., and D.B. prepared the manuscript.

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KEY WORDS:
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ARTICLE 4

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Indirect CD4+ TH1 Response, Antidonor Antibodies and Diffuse C4d Graft Deposits in Long-Term Recipients Conditioned by Donor Antigens Priming

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Priming of recipients by DST induces long-term survival of mismatched allografts in adult rats. Despite these recipients developing inducible T regulatory cells able to transfer long-term graft survival to a secondary host, a state of chronic rejection is also observed. We revisited the molecular donor MHC targets of the cellular response in acute rejection and analyzed the cellular and humoral responses in recipients with long-term graft survival following transplantation. We found three immunodominant peptides, all derived from LEW.1W RT1.D^u molecules to be involved in acute rejection of grafts from unmodified LEW.1A recipients. Although the direct pathway of allorecognition was reduced in DST-treated recipients, the early CD4$^+$ indirect pathway response to dominant peptides was almost unimpaired. We also detected early and sustained antidonor class I and II antibody subtypes with diffuse C4d deposits on graft vessels. Finally, long-term accepted grafts displayed leukocyte infiltration, endarteritis and fibrosis, which evolved toward vascular narrowing at day 100. Altogether, these data suggest that the chronic graft lesions developed in long-term graft recipients are the result of progressive humoral injury associated with a persisting indirect T helper response. These features may represent a useful model for understanding and manipulating chronic active antibody-mediated rejection in human.

Key words: Alloantibodies, allotransplantation, antibody-mediated rejection, chronic allograft rejection, complement C4d, heart allograft, transplantation

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Introduction

Long-term graft survival through manipulation of the host 'peripheral' immune response has been achieved in a number of experimental models (1–4). However, closer attention to the graft itself has recently shown that some of the recipients with long-term graft survival (>100 days), develop histological lesions of chronic rejection (5–7). Such lesions can coexist with the presence of regulatory T cells that are able to transfer the capacity of immediate graft 'acceptance' to naïve hosts, but unable to prevent the reappearance of chronic lesions (5,7,8). This reinforces the need for careful pathological analyses, particularly in the usual non-life-sustaining, heterotopic heart graft model, to assess the mechanisms leading to chronic rejection. Moreover, these models may contribute to the understanding of chronic rejection in humans.

Using molecularly defined donor-derived MHC peptides, we revisited the direct and indirect pathway responses of CD4$^+$ and CD8$^+$ T cells as well as the humoral response following the protocol of DST-induced long-term graft survival. We show that, according to previous data emphasizing transcriptional downmodulation of a number of Th1 cytokines (9), there was strong inhibition of the IFNγ response triggered by the direct pathway of allorecognition in this model. CD8$^+$ T cells, despite undergoing a strong clonal selection, lost their capacity to respond to dominant donor peptides in the indirect pathway. However, we also noted an unambiguous CD4$^+$-mediated IFNγ response by the indirect pathway of allorecognition of dominant donor MHC peptides in the week following transplantation. Furthermore, we show that long-term surviving animals developed an unbiased and sustained antidonor humoral response with diffuse intragraft C4d deposition and severe cellular inflammation associated with vascular occlusion suggestive of an 'active humoral' type of chronic rejection recently defined in humans (10). Altogether, these data

shed new light on the classical model of tolerance induction in the rat and provide evidence that this model carries several features observed in chronic humoral rejection in human transplantation.

Materials and Methods

Animals and transplantation

Naïve adult MHC-mismatched congeneic LEW.1A (RT1^a), LEW.1W (RT1^u) and BN (RT1^n) rats were purchased from Janvier (France). LEW.1A, LEW.1W or BN heart grafts were implanted heterotopically onto LEW.1A recipients using the Ono and Lindsey technique (11) and function was monitored daily by palpation through the abdominal wall. Rejection was defined as complete cessation of heartbeat.

Donor-specific transfusions

Blood (1 mL) collected from a LEW.1W donor by cardiac puncture was injected intravenously into LEW.1A recipients on pretransplant day −14 and −7 (12). The regimen induces donor MHC-specific long-term allograft survival (>100 days), but with grafts showing signs of chronic rejection at day 100 (6).

Peptide synthesis

Synthetic peptides 16 amino-acids in length and spanning the polymorphic regions of RT1.A^u (α1, α2 and α3 domains), RT1.B^u (β1 domain) and RT1.D^u (β1 domain) or the invariable regions of RT1.A (α2 and α3 domains) molecules (13) (Figure 1) were prepared by Genepep (France). Lyophilized peptides were dissolved in deionized water/0.4% DMSO and used in cultures at a final concentration of 10 μg/mL.

Cells and cell sorting

Cells from mesenteric lymph nodes and spleen cells isolated by passing through a stainless steel mesh. Graft infiltrating cells were extracted from hearts after cutting the graft into small pieces, incubated with collagenase D (2 mg/mL Boerhringer Mannheim) for 20 min at 37°C and isolated by centrifugation over Ficoll. Blood samples were collected in heparin tubes and PBMC were isolated after erythrocytes lysis by osmotic shock. Spleen T cells were prepared using nylon wool adhesion columns followed by depletion of NK cells, monocytes and B cells with specific mAbs (clone 3.2.3, clone OX42 and clone HIS24 (BD Pharmingen) respectively), followed by anti-mouse IgG-coated dynabeads (Invitrogen). CD4$^+$ or CD8$^+$ T cells were negatively sorted using anti-mouse IgG dynabeads after T-cell staining with anti-CD8 (OX8) or anti-CD4 (W3/25) mAbs (European collection of cell, Salisbury, UK). CD4$^+$CD25^{high} T cells and their negative counterparts were sorted using an ARIA flow cytometer (BD) after staining with anti-CD4 FITC and anti-CD25 Alexa 647 mAbs (W3/25 and OX39).

IFNγ ELISPOT assays

ELISPOT plates (BD Biosciences) were coated with purified anti-rat IFNγ (5 μg/mL), incubated at 4°C overnight and blocked 2 h with RPMI 1640 medium containing 10% Fetal Calf Serum (FCS) Glutamine (Sigma) and 1% Penicillin-Streptomycin-L-Glutamine (Sigma). For assessment of IFNγ-producing cell frequency, 4×10^5 total fresh cells from spleen, lymph nodes, blood or grafts from untreated or DST-treated recipients, were seeded in triplicate in the presence of irradiated donor splenocytes (direct pathway), 16 amino-acid peptides from LEW.1W MHC molecules (10 μg/mL) (Genepep) (Indirect pathway) or medium alone. To test the T-cell responses in the different subsets, 2×10^5 CD4$^+$, CD8$^+$, CD4$^+$CD25^{high}-depleted T cells, non-T cells, total recipient spleen T cells or 2.5×10^4 CD4$^+$CD25^{high} T cells were stimulated in the presence of 2×10^5 irradiated recipient splenocytes. Cells were incubated for 24 h at 37°C 5% CO_2. For detection of spots, 2 μg/mL of biotinylated antirat IFNγ were added and plates incubated for 2 h, followed by a 1-h incubation with streptavidin-horseradish peroxidase. The plates were developed using 20 μL of 3-Amino-9-ethylcarbazole (AEC chromogen) added per 1 mL of AEC Substrate. The number of spots was counted on a computer-assisted-enzyme-linked immunospot image analyzer (AID).

Serum antibody detection

For the assessment of posttransplant antidonor IgG subtypes, decomplemented sera (30 min at 56°C) from naïve, rejecting (day 7 and 10), or DST-treated animals and syngeneic recipients (day 7, 30 and 100) were incubated with donor splenocytes. Donor splenocytes were subsequently stained with purified mouse anti-rat IgG1, IgG2a, IgG2b or IgG2c mAbs (University of Louvain, kindly provided by F. Nisol) and revealed with PE-conjugated donkey anti-mouse mAbs (Jackson ImmunoResearch). At the same time, donor splenocytes were stained with Alexa 647-conjugated anti-rat TCR alpha/beat (R7–3) mAbs obtained from the European collection of animal cell culture (Salisbury, UK). Cells were collected on a FACScan and analyzed using CellQuest software (BD Biosciences). The analysis was performed on TCR positive (for anticlass I antibody detection) or negative cells (for anticlass I and class II antibody detection). Data were expressed as mean PE fluorescence intensity.

In vitro *production and characterization of alloantibodies*

Organocultures were performed as previously described (14). Microdissected heart allografts were recovered from 4 DST-treated rats 100 days after transplantation or 3 naïve hearts and placed in cold sterile RPMI 1640 medium containing Glutamine (Sigma) and 1% Penicillin-Streptomycin-L-Glutamine (Sigma) and 25 μg/mL Fungizone (GIBCO). Tissues were washed three times in fresh medium. Each sample was fragmented with a sterile razor blade, placed into 2 mL of fresh medium, and cultured in 12-well plates at 37°C. Culture supernatants were recovered after 5 days of culture. The analysis of the specificity (anticlass I and II or anticlass I) of the antibodies present in the organoculture-derived supernatants was performed by flow cytometry by using LEW.1W (donor) splenocytes. One hundred microliters of each organoculture-derived supernatant were incubated with 2×10^5 LEW.1W cells for 30 min at 4°C. The binding of antibodies on the cell surface was then determined with purified mouse anti-rat IgG1, IgG2a (University of Louvain, kindly provided by F. Nisol) and revealed with PE-conjugated donkey anti-mouse mAbs (Jackson ImmunoResearch). At the same time, cells were stained with FITC-conjugated anti-rat HIS24 mAbs (BD Pharmingen). Cells were collected on a FACScan and analyzed using CellQuest software (BD Biosciences, Le Pont de Claix, France). The analysis was performed on HIS24 negative (class I) or positive (class I and II) cells. Data are expressed as mean fluorescence intensity.

Histology and immunostaining for C4d

Heart allografts were removed and fixed in 4%-diluted fomalin. Tissue blocks were routinely processed and embedded in paraffin. Serial sections of 6 μm were obtained for hematoxylin-eosin-saffron staining. Leukocyte infiltration, fibrosis, vascular damage and luminal occlusion of arteries were evaluated.

C4d deposition was localized by immunoperoxidase staining with an affinity-purified polyclonal rabbit antibody to C4d kindly provided by K. Murata (15) (department of pathology, John Hopkins Medical Institutions, Baltimore MD) used at a 1:1000 dilution. Deparaffinized tissue sections were submitted to heat-induced antigen retrieval and processed using the Menarini revelation kit (A. Menarini diagnostics) with 3,3'-diaminobenzidine and a hematoxylin counterstain.

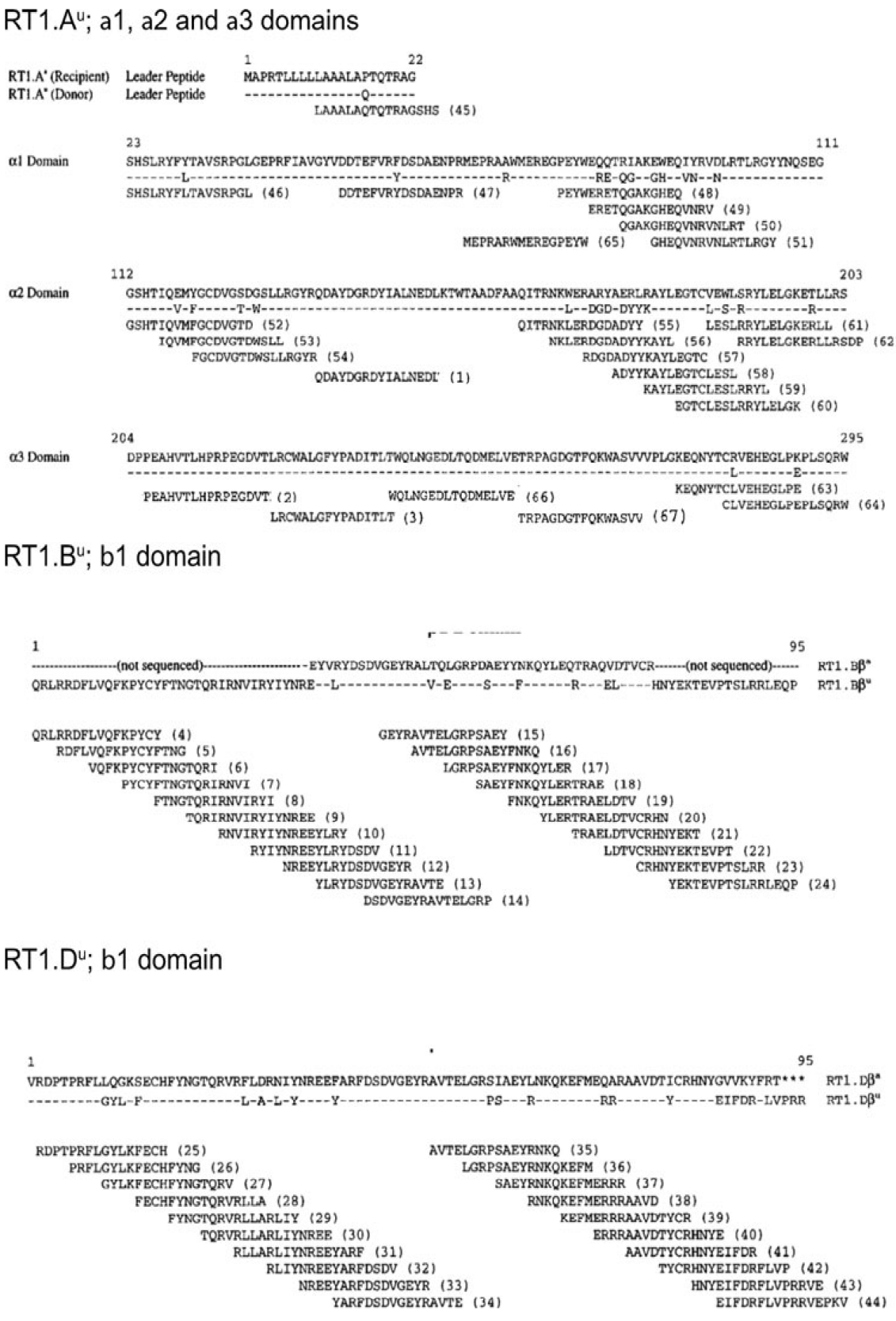

Figure 1: Sequence alignment and peptides. Sixteen amino acid-long overlapping peptides (61 peptides) were chosen along the polymorphic regions of the RT1A^u (α1, α2 and α3 domains), RT1.B^u (β1 domain) and RT1.D^u (β1 domain) molecules. Peptides 1, 2, 3, 66 and 67 were chosen in the nonpolymorphic sequences of the RT1A^u molecule.

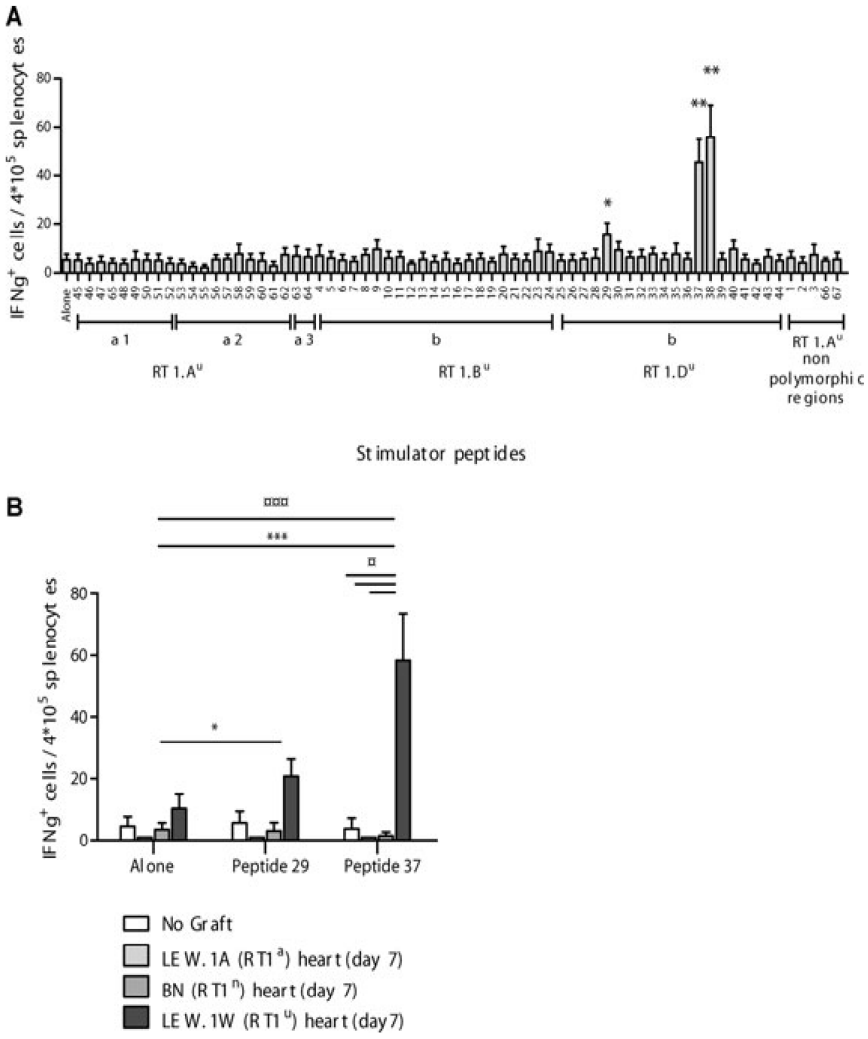

Figure 2: Immunodominant peptides. (A) *Immunodominant peptides are derived from donor RT1.D^u molecules.* Splenocytes (4×10^5) from untreated animals were obtained 7 days after transplantation and stimulated in IFNγ ELISPOT assays with peptides from donor RT1A^u (α1, α2 and α3 domains), RT1.B^u (β1 domain) and RT1.D^u (β1 domain) molecules. Bars represent the mean frequency of IFNγ-producing cells ± SEM for n = 6 animals. (B) *Peptides are specific.* LEW.1A rats received a heart allograft from syngeneic (RT1^a), BN (RT1^n) or LEW.1W (RT1^u) rats 7 days before sample collection. LEW.1A splenocytes were stimulated for 24 h in an IFNγ ELISPOT assay with peptides 29 and 37 from RT1.D^u molecules. Results ± SEM are representative of at least three animals. Statistical significance was evaluated using the Mann–Whitney test comparing peptide-stimulated cells to unstimulated cells (*$p < 0.05$ and **$p < 0.01$) or using the Kruskall–Wallis test to compare each graft type (¤$p < 0.05$).

Statistical analyses

All statistical analyses were performed using the Mann–Whitney test or Kruskall–Wallis tests, as appropriate. A $p < 0.05$ was considered significant.

Results

Identification of immunodominant peptides during acute rejection in unmodified LEW.1A recipients of LEW.1W hearts

Rejecting recipients recognize immunodominant peptides derived from donor MHC class II RT1.D^u molecules: We first assessed the indirect alloresponse following LEW.1W (RT1^u) heart transplantation to the congenic mismatched LEW.1A (RT1^a) recipients. At day 7 posttransplantation (time of rejection), a significant frequency of splenocytes responded to peptides 29, 37 and 38 (Figure 2A *$p < 0.05$ **$p < 0.01$ versus unstimulated). All of the stimulator peptides were from polymorphic regions of the β1 domain of RT1.D^u molecules. For subsequent analyses, we focused on peptides 29 and 37, since peptide 38 always displayed the same profile as peptide 37. Splenocytes harvested from untreated LEW.1A rat recipients of syngeneic (RT1^a) or BN (RT1^n) hearts at day 7 did not respond to the RT1^u dominant peptides, indicating a specific anti-RT1^u response (Figure 2B). Figure 3 shows that most of the response directed against peptide 29 in the rejecting LEW.1A recipients involved CD4$^+$ T cells and that CD8$^+$ were much more efficient than CD4$^+$ splenocytes at mounting a response against peptide 37 (Figure 3 *$p < 0.05$).

Indirect pathway responses against dominant RT1^u peptides are detectable as early as day 5 in rejecting animals: We next assessed the kinetics of the indirect pathway response, from day 3 to 14 after transplantation in the spleen, lymph nodes, blood and graft from LEW.1A rejecting recipients and compared it to the direct response (Figure 4A, B). The response toward peptide 29 was dominant in the spleen, reaching a peak 5 days after transplantation (Figure 4B ¤$p < 0.05$). The responses in the three other compartments tested presented a similar profile although the detectable IFNγ response did not reach significance in the lymph nodes or in the graft (Figure 4B). The most

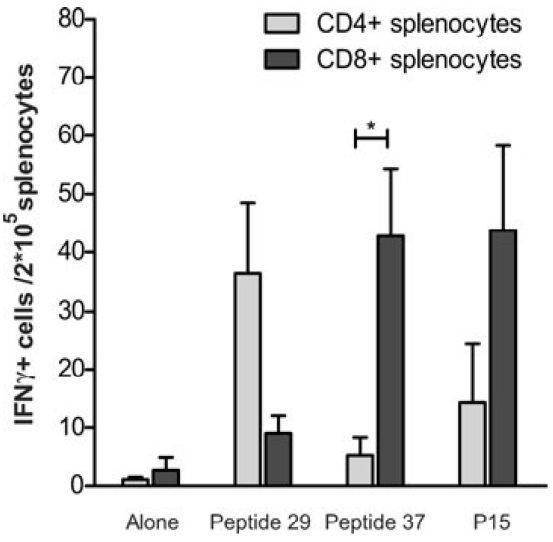

Figure 3: The responses toward peptides 29 and 37 are mediated by CD4$^+$ and CD8$^+$ T cells, respectively. CD4$^+$ or CD8$^+$ T splenocytes were purified from untreated recipients 5 days after transplantation. The 2×10^5 cells were stimulated for 24 h in an ELISPOT assay with peptides in the presence of 2×10^5 irradiated recipient splenocytes. Statistical significance was evaluated using the Mann–Whitney test (*$p < 0.05$). Results ± SEM are representative of six animals.

vigorous response toward peptide 37 was observed in the blood, reaching a peak at day 7 (Figure 4B ¤$p < 0.05$ ¤¤$p < 0.01$). The response to peptide 37 peaked on day 7, except in the graft where a peak was observed on day 5. The response toward irradiated donor cells (direct pathway) was more vigorous, reaching a peak at day 5 in the spleen, lymph nodes and on day 7 in the blood (Figure 4A ¤$p < 0.05$ and ¤¤$p < 0.01$). In the rejecting hearts, a direct response was detected as early as day 3, was maximal on day 5 and persisted until day 14 (Figure 4A).

Early activation of CD4$^+$ T cells through the indirect pathway in DST-treated recipients

We then assessed the effect of DST treatment on the cellular response.

First, DST alone (without transplantation) did not trigger a measurable indirect response (Figure 4B, day 0). However, we observed an increased response toward irradiated donor cells (direct pathway) in the blood before transplantation in DST-treated animals (Figure 4A *$p < 0.05$).

In the first days following transplantation, a significant T-cell response toward peptide 29 was still detected in the spleen from DST-treated recipients of LEW.1W hearts (Figure 4B ¤$p < 0.05$). However, the peak of the response was delayed to day 7 compared to that in untransfused recipients. As expected, this response was CD4$^+$ T-cell-mediated (Figure 5A *$p < 0.05$). In the hearts from DST-treated recipients, the frequency of peptide 29-specific cells was similar to that observed in untransfused rejecting animals, although delayed to day 7 (Figure 4B). The indirect response toward peptide 29 was inhibited in the lymph nodes and in the blood (*$p < 0.05$ **$p < 0.01$). A strong inhibition of the T-cell response to peptide 37 was seen in the graft compared to untransfused recipients on day 5 (Figure 4B *$p < 0.05$). The inhibition, which was also seen in the spleen, could not be restored by depletion of CD4$^+$CD25^{high} T cells (Figure 5B). The direct pathway of allorecognition was also reduced in the spleen (Figure 4A day 3 and 7 *$p < 0.05$ **$p < 0.01$) and in the lymph nodes and blood (Figure 4A day 5 and 7 **$p < 0.01$). In the hearts, the IFNγ responses was significantly reduced compared to rejecting animals (Figure 4A day 5 and 7 *$p < 0.05$).

Hundred days after transplantation, no response to donor peptides or irradiated donor cells could be detected in the spleen, lymph nodes or blood (Figure 4B). In the hearts, however, we found a strong frequency of graft infiltrating cells producing IFNγ without the need for further stimulation *in vitro* with peptides or donor cells (Background DST, gray line, Figure 4A, B) suggesting an 'exhaustive' response *in situ*.

Taken collectively, these data show that despite a strong reduction in the CD4$^+$ direct pathway and the CD8$^+$ response, the indirect allorecognition pathway of CD4$^+$ T cells still persisted in apparently DST-treated recipients 7 days after transplantation.

Unbiased and sustained antidonor IgG antibodies in the sera of DST-treated recipients

Anticlass I and II IgG subtypes were analyzed in the blood of naive, untransfused (day 7, time of rejection), syngeneic (LEW.1A recipients of LEW.1A heart) or DST-treated recipients (days 7, 30 and 100 posttransplantation). Seven days after surgery, antidonor class I and antidonor class I and II IgG1 (Th2), IgG2a, IgG2b and IgG2c (Th1) levels were roughly similar to those observed in syngeneic animals (Figure 6). However, the quantity of antidonor antibodies of each subtype increased until day 100. The quantity of anticlass I and anticlass I and II antibodies was higher in long-term surviving DST-treated animals compared to DST-treated animals on day 7 ($p < 0.05$ for anticlass I and anticlass I and II IgG1 and IgG2a) and to syngeneic controls tested at the same time ($p < 0.05$ for anticlass I IgG1, IgG2a and IgG2b and for anticlass I and II IgG1, IgG2a, IgG2b and IgG2c).

Both antibodies against class I and II molecules are produced in heart allografts with chronic rejection

LEW.1W heart allografts from DST-treated recipients (day 100) or naïve LEW.1W hearts were cultured so as to collect immunoglobulins produced within these tissues. The supernatants of these cultures were tested on LEW.1W donor splenocytes. Reactivity of antibodies

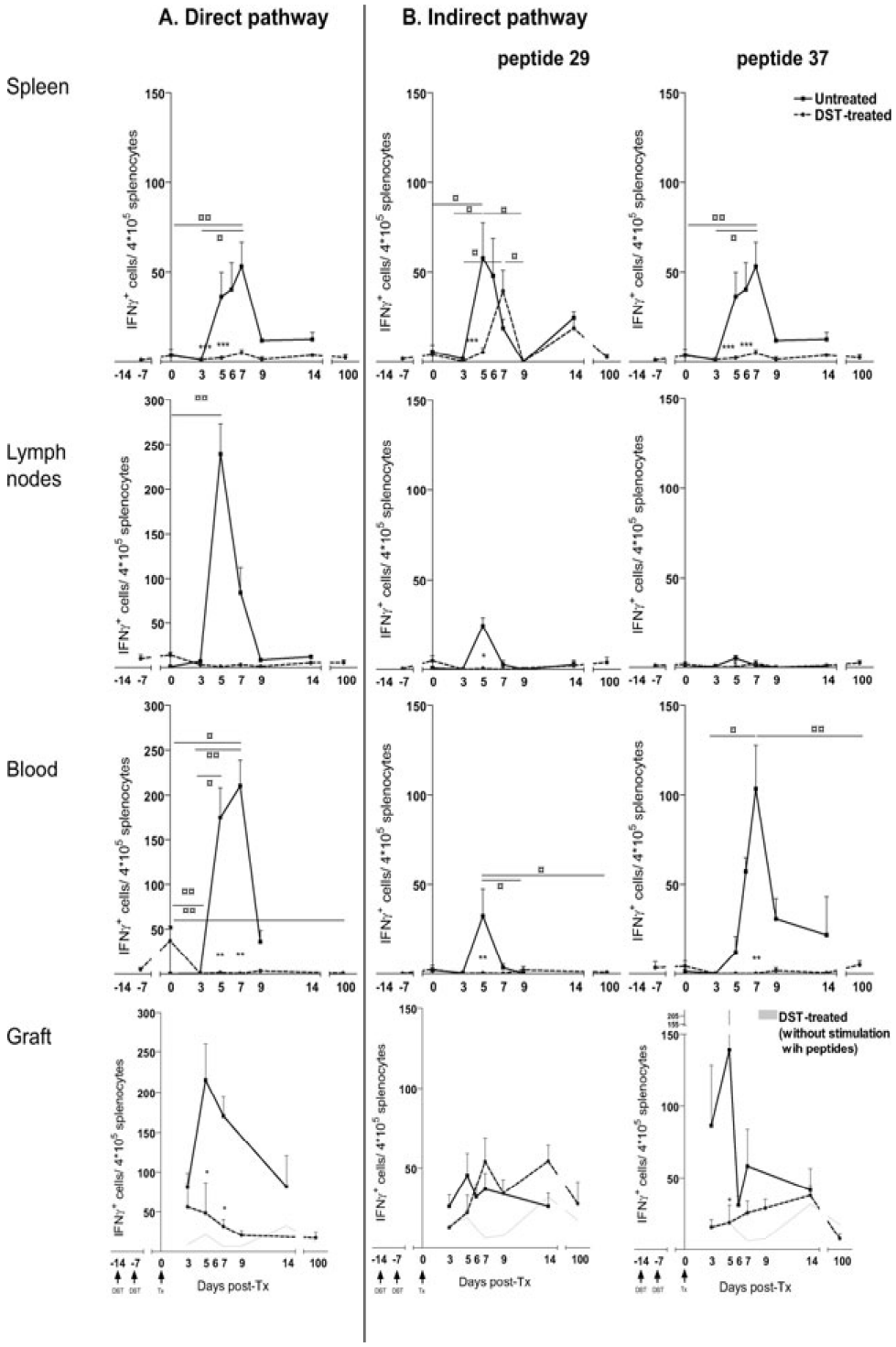

Figure 4: Direct and indirect pathways of allorecognition in rejecting or DST-treated animals. The 4×10^5 cells harvested from spleen, lymph nodes, blood and grafts before transplantation (day −14, −7 and day 0) and from day 3 to 100 after transplantation of LEW.1W heart allografts, were stimulated 24 h in IFNγ ELISPOT assay. (A) *Direct pathway*. LEW.1A cells were stimulated with donor irradiated splenocytes (1×10^5). (B) *Indirect pathway*. Cells were tested for their reactivity against peptides 29 and 37 derived from donor MHC class II (RT1.D^u) molecules. (A) and (B) DST-treated recipients (circles, dotted lines), untreated recipients (squares, full lines). The gray line in the graphs showing the response in the graft, represent the frequency of IFNγ-producing cells in the absence of stimulation *in vitro* (Background DST). Note that because frequencies of activated cells were higher in the direct than in the indirect pathway, scales are different. Significant differences were calculated by the Kruskall–Wallis test (¤) and by the Mann–Whitney U test (¤ and $*p < 0.05$; ¤¤ and $**p < 0.01$). Results at each day ± SEM are representative of at least three animals.

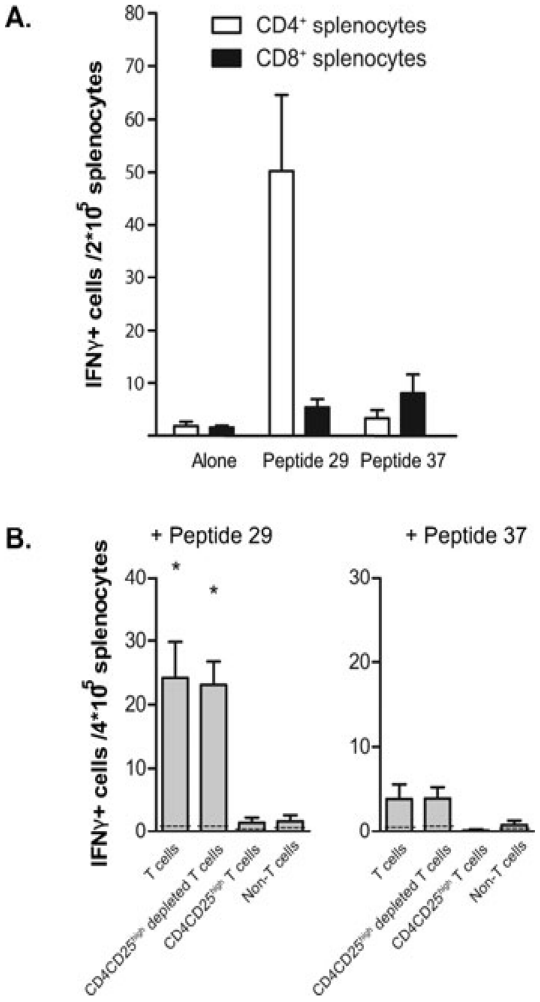

Figure 5: Indirect response in DST-treated animals. (A) *The indirect response toward peptide 29 is mediated by CD4+ T cells in DST-treated recipients.* $CD4^+$ or $CD8^+$ T splenocytes were purified from DST-treated (day 7) recipients. The 2×10^5 cells were stimulated for 24 h in an ELISPOT assay with peptides 29 and 37 in the presence of 2×10^5 irradiated recipient splenocytes. Statistical significance was evaluated using the Mann–Whitney test ($^*p < 0.05$). Results ± SEM are representative of 5 animals. (B) *$CD4^+CD25^{high}$ depletion had no effect on the indirect response.* The 4×10^5 enriched T cells, 4×10^5 $CD4^+CD25^{high}$ depleted T cells or 2.5×10^4 $CD4^+CD25^{high}$ or non-T cells from DST-treated recipients (day 7) were stimulated in the presence of 1×10^5 irradiated recipient splenocytes with peptides for 24 h in an IFNγ ELISPOT assay. Results ± SEM are representative of seven animals. Statistical significance was evaluated using the Mann–Whitney test ($^*p < 0.05$).

against these cells was evaluated by flow cytometry. The amounts of IgG1 or IgG2a antibodies against the donor MHC I and II molecules were far higher in heart allografts from DST-treated animals than in hearts from naïve LEW.1W hearts (Figure 7 $p < 0.05$).

Chronic rejection lesions are associated with C4d deposits in long-term surviving heart allografts

We next investigated the endomyocardial histology of biopsies harvested from day 7 to 320 in DST-treated recipients. An intense mononuclear cell infiltrate appeared on day 7 after transplantation (Table 1(column A) grade 4), reached a maximum on day 30 and then became moderate until day 320 (Table 1(column A)). A minor mononuclear cell infiltrate was observed in control, syngeneic grafts (Table 1(column A), Figure 8A). Focal fibrosis was detected as early as day 14 (Table 1(column B)) and became progressively diffuse and marked until day 320 (Table 1(column B)). Mild intimal arteritis, defined as lymphocyte infiltration beneath the endothelium, was first observed on day 14 (Table 1(column C)) and persisted until day 60 (Table 1(column C), Figure 8C). Intimal arteritis became more severe on day 100 with the beginning of myofibroblastic cell proliferation (Table 1(column C)), associated with fibrointimal thickening on day 240 and 320 (Table 1(column C)), leading to 80% of luminal occlusion in most arteries (Table 1, Figure 8D). No C4d binding was observed in syngeneic controls (Figure 8E). Figure 8F shows diffuse and abundant C4d deposits along heart capillaries (Figure 8 arrow a) and arteries (arrow b) as early as day 7, as in rejecting animals, indicating persisting complement activation in the grafts of DST-treated animals.

Discussion

Much attention has recently been paid to the histological lesions of long-term surviving recipients (>100 days) after tolerance induction. It has been established that chronic rejection progressively develops following tolerance induction procedures such as donor blood transfusions or costimulatory blockade (16,17), even when long-term surviving recipients harbor regulatory T cells capable of transferring to a secondary naive host, the ability to accept a graft (5,7,8,18,19). In this study, long-term survival of LEW.1W heart allografts was induced by DST priming 14 and 7 days before transplantation in LEW.1A recipients (12). We demonstrated that recipients of long-term accepted grafts developed in the first days following transplantation an indirect $CD4^+$ IFNγ response, associated with the chronic production of antidonor antibodies. Furthermore, we showed that long-term surviving grafts exhibited lesions of chronic rejection with diffuse C4d deposits on donor tissue capillary walls. We hypothesize that this model may be useful for understanding antibody-mediated chronic rejection in human allografts and for testing the efficacy of therapeutic strategies aimed at blocking antibody-mediated rejection.

To assess the cellular response in DST-treated recipients, we first needed to precisely characterize the dominant donor MHC peptides eliciting a T-cell response during acute rejection in unmodified LEW.1A recipients of MHC incompatible LEW.1W hearts. We measured the cellular response using the ELISPOT assay that enables

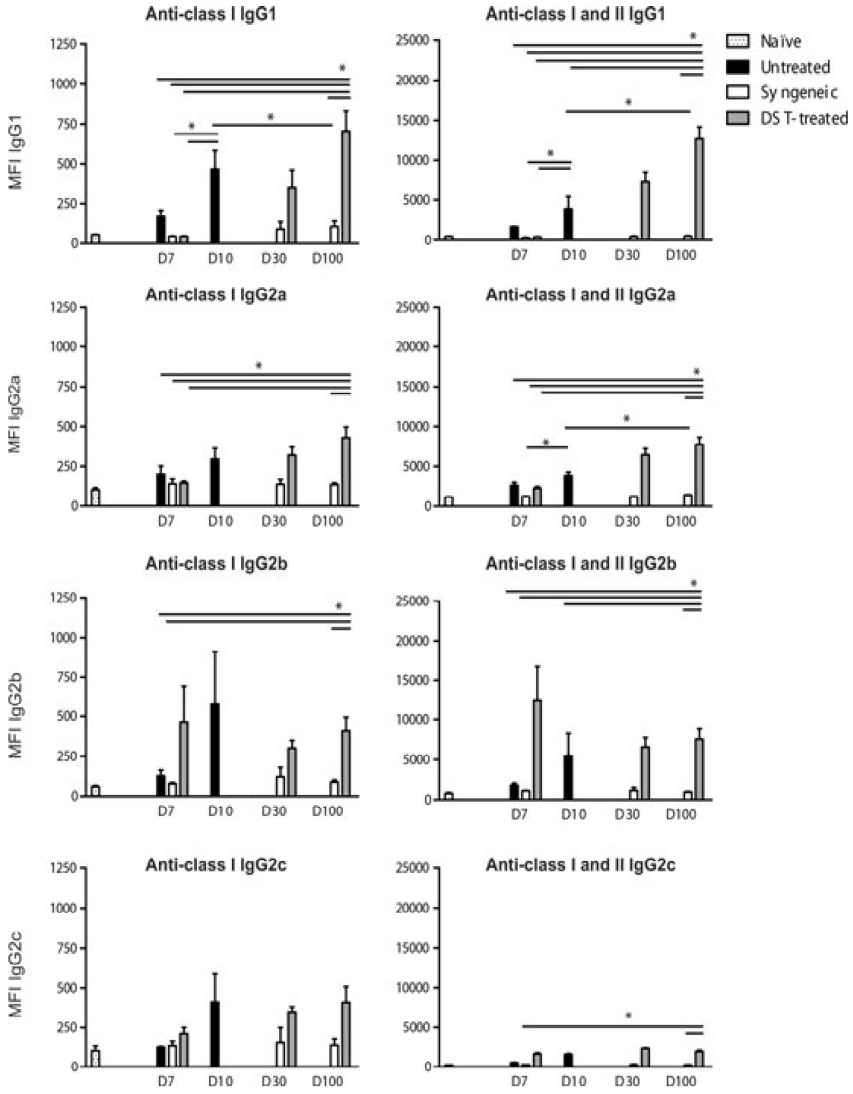

Figure 6: Unbiased and sustained circulating antidonor MHC class I and class II antibodies in DST-treated animals. Assessment of posttransplant antidonor class I and class I and II IgG subtypes (IgG1, IgG2a, IgG2b, IgG2c) in the sera of 2 naïve, 3 rejecting (day 7), 5 rejecting (day 10) or 3 DST-treated animals and 3 syngeneic recipients (day 7, 30 and 100). For anticlass I and II antibody detection, decomplemented sera ($1/20^{e}$) were incubated with donor splenocytes. Cells were then stained with purified mouse anti-rat IgG1, IgG2a IgG2b or IgG2c mAbs and revealed with PE-conjugated donkey anti-mouse mAbs. At the same time, splenocytes were stained with Alexa 647-conjugated anti-rat TCR (R73) mAbs (T cells). Cells were analyzed using a FACScan with CellQuest software (BD Biosciences). The analysis was performed on TCR positive cells (for antidonor class I antibody detection) or TCR negative cells (for antidonor class I and class II antibody detection). Data are expressed as mean PE fluorescence channel. Statistical significance was evaluated using the Mann–Whitney test (*$p < 0.05$).

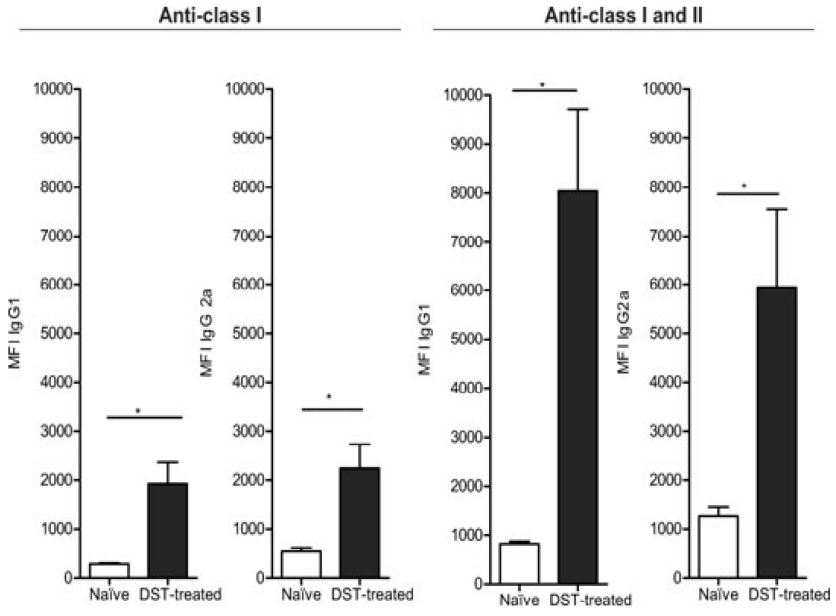

Figure 7: IgG1 and IgG2a antibodies against class I and II MHC molecules are locally produced in hearts presenting chronic rejection. The presence of antidonor class I and II IgG1 and IgG2a antibodies in the supernatant of organocultures of hearts from DST-treated animals was assessed by flow cytometry using LEW.1W donor splenocytes and compared to that observed in naïve LEW.1W animals. Statistical significance was evaluated using the Mann–Whitney test (*$p < 0.05$).

Table 1: Histological analyses of heart allografts

Groups	Leukocytes (A)	Fibrosis (B)	Vascular damage (C)	Luminal occlusion (D)	% occluded arteries	Number of arteries examined	C4d staining DST/ syngeneic
Syngeneic D140	1;1;1	1;0;0	0;0;0	0;0;0	0;0;0	12;12;10	
DST D7	4;4;4	0;0;0	0;0;0	0;0;0	0;0;0;	21;26;22	± (×3)
DST D14	4;4	0;1	1;0	2;0	30;0	40;42	± (×2)
DST D30	4;4;3	1;1;3	1;0;1	1;0;1	3;0;4	36;57;56	± (×3)
DST D60	3;2–3;2–3	2;2;4	1;0;1	2;0;1	5;0;8	18;19;13	± (×3)
DST D100	2;2;2–3	3;2;4	2;0;2	2;0;4	4;0;81	28;20;16	± (×3)
DST D140	2;2;3	2;1;3	2;2;2	4;2;3	44;37;76	9;8;17	
DST D240	1;1;2	2;2;2	2;3;3	3;2;2	73;7;50	8;14;8	
DST D320	3;2;3	3;3;4	2;2;3	3;4;4	50;33;70	10;6;10	

Hematoxylin-eosin-saffron staining of heart allografts from DST-treated (day 7, 14, 30, 60 and 100) or syngeneic (day 140) animals. (A) Leukocytes: none = 0; minor = 1; moderate = 2; dense = 3; intense = 4. (B) Fibrosis: none = 0; focal = 1; diffuse minor = 2; diffuse moderate = 3; diffuse marked = 4. (C) Vascular damage: normal = 0; leukocyte adhesion to endothelial cells = 1; inflammatory intimal proliferation = 2; fibrointimal thickening = 3; atheroma = 4. (D) Luminal occlusion: none = 0; <20% = 1; 20 to 50% = 2; 50 to 80% = 3; >80% = 4.

quantification of the frequency of T cells already committed to antigen. IFNγ was chosen since it has been shown to offer the best correlation to rejection in various studies (20–22). Using this method, we first identified three 16 amino-acid long immunodominant peptides from the $RT1^u$ MHC of LEW.1W donors (referred to as peptides 29, 37 and 38), which triggered a potent IFNγ response during LEW. 1W heart rejection as early as day 5 after transplantation for peptide 29 and day seven for peptides 37 and 38. Testing of purified $CD4^+$ or $CD8^+$ subsets revealed that the responses toward peptides 29 and 37 were $CD4^+$ and $CD8^+$-mediated, respectively. Several studies have previously shown that indirectly primed T cells are involved in allograft rejection in many experimental models (23–26) and in humans (27,28). We have also shown that in the same strain combination, LEW. 1W hearts depleted of resident DC are acutely rejected following the same tempo, indicating that the indirect pathway can rapidly develop in unmodified LEW. 1A hearts (29,30). The dominant peptides concerned the donor class II $RT1.D^u$ molecule sequence, in agreement with data obtained in different strain combinations (31–33). None of these peptides activated cells harvested from naive LEW. 1A, syngeneic graft recipients (LEW. 1A to LEW. 1A) or $RT1^n$ BN heart recipients, indicating a specific response. A higher frequency of alloreactive cells was found to result from the direct pathway of activation, notably on day five and seven after transplantation. This was in agreement with previous data from Benichou et al. who showed that acute rejection was principally governed by the direct pathway.

Hyporesponsiveness of directly activated alloreactive T cells (9,29) associated with an overall downmodulation of Th1 cytokines (34) and inflammatory monokines (35) was observed in the first days following transplantation in the same strain combination. We confirmed a strong reduction in the direct allorecognition pathway in DST-treated recipients in the first days following transplantation in all tested compartments. Considerable inhibition of the indirect pathway of allorecognition mediated by $CD8^+$ IFNγ- producing cells was also observed in all compartments tested from DST-treated recipients in the first days after transplantation. Because depletion of $CD4^+CD25^{high}$ regulatory cells could not restore the $CD8^+$ IFNγ response, it is unlikely that naturally occurring T regulatory cells are involved in this inhibition. Our data also show an early activation of $CD4^+$ T cells specific to peptide 29 in the spleen and graft of DST-treated recipients in the first days following transplantation, and this response was as robust as in rejecting animals.

Because several studies have shown a close relationship between chronic rejection and the indirect pathway of allorecognition (28,36), we also analyzed T-cell responses 100 days after transplantation, when chronic lesions are well established. We found the persistence of IFNγ-producing graft infiltrating cells without the need for further stimulation *in vitro* with donor cells or peptides suggesting that graft infiltrating cells themselves are strongly activated.

We then asked whether IgG alloantibodies could be detected in this model and could be implied in the chronic rejection process. In the RA ($RT1^p$) to PVG ($RT1^c$) model of 'tolerance' induction by DST priming, Th2 polarized IgG1 production associated with Il4 production was reported to occur 30 days after transplantation (5). In our model, no increase in Il-4 or Il-13 protein was detected in any of the serum samples from DST-treated recipients tested following transplantation (data not shown), suggesting an absence of Th2 polarization. This corroborated the sustained production of both Th1 and Th2 antidonor alloantibody IgG subtypes against class I and II MHC antigens, which reached a maximum 100 days after transplantation. Because previous data emphasized the role of the lymphoid neogenesis in the graft itself during chronic rejection (14), we also investigated whether locally produced antibodies directed against donor MHC molecules could be detected

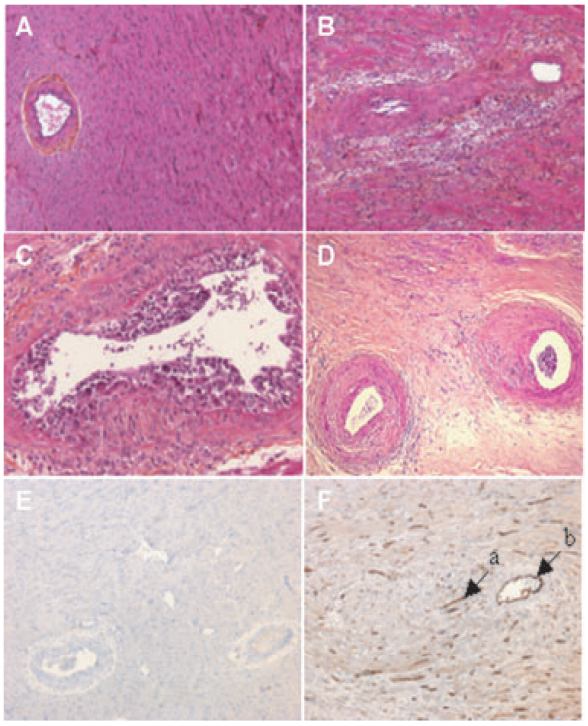

Figure 8: Pathologic findings in DST-treated animals. Hematoxylin-eosin-saffron staining of heart allografts paraffin sections (syngeneic or DST-treated recipients) (A) Normal artery observed in a syngeneic animal at day 60 (×10). (B) Acute lesions with intimal leukocyte adhesion and infiltration in hearts from DST-treated animals at day 60 (×10). (C) Inflammatory intimal and perivascular fibrosis on day 100 (×20). (D) Intimal thickening and myofibroblast proliferation associated with perivascular fibrosis on day 140 (×10). C4d staining. (E) No complement binding in syngeneic hearts from day 7 to 100 after transplantation (×10). (F) C4d deposits in capillaries (a) and arteries (b) in DST-treated hearts from day 7 to 100 posttransplantation (×10).

at day 100 in the grafts from DST-treated recipients. The fact that the amounts of antidonor class I and II antibodies produced in the organocultures supernatants were higher in animals presenting chronic rejection than in naïve hearts, suggest that local germinal center formation could also be involved in the process leading to chronic rejection in this model. Although relatively few studies have linked C4d staining with antibody-mediated rejection in human cardiac transplantation (37–40), this by-product of C3 convertase degradation is a well-defined correlate of antibody-mediated injury in renal transplantation (10). In a rat heart transplantation model, C4d staining has already been detected in the vascular endothelium following DST treatment in C6-deficient animals with chronic rejection (41). In our model, diffuse C4d deposits were detected in capillary and artery walls as early as day 7 and persisted in long-term accepted grafts (analyzed up to day 100). Notably, such staining was absent from syngeneic grafts. Histological analysis revealed that in the first days, hearts from DST-treated recipients presented histological patterns of subacute rejection, with endothelialitis and leukocyte infiltration. This pattern was progressively enriched by typical lesions of chronic rejection associating severe intimal fibrosis, vascular occlusion and mononuclear cell infiltration in the grafts from day 100 to 320.

Altogether, we hypothesize that the chronic rejection lesions developing in this model are the consequence of synergistic cellular and humoral events. Chronic rejection is probably initiated in the early phases following transplantation by indirectly primed $CD4^+$ cells providing help to B cells for antidonor class I and II alloantibody production, leading to vascular damage through complement activation. Nevertheless, establishing a precise link between antidonor antibody production and chronic vascular lesions would require passive transfer, although these experiments would not mimic the alloantibody production that also occurs in the graft itself. Although we cannot make firm conclusions, this model presents features common with the chronic active antibody-mediated rejection described in human renal transplantation, involving antidonor antibody production, diffuse C4d deposits in the graft capillaries and histological vasculopathy. TRIB1, recently identified as a biomarker of chronic antibody-mediated rejection in human kidney recipients, was also upregulated in the blood and graft in our model (42). Collectively, we suggest that DST-induced long-term survival represents a pertinent model for testing new strategies aimed at controlling chronic active antibody-mediated rejection in humans.

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Inhibition du protéasome et du récepteur de l'interleukine-7 en transplantation d'organe

Le rejet de la greffe d'organe est classé en deux catégories principales : le rejet dépendant des cellules T et le rejet dépendant des anticorps, bien que ces deux mécanismes soient souvent associés chez un même patient. Les immunosuppresseurs actuels ont des effets secondaires importants, notamment la néphrotoxicité des inhibiteurs de la calcineurine (CNIs). Les nouveaux médicaments qui cherchent à remplacer les CNIs sont souvent moins efficaces en prévention du rejet aigu. L'objectif de nos recherches est de trouver de nouveaux médicaments efficaces qui ciblent chacun de ces deux mécanismes du rejet. Dans la première partie de notre travail, nous avons démontré que le Bortezomib, un inhibiteur du protéasome, prolonge la survie du greffon cardiaque et diminue la formation des allo-anticorps dans des modèles de rejet aigu et chronique chez le rat. Nous avons ensuite présenté nos expériences sur l'utilisation du Bortezomib comme traitement du rejet dépendant des anticorps chez quelques patients transplantés rénaux. Dans la deuxième partie de cette thèse, nous avons démontré que chez la souris, un anticorps dirigé contre le récepteur de l'interleukine-7 (IL-7) diminue le nombre de presque toutes les sous-populations de lymphocytes T, y compris les T mémoires et augmente le pourcentage des T régulateurs. L'anticorps anti-récepteur de l'IL-7 prolonge la survie des greffes d'îlots pancréatiques chez la souris, et en combinaison avec une déplétion des cellules T par des anticorps déplétants, il prolonge également la survie des greffes de peau chez la souris, ce dernier étant un modèle très rigoureux. Le blocage du récepteur de l'IL-7 après une déplétion des cellules T est un traitement immunosuppresseur puissant, qui peut être envisagé chez les malades allo-immunisés ou combiné avec des immunosuppresseurs non-CNI pour renforcer leur efficacité anti-rejet.

Mots clés : transplantation, rejet, anticorps, protéasome, bortezomib, interleukin-7.

Proteasome inhibition or interleukin-7 receptor blockade in organ transplantation

Allograft rejection is currently classified into two main categories: T-cell mediated or antibody-mediated rejection (AMR), although these two mechanisms usually coexist in the same patient. Most of the therapies used in organ transplantation target T cells, only few of them can directly decrease alloantibody formation. Moreover, current immunosuppressive drugs have important side effects, the most well-known of which is the nephrotoxicity caused by calcineurin inhibitors (CNIs). However, non-CNI protocols are usually less effective in the prevention of acute rejection. The aim of our study is to find new drugs that target each of these two main mechanisms of graft rejection. In the first part of our study, we have demonstrated that bortezomib, a proteasome inhibitor prolonged cardiac allograft survival and abrogated alloantibody formation in rat models of acute and chronic rejection. We then presented our experience with the use of bortezomib in the treatment of AMR in some kidney transplant patients. In the second part of our study, we have showed that in mice, anti-IL-7R monoclonal antibody (mAb) decreased the number of almost all T cell subsets, including memory T cells, and increased the percentage of regulatory T cells. Anti-IL-7R mAb prolonged mouse islet allograft survival, and when combined with a T cell depletion therapy, also prolonged graft survival in a stringent model of mouse skin allograft. IL-7R blockade following a T cell depletion therapy is a very powerful immunosuppressive protocol which can be used in hypersensitized patients or in combination with non-CNI-based therapies in order to reinforce their anti-rejection efficacy.

Key words: transplantation, rejection, antibody, proteasome, bortezomib, interleukin-7.