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Utilisation de lymphocytes T en thérapie cellulaire pour le traitement de la néphropathie au polyomavirus BK chez les greffés rénaux

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RÉSUMÉ

Le polyomavirus BK est un virus très prévalent qui demeure normalement en phase de latence dans l'uroépithélium sans entrainer de complications. Chez les greffés rénaux, il peut cependant se réactiver et mener à une néphropathie pouvant nuire à la survie du greffon. L'immunité du receveur est la pierre angulaire de la prévention et du traitement de cette néphropathie, puisque le seul traitement démontré efficace est une diminution de l'immunosuppression. Cependant, une augmentation non spécifique de l'immunité augmente également le risque de rejet. Notre objectif était donc d'adapter et de valider un protocole transférable en clinique d'immunothérapie adoptive antivirale nous permettant de produire des lignées de lymphocytes T BK-virus spécifiques à partir du sang de patients greffés virémiques, afin de prévenir et traiter ces néphropathies. Nous avons tout d'abord comparé les lignées cellulaires produites à partir de donneurs sains à celles de patients immunosupprimés soumis à une immunosuppression chronique. Par la suite, nous avons adapté le protocole en ajoutant une stimulation à l'aide de cellules dendritiques afin de maximiser l'expansion cellulaire, le statut de différentiation et la spécificité. Bien que les lignées étaient polyclonales, elles n'ont pas démontré de potentiel alloréactif *in vivo* et *in vitro,* et ce, malgré une persistance et une prolifération *in vivo*. Nous avons donc élaboré un protocole qui est prêt à être transféré en étude clinique de phase I/II et qui pourrait nous permettre de prévenir et traiter la néphropathie associée au polyomavirus BK, sans augmenter le risque de rejet.

Mots clés: BK polyomavirus, transplantation rénale, immunothérapie, thérapie cellulaire, néphropathie associée au polyomavirus

ABSTRACT

More than 75% of the population has been exposed to BK polyomavirus and carries latent virus in the uroepithelium without any complications. However, it can reactivates in kidney transplant recipients (KTR) and lead to a nephropathy affecting graft survival. Recipient anti-viral immunity is the cornerstone of BK-virus associated nephropathy prevention and treatment and thus, reduction of immunosuppression is the only well-accepted treatment. Adoptive immunotherapy is a promising solution to this problem, allowing a specific T cell mediated response against this virus without the alloreactive risk. It was demonstrated efficacious for other viral infections in immunocompromised hosts but it has not been used in this specific context. Our objective was to adapt and validate a clinical-compliant protocol to obtain BK-specific T cell lines from viremic KTR and to compare their expansion, differentiation and specificity to ones obtained from healthy donors. Although comparable specificity and differentiation status, cell expansions form KTR were not systematically sufficient for a therapeutic dose. The addition of a stimulation with dendritic cells improved cell expansion in addition to favors a central memory phenotype and refined BK-specificity. Despite polyclonality, T cell lines didn't demonstrated alloreactivity in a chromium release assay and *in vivo*. Furthermore, T cell lines could persist and proliferates *in vivo*. This protocol is ready for a phase I/II clinical trial. This opens the possibility to solve the current conundrum and treat PVAN without increasing rejection risk.

Keywords: BK polyomavirus, kidney transplant, cellular therapy, immunotherapy, polyomavirus-associated nephropathy

RÉSUMÉ DE VULGARISATION

La greffe rénale permet d'améliorer la survie des patients souffrant d'insuffisance rénale terminale. Dans les dernières décennies, la survie du greffon s'est beaucoup améliorée, notamment grâce à des immunosuppresseurs (anti-rejets) plus puissants. Cependant, l'affaiblissement du système immunitaire entraine une augmentation du risque de certaines infections, notamment le polyomavirus BK.

Une grande proportion de la population a contracté ce virus durant l'enfance et il demeure latent (inactif) dans les reins et les uretères (conduits entre les reins et la vessie), sans entrainer de complications. Par contre, chez les greffés rénaux, l'affaiblissement du système immunitaire fait en sorte que le virus peut se réactiver et entrainer des dommages pouvant évoluer jusqu'à la perte du greffon. Pour l'instant, le seul traitement démontré efficace consiste à diminuer les immunosuppresseurs, mais cela augmente le risque de rejet. Une solution consisterait à prélever les lymphocytes (globules blancs), de les éduquer à combattre le virus en laboratoire et ensuite de les ré-injecter au patient. Il s'agit donc de redonner au patient son propre système immunitaire, mais apte à contrôler le virus. Nous avons donc développé un protocole nous permettant de produire des lignées de lymphocytes T éduqués contre le polyomavirus BK (spécifiques), de bonne qualité et en quantité suffisante pour traiter un patient, à partir du sang de patient greffé ayant un virus actif. De plus, elles sont sécuritaires, donc n'entraineraient pas de rejet de greffon. Nous sommes maintenant prêts à en faire une étude clinique pour le tester chez des patients.

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LISTE DES ABRÉVIATIONS

BrdU, 5-Bromo-2′-deoxyuridine

CMH, Complexe majeur d'histocompatibilité

CMV, Cytomégalovirus

DC, Cellule dendritique

EBV, Epstein-Barr virus

ELISpot, Essai d'immunospot enzymatique

ERE, Élément de réponse oestrogénique

GRE/PRE, Élément de réponse glucocorticoide et/ou progestatif

GVHD, Maladie du greffon contre l'hôte

HC, Control sain

HCV, Virus de l'hépatite C

HSV, Virus de l'herpes simplex

HLA, Antigènes des leucocytes humains

HPV, Papillomavirus humain

HSCT, Transplantation de cellules souches hématopoïétiques

ICAM, Molécule d'adhésion intracellulaire

IFN-γ, Interferon-γ

IRT, Insuffisance rénale terminale

IVIG, Immunoglobuline intraveneuse

KDIGO, Kidney Disease | Improving Global Outcomes

KIR, Récepteur *killer cell immunoglobulin-like*

KTR, Receveur d’une transplantation rénale

LT, Large tumoral

LTA, Antigène large tumoral

MCP, Protéine chimiotactique monocytaire

MIP, Protéines inflammatoires de macrophages

MPA, Acide mycophénolique

mRNA, ARM messager

NCCR, Région de contrôle non-codante

NK, *Natural killer*

NSG, NOD/SCID/IL2Rγ^{null}

PBMC, Cellules mononucléaires du sang périphérique

PCR, Réaction de polymerase en chaine

PTLD, Syndrome lymphoprolifératif post-transplantation

PVAN, Néphropathie associée au polyomavirus

RCITO, Registre canadien des insuffisances et des transplantations d’organes

SV40, Simian virus 40

TCR, Récepteurs des lymphocytes T

Tcm, Lymphocyte T central mémoire

Tem, Lymphocyte T effecteur mémoire

TGF, *Transforming growth factor*

Tn, Lymphocyte T naif

TNF-α, *Tumor necrosis factor-α*

Tscm, Lymphocyte T cellule souche mémoire

VIH, Virus d’immunodéficience humaine

VP, Protéine encodant le virus

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INTRODUCTION

1 - La transplantation rénale

1.1 Mise en contexte

L'insuffisance rénale chronique est définie comme la présence d'anomalies dans la structure ou la fonction des reins, présents pour une période de plus de 3 mois et ayant des implications pour la santé (1). L'insuffisance rénale terminale (IRT), quant à elle, se définit comme une fonction rénale insuffisante pour maintenir un individu en vie. Afin de survivre, une thérapie de remplacement rénal sera nécessaire, soit sous la forme de dialyse ou de transplantation rénale (2). En 2013, au Canada, selon le dernier rapport du registre canadien des insuffisances et des transplantations d'organes (RCITO), plus de 40 000 Canadiens souffraient d'IRT. De ces derniers, 57,5% recevaient un traitement de dialyse et 42,5% avaient un greffon rénal (3). Les causes principales d'IRT au Canada sont le diabète, les glomérulonéphrites et les maladies vasculaires rénales (3).

Malgré ces thérapies de remplacement de la fonction rénale, l'IRT demeure une maladie chronique morbide et mortelle. En effet, la survie à 5 ans en dialyse n'est que de 44,8% selon les dernières données canadiennes (3), ce qui est inférieur à la survie avec un cancer du sein pour les stades non métastatiques (0-III) (4). Après une transplantation rénale, cette survie augmente à 89,2% pour les patients ayant reçu un rein provenant d'un donneur vivant et 82,6% pour un donneur décédé (3). Bien que les patients admissibles à une transplantation sont généralement en meilleure santé et ont une espérance de vie en dialyse supérieure aux patients qui ne sont pas

admissibles, leur risque relatif de mortalité est diminué de 32% à 18 mois seulement après une transplantation (5).

Au Québec seulement, en 2015, il y a eu 297 reins transplantés. Cependant, encore 613 patients étaient en attente de transplantation rénale (6). La survie de la greffe n'est pas éternelle. En effet, en 2005 aux États-Unis, la demi-vie d'un greffon était de 8.8 ans pour les greffes de donneurs décédés et 11.9 ans pour celles de donneurs vivants (7). De plus, environ 13% des patients inscrits sur la liste d'attente de transplantation aux États-Unis le sont suite à la perte de leur greffon (8). Considérant l'avantage de la transplantation rénale, l'énorme besoin au sein de notre population et la rareté de la ressource, il est d'autant plus important de combiner nos efforts afin de prolonger la longévité d'un greffon.

1.2 Alloréactivité

Après une transplantation allogénique, donc entre deux membres non identiques d'une même espèce, le principal risque à la survie de l'organe est son rejet. De façon précoce après la transplantation, il y aura d'abord une atteinte par le système immunitaire inné et plusieurs médiateurs inflammatoires, suivi par une réponse immunitaire adaptative, antigène spécifique.

Le risque de rejet est accru dans les premiers jours suivant la transplantation puisque le prélèvement de l'organe et la transplantation entrainent inévitablement des lésions d'ischémie-reperfusion menant à une augmentation de l'expression des antigènes

des leucocytes humains (HLA) et la relâche de chimiokines telles que GRO/CXCL1, MCP1, MIP-1 et IP-10, de cytokines telles que TNF-α, IFN-γ, IL-2 et IL-6, et des molécules d'adhésions dans la greffe telles que le CD11/CD18 et ICAM-1 (9, 10). L'activation des cellules immunitaires innées causera alors du dommage soit directement ou indirectement via l'activation et le recrutement de lymphocytes T.

Les cellules dendritiques (DC) seront les principales cellules présentatrices d'antigènes pour activer les lymphocytes T. Lorsque matures, les DC du donneur (voie directe) ou du receveur (voie indirecte) migrent dans les organes lymphoïdes secondaires et présentent un allo-antigène aux récepteurs des lymphocytes T (TCR) via leur complexe majeur d'histocompatibilité (CMH). Le contact entre le TCR, le peptide et le CMH correspond au premier signal d'activation des lymphocytes T. Pour compléter l'activation, ils fourniront également des signaux de costimulation (signal 2). La production de cytokines est également importante (signal 3).

Les lymphocytes T CD4+ ayant été activées contribueront également à l'activation d'autres cellules telles que les macrophages, les lymphocytes B et les lymphocytes T CD8+, perpétuant ainsi l'inflammation et le dommage cellulaire à la greffe.

Normalement, un lymphocyte T ne reconnait pas un antigène directement, mais seulement lorsqu'elle est présentée dans un CMH du soi. Cependant, puisque les variations alléliques entre les différents CMH sont petites, les TCR du receveur peuvent avoir une forte affinité pour un CMH intact du donneur et peuvent le

reconnaitre directement. Normalement, seulement 1 lymphocyte T sur 10 000 ou sur 1 000 000 va répondre à un certain antigène. Cependant, en transplantation, 5-10% des lymphocytes T vont répondre à une molécule CMH étrangère (11). Ce phénomène est particulièrement important en greffe rénale puisque les HLA ne sont que rarement appareillés entre le donneur et le receveur. Voilà pourquoi la plupart des traitements immunosuppresseurs en transplantation ciblent les lymphocytes T.

Le rejet d'un organe après la transplantation ne semble pas être la seule conséquence d'une reconnaissance du système immunitaire de l'organe comme le ¨non-soi¨. En effet, notre système immunitaire tolère plusieurs protéines étrangères inertes (par exemple dans l'alimentation) ainsi que plusieurs protéines du soi qui n'étaient pas présentes lors de la sélection négative qui permet d'éliminer les lymphocytes réagissant contre des antigènes du soi dans le thymus (tel que les hormones lors de la puberté). En effet, selon la théorie du danger élaborée par Polly Matzinger, le système immunitaire est d'abord activé par la perception de signaux de dangers (12). Ces signaux d'alarme sont multiples et peuvent être relâchés suite à un stress, un dommage ou lors de la nécrose cellulaire. Il peut s'agir de protéines de choc thermique, de nucléotides, de produits de dégradation de la matrice extracellulaire, de cytokines, etc (13). Lors d'une transplantation, les cellules présentatrices d'antigènes sont donc activées par ces signaux d'alarmes endogènes libérés par les cellules en détresse ou détruites, inévitablement présents suite au dommage d'ischémie-reperfusion. Cette théorie permet d'expliquer pourquoi une transplantation utilisant un organe d'un donneur vivant a un meilleur pronostic qu'une

transplantation utilisant un organe d'un donneur cadavérique pour un même degré de concordance HLA (14).

1.3 Immunosuppression

Une grande partie du succès de la transplantation est secondaire au succès de l'immunosuppression utilisée en induction et en maintenance. En effet, au cours des dernières décennies, la survie moyenne des greffons s'est nettement améliorée, avec une probabilité de perte de greffon à 5 ans qui est passée de 36,2% en 1996 à 26,9% en 2008 (8) et le taux de rejet aigu a diminué à environ 15-20% (15). En général, les différents immunosuppresseurs diminuent ou bien le nombre absolu de lymphocytes, tel que les immunoglobulines anti-thymocytes humains, ou leur fonction, en altérant un des 3 signaux précédemment décrits. La figure 1 démontre les différents immunosuppresseurs utilisés en greffe rénale, selon ce modèle.

De façon courante, les anticorps monoclonaux dirigés contre la sous-unité alpha du récepteur de l'IL-2 (CD25) (basiliximab) ou les immunoglobulines anti-thymocytes sont utilisés en association avec les corticostéroïdes comme agents d'induction selon le risque immunologique du patient. Par la suite, le patient est mis sous une triple thérapie consistant en de la prednisone, un inhibiteur de la calcineurine (préférentiellement le tacrolimus) et un anti-métabolite, souvent l'acide mycophénolique (MPA).

Cependant, en plus de leur action thérapeutique (diminution du risque de rejet), l'utilisation d'immunosuppresseurs plus puissants entraine des conséquences liées à l'immunodéficience tel que les infections et les cancers ainsi que de la toxicité cellulaire non liés à leur effet immun (hypertension artérielle, diabète…).

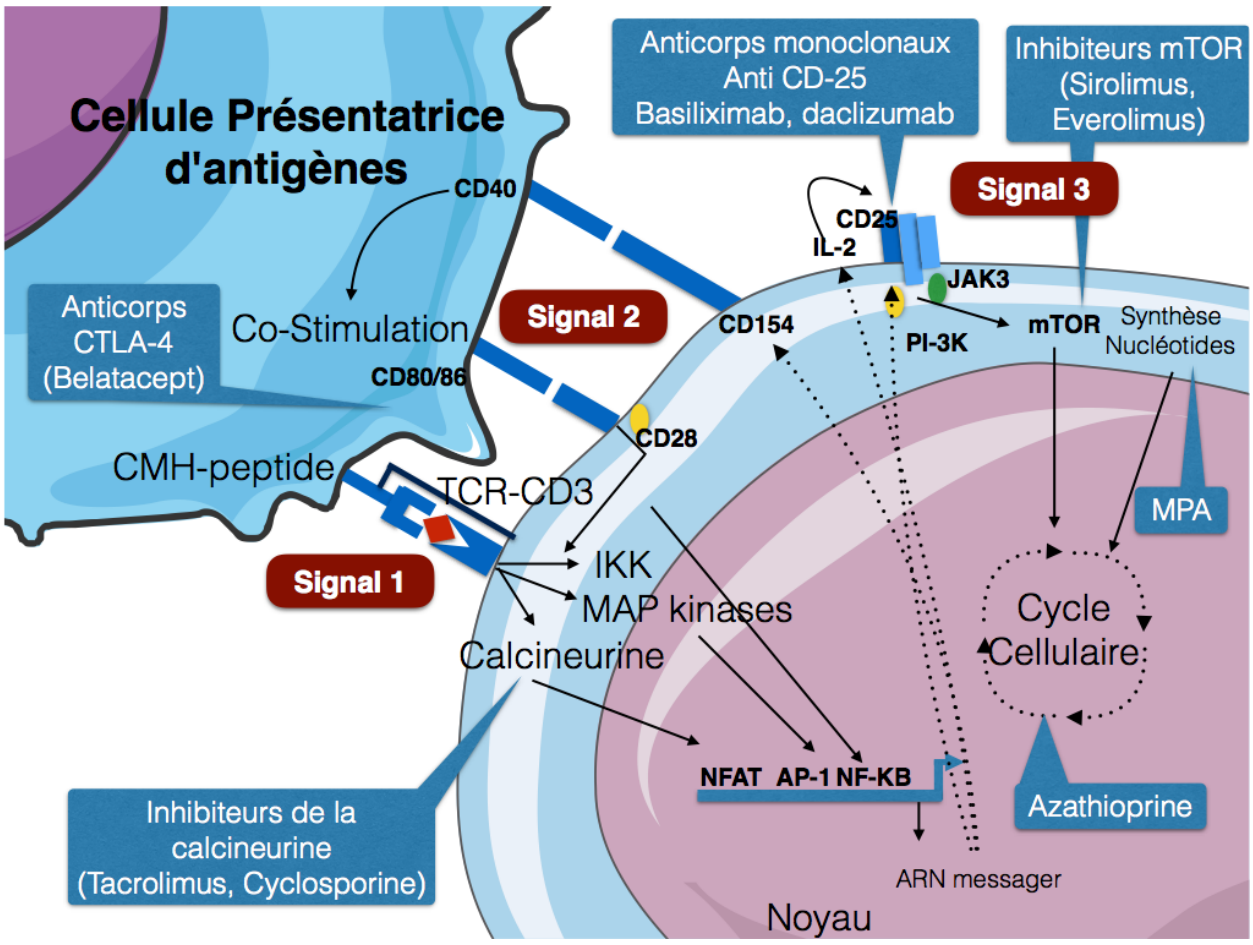

Figure 1. Médicaments immunosuppresseurs utilisés en greffe rénale et leur site d'action selon le modèle des trois-signaux, *adapté de Halloran PF, NEJM 2004* (16). MPA, Acide mycophénolique.

1.4 Infections

Les infections sont une cause importante de mortalité et morbidité après une transplantation. En effet, ils sont la deuxième cause de mortalité chez les patients avec un greffon fonctionnel (17). Le taux d'infection par année durant les trois premières années après la greffe est d'environ 45% (18). L'utilisation d'immunosuppresseurs augmente le risque de toutes sortes d'infections, soit virale, bactérienne, fongique et parasitaire, de façon dépendante du temps après la greffe. Dans le premier mois après la greffe, il y a surtout le risque d'infection nosocomiale, pneumonie d'aspiration, infection de cathéter ou de plaie, colite à *C. difficile*, le risque d'infections provenant du donneur tel que le VIH ou l'HSV ou encore les infections reliées au receveur s'il était colonisé avant la greffe avec de l'aspergillus ou du pseudomonas. Normalement, les donneurs infectés par le VIH ou l'hépatite C ne sont pas utilisés en transplantation. Malheureusement, si l'infection est très récente, elle peut être manquée au moment du dépistage et être transmise au receveur. Entre 1 et 6 mois, les infections les plus fréquentes sont virales, telles que de la famille des herpesvirus (HSV, VZV, cytomégalovirus (CMV), Epstein-Barr virus (EBV)), l'HPV, le BK, l'adénovirus et l'influenza. Il peut s'agir de la réactivation d'un virus latent, d'un virus transmis par le donneur ou plus rarement d'une infection acquise dans la communauté. Également, il y a le risque de *Pneumocystis*, *C difficile*, *Cryptococcus*, *Mycobacterium tuberculosis*, *Listeria*, *Nocardia*, toxoplasmose, strongyloïdes, leishmaniose ou *Toxoplasma cruzi.* Afin de minimiser la transmission de virus par le donneurs, la présence de plusieurs virus est évaluée avant la transplantation et les donneurs peuvent être rejetés sur la base de la présence de certains virus tels le VIH

et l'hépatite C. Cependant, il arrive parfois que la présence de ces virus ne soit pas démasquée, principalement si l'infection est très récente. Finalement, après 6 mois il y a les pneumonies acquises en communauté, les infections urinaires, l'aspergillus, les moisissures atypiques, le mucor, la *Nocardia*, le *Rhodococcus* et toujours plusieurs infections virales telles que le CMV, HBV, HCV, HSV, JC et EBV (19).

L'utilisation d'immunosuppresseurs plus puissants a altéré la prévalence et la cinétique de plusieurs infections. De plus, cela a amené l'émergence de nouveaux types d'infections, telle la néphropathie au polyomavirus BK, qui peut être un réel danger pour le greffon (20).

Puisque le rejet représente le risque le plus important pour la survie du greffon et que la dose minimale d'immunosuppresseurs permettant de prévenir cette complication pour un patient donné n'est pas connue, la pratique actuelle est souvent de sur-immunosupprimer les patients aux dépens du risque infectieux et néoplasique (21).

2 - Le polyomavirus BK

2.1 *BK polyomavirus and the transplanted kidney; immunopathology and therapeutic approaches*

Le polyomavirus BK est un virus acquis durant l'enfance demeurant latent dans l'uroépithélium. Un système immunitaire compétent permet de contrôler sa réactivation. Par contre, chez les greffés rénaux, ce virus peut se réactiver et entrainer une néphropathie affectant jusqu'à 10% d'entre eux (22). Un ensemble de facteurs agissent simultanément dans cette population afin de créer les conditions parfaites à sa réactivation. Tout d'abord, nous croyons que l'infection chez les greffés provient des donneurs. Il s'agit donc d'une souche qui peut être différente de celle contre laquelle le receveur a développé une immunité. Ensuite, nous savons maintenant que l'environnement inflammatoire présent tôt après la greffe peut favoriser la transcription virale. De plus, les lésions d'ischémie-reperfusion peuvent libérer le virus. Finalement, les immunosuppresseurs utilisés après la transplantation ciblent préférentiellement les lymphocytes T, qui s'avèrent primordiaux pour le contrôle de la réactivation virale. Lorsque le virus se réactive, on le retrouve dans l'urine puis dans le sang de façon séquentielle avant de causer une néphropathie. À ce jour, le seul traitement démontré efficace est une diminution de l'immunosuppression, ce qui peut cependant augmenter le risque de rejet. Il est donc important de mieux comprendre les interrelations le virus, l'environnement et le système immunitaire afin d'améliorer notre approche face à cette problématique.

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BK polyomavirus and the transplanted kidney; immunopathology and therapeutic approaches

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Abbreviations:

DC, dendritic cells

ELISpot, Enzyme-linked immunospot assay

ERE, estrogen response element

GRE/PRE, glucocorticoid and/or progesterone response element

HLA, human leucocyte antigen

HSCT, hematopoietic stem cell transplantation

IFN-γ, interferon-γ

IVIG, intravenous immunoglobulin

KDIGO, Kidney Disease | Improving Global Outcomes

KIR, killer cell immunoglobulin-like receptors

LT, large tumor

mRNA, messenger RNA

NCCR, non-coding control region

NK, natural killer

PCR, polymerase chain reaction

PVAN, polyomavirus-associated nephropathy

SV40, simian virus 40

TGF, transforming growth factor

TNF-α, tumor necrosis factor-α

VP, virus-encoded protein

Abstract

BK polyomavirus is ubiquitous, with a seropositivity rate of over 75% in the adult population. Primary infection is thought to occur in the respiratory tract, but asymptomatic BK virus latency is established in the urothelium. In immunocompromised host, the virus can reactivate but rarely compromises kidney function except in renal grafts, where it causes a tubulo-interstitial inflammatory response similar to acute rejection. Restoring host immunity against the virus is the cornerstone of treatment. This review covers the virus-intrinsic features, the post-transplant micro-environment as well as the host immune factors that underlie the pathophysiology of PVAN. Current and promising therapeutic approaches to treat or prevent this complication are discussed in relation to the complex immunopathology of this condition.

Introduction

Polyomaviruses were first discovered by Ludwig Gross in 1953 as murine leukemia viruses. Notably, newborn mice injected with cell-free extracts of murine leukemia tissues developed adenocarcinomas of the parotid gland in addition to leukemia, suggesting that an infectious agent was the cause of the malignancies[1]. The infectious agent was named using the Greek words for many (poly) and cancer (oma)[2]. So far, about 30 species of polyoma viruses have been identified in birds and mammals, including thirteen in humans: BK, JC, KI, WU, Merkel cell polyomavirus, H6, H7, H9, H10, H12, STL, trichodysplasia spinulosa-associated polyomavirus and NJ[3]. BK polyomavirus was first isolated by Gardner and al. in 1971 from the urine of a renal allograft recipient and was named after the patient's name[4]. Whether BK virus is oncogenic is controversial, but a role in the development of urothelial cancers has been proposed in immunocompromised patients[5]. In immunocompetent patients, the presence of BK virus DNA was found in numerous cases of bladder, urothelial and other tumors[6,7]. However, given the high prevalence of BK virus infection and latency in those tissues, the detection of BK in tumors does not imply a causal relationship[8].

It is estimated that at least 75% of the adult population is latently infected with BK virus[9]. Immunocompetent subjects are usually asymptomatic, but immunocompromised hosts can suffer BK-related complications. In kidney transplant recipients, BK and possibly other polyoma viruses can cause nephropathy and ureteral stenosis[10], while hemorrhagic cystitis is prevalent in hematopoietic stem cell transplantation (HSCT) patients[11]. Rare cases of BK disseminated disease

(tubulointerstital nephritis, desquamative pneumonitis, meningoencephalitis and retinitis) have also been described[12], especially in patients with acquired immune deficiency syndrome[13].

Such wide range of presentations associated with BK virus suggests that condition-specific interactions between host and virus factors ultimately dictate the clinical complications related to BK-virus reactivations. As an illustration, polyomavirus-associated nephropathy (PVAN) in kidney transplantation patients and hemorrhagic cystitis occurring after HSCT are both associated with risk factors that are specific for these conditions. In HSCT, risk factors notably include myeloablative conditioning [14], CMV viremia[14], recipients of cord blood units[15] and acute graft-versus-host disease[15], which may relate to two main factors; urothelial damage (myeloablative conditioning) and profound immunosuppression. Immunosuppression following kidney transplantation is likewise necessary for the development of PVAN as well as specific features uniquely associated with renal transplants. As opposed to HSCT where BK reactivation occurs in host tissues, in kidney transplantation the virus reactivates in the graft and the infection is mostly donor-derived[16]. Bohl and colleagues have shown a concordance in BK virus infection in receiving pairs from the same donor, with a match in sequences of segments of the two genes (non-coding control region (NCCR) and virus-encoded protein (VP1)) in these patients compared to recipients from different donors[17], strongly supporting a donor origin of the virus in this case. Moreover, there is also a higher rate of reactivation in recipients from BK virus seropositive donors[16].

This review focuses on the virology and BK-specific immunity in the context of renal transplantation, highlighting the interplay between three major variables; the virus, the kidney graft environment and the immune system. The rationale and merit of actual as well as plausible future prevention and treatment approaches for PVAN occurring following BK-virus reactivation are discussed in relation to BK-associated immunopathology.

1. Epidemiology and diagnosis of PVAN

Reactivation of BK virus in the transplant kidney can lead to PVAN in up to 10% of kidney transplant recipients[18]. BK virus reactivation is first observed with the appearance of decoy cells or BK virus DNA in the urine preceding viremia by a median of 4 weeks[18]. Decoy cells are virally infected uroepithelial cells that can be observed with standard light microscopy. They can be used as a screening method for PVAN, but their positive predictive value is weak (11,7%)[19]. These early findings are followed by BK viremia, which precedes nephropathy by a median of 8 weeks. As such, viremia has a better positive predictive value for nephropathy than viruria, especially if viral load is more than 10000 copies/mL[20,21], with the caveat that BK virus polymerase chain reaction (PCR) assays are not standardized across centers[22]. Fifty percent of all detectable viremia occurs in the first 2 months and 95% in the first two years after transplant[18]. This timing for reactivation may be related to several factors including intense immunosuppression, tubular injury and ensuing inflammation that characterize the early post-transplantation period.

The intensity of immunosuppressive regimens is a risk factor for the development of PVAN[23]. The occurrence of PVAN correlates with the use and dosage of tacrolimus[23,24] and/or mycophenolate mofetil[23,25], anti-thymocyte globulin induction[25] and anti-rejection treatment[20]. Other risks factors are less consistently reported in the literature, but include various recipient-related factors (older age, male sex[26]), donor factors (degree of human leucocyte antigen - HLA mismatches and BK virus seropositive status[27]), and factors associated with renal injury (cold ischemia time, delayed graft function and ureteral stent placement)[27]. The diagnosis of PVAN is highly suggested by the detection of viral inclusion bodies on kidney biopsy but is confirmed with immunohistochemical staining for simian virus 40 (SV40) large T antigen and/or in-situ hybridization for BK virus genetic sequences[28]. According to the Banff classification, the histopathological findings further categorize PVAN into three stages. Grade A refers to inflammatory changes without acute tubular necrosis, while grade B is defined by tubular epithelial cell lysis and acute tubular necrosis. Finally, the presence of interstitial fibrosis characterize grade C PVAN[29,30]. Graft prognosis correlates with PVAN severity as 2-year graft survival is 90% for grade A, but only 70% and 50% for grade B and C respectively[31]. Several biomarkers have been developed to assess intrarenal viral disease. The urinary polyomavirus-haufen test which relies on the detection of urinary casts composed of uromodulin, lysed tubular cell and virions is reported to predict PVAN onset, intrarenal viral activity and resolution [31]. Urinary VP1 mRNA is also proposed as a new biomarker to identify PVAN[32] which may be used with granzyme B mRNA, proteinase inhibitor-9 mRNA and plasminogen activator inhibitor-1 mRNA to predict graft failure risk[33,34]. Although

promising, these tests require further validation before widespread clinical use[31]. Furthermore, granzyme B and proteinase inhibitor-9 mRNAs are not specific for PVAN and they were used as biomarkers in the CTOT-O4 study to predict acute cellular rejection[35]. Interestingly, the diagnostic signature elaborated in this study was also associated with BK virus infection[35] indicating that non-specific surrogate markers of immune activation can only be used in conjunction with other diagnostic information.

2. Virology and pathogenesis of BK

BK polyomavirus genome shares about 72% nucleotide homology with JC virus and 70% with SV40. It consists of a single molecule of circular viral DNA of 5300 base-pairs, complexed with cellular histones (H2A, H2B, H3, H4) and surrounded by an icosahedral capsid containing three virus-encoded proteins, VP1, VP2 and VP3[36]. BK virus genome contains three functional regions; NCCR which regulates viral replication and transcription, the early and the late regions[37]. The early region contains large tumor (LT) and small tumor antigen proteins, which are derived by alternative splicing of a common precursor[36] and are believed to be the first proteins expressed. The late region contains genetic information for the three virus-encoded proteins (VP) and agnoproteins[37].

Role of the viral proteins

BK polyomavirus binds to the target cells through interaction with two ganglioside receptors, GT1b and GD1b and then uses caveolae-mediated endocytosis to reach the endoplasmic reticulum[38] (Figure 1). Following partial uncoating of the virus by

reduction and isomerization of the disulfide bonds that link VP1 proteins, BK virus retrotranslocates to the cytosol for a second rearrangement of the capsid, thereby enabling a liaison to the nuclear pore and passage of viral DNA into the cell nucleus[39], possibly facilitated by nuclear localization signals on the minor capsid proteins, VP2 and VP3[40]. Following infection of human kidney epithelial cells *in vitro*, LT expression is observed at 36 hours before VP1 expression and viral DNA replication[41] and is required for viral DNA replication and expression of the late genes[42]. It can also induce an oncogenic effect by specifically binding and inactivating tumour suppressor proteins, including retinoblastoma family genes and p53 (Figure 1). Thus, it can promote the transition of the cell into the S phase[43]. In an elegant study, Seemayer and colleagues demonstrated with indirect immunofluorescence that the enlargement of nucleus seen in polyomavirus-infected tubular cells is associated with viral replication, large T antigen expression and p53 accumulation. This observation correlates with an activation of the cycle cell, as seen by the expression of Ki-67. Viral replication can also lead to cellular demise. The absence of caspase 3, bcl-2 as well as a regular distribution of nuclear DNA indicates that cells die mostly by necrosis and not apoptosis[44], in accordance with the previous observation made in PVAN patients by light and electronic microscopy[45].

Viral strains

We can classify BK virus as “archetype” or “rearranged” types, based on the genotype of the NCCR. The NCCR regulates viral replication and transcription[46]. The rearranged variant implies numerous mutations in the NCCR region which can

amplify the replication potential[47], a phenomenon that has been validated *in vivo*[48]. In their meta-analysis, Sharma and colleagues concluded that there is a correlation between the rearranged variant and the development of nephritis[49]. Two hypotheses have been suggested to explain this relationship; 1) the rearranged variant is more virulent and leads easily to nephritis, or 2) nephritis is associated with a more rapid viral turnover which favors the development of mutations[49]. Chatterjee and al found both virus types can be found in peripheral blood cells of healthy individuals. Hence, they proposed that leukocytes may play a role in the NCCR rearrangement process and transport of BK virus[46, 50].

Transmission,

The exact route of transmission from human to human is unknown[51]. Oral transmission has been proposed [52], but the most accepted hypothesis is that BK is spread through the respiratory tract[53]. Primary infection is indeed associated with upper respiratory symptoms in one third of children[54]. Moreover, Goudsmith *et al.* have demonstrated that BK seroconversion is present in 8% of children admitted to the hospital for any upper respiratory tract illness, compared with 15% for adenovirus, influenza A, parainfluenza, respiratory syncytial virus and mycoplasma pneumoniae combined[53] further strengthening the hypothesis that primary BK infection occurs through the respiratory tract.

Latency

After primary infection, BK virus persists mostly in the renal tubular epithelial cells and the uroepithelium, in a latent form. BK virus DNA was found in 33% of kidneys by

DNA-DNA hybridization in normal subjects [55] and in 25% of fresh frozen prostate specimens of patients with prostate adenocarcinoma, using nested PCR[56]. It was also found in 2/67 autopsy brain specimens with southern blot[57] and in 17/18 healthy donors peripheral blood leucocytes by PCR amplification with in situ hybridization[58]. Interestingly, Dolei and colleagues detected BK virus NCCR DNA by nested PCR in 22% of healthy donors, but the presence of VP1 DNA in only 7% of subjects, a prevalence that was declining with age. NCCR positive prevalence in peripheral blood monocytes cells was 37,5% in the less than 20 years old group, to 12,5% in the 21 to 40 years old and 0% in the more than forty years old[59]. Therefore, they hypothesized that blood cells do not host biologically active BK virus for a long time after acute infection or reactivation[59].

Reactivation

Seroprevalence in general population is about 50% in children of four years old and more than 75% in adults[9]. Newborns have maternal antibodies that decline with a nadir at 6 months[9]. Viruria, which may represent the first evidence of reactivation, can be detected in both healthy and immunocompromised subjects. Pregnant women can have asymptomatic viruria[60], a possible consequence of hormones (mostly glucocorticoid and the combination of oestrogen and progesterone) on viral replication[61]. Viruria has also been noted in 7% of asymptomatic healthy blood donors[62] and in more than 60% in immunocompromised patients[63]. Indeed, in addition to kidney transplant and hematopoietic stem cell transplant recipients, viral reactivation has been described in patients with HIV[13], lupus erythematosus

patients[64], Wiskott-Aldrich syndrome[7], hyperimmunoglobulin M immunodeficiency[65], cartilage-hair hypoplasia and Hodgkin's disease[66], in non-renal solid transplant[67] and in multiple sclerosis patients receiving Natalizumab therapy[68].

In kidney transplant recipients the virus initially replicates in the distal tubular epithelial cells, leading to necrosis and initiation of local damage and inflammation. The spread of virus in the adjacent environment will result in viruria and the infection of adjacent cells. Following this initial insult, denudation and dissolution of the tubular basement membrane occurs, allowing infection to spread in the intertubular space and by peritubular capillaries resulting in viremia[45] (Figure 2). This is followed by recruitment of inflammatory cells in the tubulo-interstitial space and viral spreading to proximal cells. Infection control will normally occur with the reestablishment of immune competence. A two-hit phenomenon is usually required for BK virus associated nephropathy development: environmental factors promoting viral replication and immunodeficiency.

3.1 Environmental factors and the inception of PVAN

PVAN occurs early after transplant, likely in the context of a “perfect storm” where immunosuppression is at its peak and active tubular lesions from ischemia-reperfusion or surgical trauma coincide. Indeed, electron microscopic data of kidney biopsies from patients with BK nephropathy demonstrated extensive tubular necrosis, even of the non-infected cells. Therefore, a question is whether tubular injury can

trigger BK-mediated nephritis, or does PVAN requires an environment conducive to tubular injury[45].

Mouse model of polyomavirus infection demonstrated that kidney damage, either chemical or ischemic, can promote viral replication[69]. Viral replication is controlled by the NCCR and can be regulated by numerous cellular transcription factors, including nuclear factor I, Sp1, NFAT, AP1, Smad3, estrogen response element (ERE), glucocorticoid and/or progesterone response element (GRE/PRE), p53, NF-κB, C/EBP and maybe PEA3, AP-2, CREB and GM-CSF (as reviewed by Liang and colleagues)[70]. Several of these molecules articulate numerous pro or anti-inflammatory pathways that are active following kidney injury and that could link ischemia/reperfusion, as well as inflammatory responses, to BK virus replication. As examples, factors such as TGF-β and tumor necrosis factor alpha (TNF-α) that can directly enhance transcriptional activity and promote viral replication[70]. Microenvironmental factors can therefore explain the particular vulnerability of the transplanted kidney. In addition, immunosuppression strategies could also directly activate viral replication. Hence, glucocorticoid pulses often used for treatment of acute rejection are well known risk factors for BK virus reactivation[20]. Independently of their effects on the immune system, steroid hormones can increase virus transcription by their action on GRE/PRE and ERE transcription factors on NCCR[71]. Once the virus has started to replicate, it must be held in check by a proficient immune system.

3.2 Immunology of PVAN

3.2.1 Cellular

T cells, especially CD8+, are pivotal to the anti-BK response and surveillance as they can detect and kill infected cells. The presence of BK virus specific T cells in the blood of seropositive healthy patients was demonstrated by T cell production of interferon-γ (IFN-γ), TNF-α, granzyme A and B and CD107 expression following stimulation with BK's VP1 and LT antigens[72]. This was also demonstrated in patients with BK viremia and nephropathy by assessing IFN-γ producing cells by flow cytometry and multiplex analysis of the supernatant of peripheral blood mononuclear cells stimulated with BK VP1 peptide mix[73]. Cellular immune responses against LT and VP1 antigens are also higher in patients with decreasing or past viremia, compared to those with increasing or persisting viremia[74], or BK nephropathy[75], suggesting again that they play a role in the control and resolution of BK virus reactivation. Additional evidence of T cell activation during viremia or PVAN include the expression of messenger RNA (mRNA) associated with a cytotoxic program in T cells[76].

In a study by Comoli and colleagues, transplant recipients with or without BK viruria had lower BK specific T cells evaluated by enzyme-linked immunospot assay (ELISpot) compared to healthy patients, which may suggest an impact of immunosuppression on BK immunosurveillance[77]. However, Chakera *et al.* failed to demonstrate a correlation between BK-specific T cells against any of BK peptides by ELISpot assays and tacrolimus trough levels or the total burden of immunosuppression, suggesting other factors must contribute to the lack of specific immunity post transplant[78]. In the study by Comoli *et al.*, viremic patients had

undetectable CD4 and CD8 for BK virus. Appearance of BK reactive T cells coincided with graft function improvement and resolution of viremia[77] results that had been confirmed by at least two other groups[78,79]. Compared to viremic patients without BK nephropathy, patients recovering from an episode of viremia had improved T cell response, as evaluated by ELISpot[79]. With the inherent limitations associated with testing peripheral blood and not lymphoid organs or the kidney, these data nonetheless suggest that the restoration of immune competence is central to viral control.

Mueller and colleagues have found that the five BK virus specific proteins (VP1, VP2, VP3, LT, sT) were able to elicit memory T cell response, demonstrated by specific production of IFN-γ, IL2 and TNF-α by flow cytometry analysis. All patients with a history of PVAN had a response to at least VP3 and 74% had a response to all five[80]. Also, these patients had a greater CD4 response than patients with asymptomatic viremia, as seen by a greater production of IL-2 and INF-γ[80]. However, T cells producing three cytokines (IFN-γ, IL2 and TNF-α), were more frequent in patients with asymptomatic viremia or no BK virus reactivation compared to PVAN patients, suggesting a possible protective role, or that strong T cell activation in PVAN leads to exhaustion and loss of polyfunctional responses[80]. In a study by Schmidt *et al.*, transplant recipients with BK virus complications had more BK-specific T cells but less polyfunctional compared to transplant recipients without BK complications, suggesting also exhaustion of those T cells[81].

T cells recognize peptide antigens presented by HLA molecules. HLA matching could therefore be important to elicit an optimal response. Whether HLA mismatching has an impact on PVAN is controversial. While some studies found an association between BK virus nephropathy and HLA mismatch[82], others did not[83]. With the significant caveat that patients with many HLA mismatches are more aggressively immunosuppressed, thereby impeding anti-viral T cell responses, HLA mismatching could further limit viral antigen recognition on mismatched HLA molecules. Matching of HLA-A2, B44 and DR15 may be protective against BK viremia[84], and the absence of C7 in either the donor or the recipient may be a risk factor for BK infection[17], a result that was not confirmed in another cohort[85].

Little is known about the resolution process of PVAN. There is an inflammatory response resembling histologically and genetically to acute rejection[76,86]. Whether this response is appropriate or is overwhelming, as an immune reconstitution syndrome, is not known. Two questions remain; does this process trigger fibrosis[87] and/or allospecific damage? In the study by Menter and colleagues, PVAN resolution was not associated with fibrosis, but all biopsies were obtained relatively early after PVAN resolution (within one year)[86]. Despite the risk of alloreactive damage, the central tenet of PVAN prevention and treatment is a reduction in iatrogenic T cell immunosuppression.

3.2.2 Humoral

Many studies used serological testing as a surrogate maker for B cells activity in BK virus infection. However, two critical elements must be considered; i: no current serological assay is standardized[88] and ii; seropositivity indicates that a patient has been in contact with the virus and seroconverted, but this does not imply the development of effective anti-BK T cell memory responses which are principally needed to control BK reactivation[77].

Qualitative and quantitative serostatus

Pediatric studies demonstrated a correlation between seronegative status and an increase risk of viruria[89] and PVAN[90]. However, this correlation is controversial in adults. Two hypotheses have been proposed to explain the difference between these two patient populations. First, seropositivity may decline with time[91]. Shah and colleagues reported 100% seropositivity at 10-11 years old and 67% after 35 years old[92]. Hence, antibodies may be present, but under the threshold of detection. Second, adults have been exposed to many different viruses and may have acquired a cross-reactive protection[89]. Bohl demonstrated that a seropositive status in adults pre-transplant does not prevent viremia[93] and Hirsch showed that seronegativity in patients before transplantation is not a risk factor for PVAN[20]. However, another group found a higher risk for BK viremia in seronegative recipients who received a kidney from a seropositive donor[94]. These discrepancies may be accounted by variability in the assays used to detect BK-specific antibodies and quantitative differences in anti-BK antibody titers. It was previously shown that viremic patients had a lower antibody level pre-transplant than those who never developed BK viremia[93]. Moreover, kidney

recipients from a seropositive donor will have a larger increase in antibody titers than those receiving a graft form a seronegative donor[16], regardless of their own status. This suggests that BK virus transferred through the transplanted kidney can elicit a host primary or recall humoral response. Finally, there is an increase in IgG titer with PVAN resolution, suggesting humoral immunity could play a role in viral control[95].

3.2.3 Innate immune response

Natural killers

Natural killers (NK) cells play an important role in the innate immune response against viral infections, and probably in polyoma infection/reactivation as well[76]. NK cell activity is controlled by opposing signals that come from a balance between activating and inhibitory receptors and can contribute to the orchestration of the adaptive immune response as well as mediating direct killing of infected cells. Many strategies are developed by viruses to avoid recognition by NK cells[96]. For example, BK virus miRNA can mediate down regulation of the NKG2D ligand ULBP3[97]. Trydzenskaya and colleagues found a relation between activating killer cell immunoglobulin-like receptors (KIR) genotype and the control of BK virus infection as well as nephropathy in kidney transplant recipients[98]. NK from PVAN patients had lower activating receptors compared to the control group. However, they did not find any correlations between KIR, HLA compatibilities and BK virus infection[98]. Although less studied than in T cells, the impact of immunosuppressive therapy on NK function reveals that NK cells are inhibited by currently used medications. Cyclosporin A affects NK-cell function, phenotype[99] and proliferation[100], while prednisolone inhibits

their proliferation when exposed to allogenic tubular epithelial cells and tacrolimus may counter their capacity to degranulate in the same context[101]. Also, mycophenolate mofetil possibly inhibits proliferation induced by IL-2[100]. However, the relative importance of NK cells relative to other immune effectors remains to be defined and whether NK cells could be mobilized for prevention or therapy of BK-related diseases is unclear.

Dendritic cells

Dendritic cells (DC) are central to the adaptive cell response, as they are efficacious antigen-presenting cells. Kidney transplantation and chronic immunosuppression lead to an absolute decrease in DC counts in the peripheral blood[102,103]. Transplant surgery in itself induces a strong decline in the number of DC (and possibly with a greater reduction for plasmatoid DC[103]), in kidney transplant recipients as well as in kidney donors. This decline can last up to 3 months after surgery[102]. As opposed to donors, patients on chronic immunosuppression fail to recover normal counts[102]. Hackstein and colleagues demonstrated that all DC subtypes were lower in patients treated with long term immunosuppression (more than a year) in kidney transplant recipients compared to age and sex matched controls, independently of total leucocyte count[104]. Despite this possible DC deficiency, Yapici and colleagues found significant amount of myeloid DC in PVAN biopsies and those cells were found closely to BK virus infected tubules[105], suggesting a role in PVAN physiopathology.

Pre-transplant DC deficiency, both absolute and functional, is associated with an increased BK viremia risk after transplant, even after adjustment for ureteral stent, tacrolimus and cyclosporine use[106]. Functional DC deficiency was evaluated by the production of IL-12 of a pool of peripheral blood mononuclear cells after lipopolysaccharide (LPS) stimulation. Furthermore, the absolute DC number in PVAN patients is reduced compared to other kidney recipients, despite the presence of ureteral stent and the use (not trough level) of tacrolimus[103]. Whether these findings reflect a direct impact of DC deficiency on BK reactivation is unclear. Nonetheless, DC levels and function could be further studied as biomarkers for the prediction of BK reactivation and disease.

Monocytes/macrophages

Little is known about monocytes' role in BK nephropathy. Patients with BK viruria (not PVAN) have increased soluble interleukin-1 receptor antagonist levels in their urine, a counter regulator of monocyte activation which can be produced by monocytes (as well as other cell types, as endothelial and epithelial cells upon inflammatory stress)[107]. More research is needed to decipher the role of inflammatory macrophages (M1) and anti-inflammatory (M2) macrophages in PVAN, as they could respectively propagate the initial immune response and orchestrate the resolution of inflammation as well as the development of fibrosis.

4. Current Therapeutic approaches

A first strategy to prevent BK reactivation would be to tailor immunosuppressive regimen according to BK virus reactivation risk. Although promising, there is currently not enough evidence to recommend such approach currently[108,109]. Hence, a pre-emptive strategy is used. To date, the best preventive PVAN strategy is to routinely monitor BK virus reactivation and to reduce immunosuppression pre-emptively if needed. According to the KDIGO (Kidney Disease | Improving Global Outcomes) recommendations, BK screening should be performed monthly early after transplant (first 3-6 months), then every 3 months until the end of the first year post-transplant[110]. Testing should be repeated and performed at increased frequency if there is an unexplained rise in serum creatinine and after treatment for acute rejection. PCR quantification of BK viremia is recommended as the screening method as it has the best sensibility and specificity[19]. If not accessible, urinary cells or urinary PCR are acceptable surrogate markers of BK reactivation[19,111]. Kidney biopsy of patients with viral load of 10000 copies/mL should be performed as it is highly associated with PVAN [20,21]. Absence of histological changes associated with PVAN, associated with viremia over 10000 copies/mL may be called ¨presumptive PVAN¨. The conventional approach is to treat those patients as definitive PVAN. However, to minimize the risk of acute rejection associated with a reduction in immunosuppression in patients who might not develop definitive PVAN, Nickeleit and Singh recently proposed to better stratify these patients using the urinary polyomavirus-haufen test and urinary mRNA in order to personalize therapeutic interventions and avoid under

treating BK reactivation in the kidney [31]. These complementary analyses are not available to all centres and have not made their way into the KDIGO recommendations.

When there is viral reactivation, the only recommended treatment is a reduction in immunosuppression (KDIGO), but it comes with the risk of acute rejection[112]. These approches include to first reduce the calcineurin inhibitor [83,113,114,115], or reduce/ discontinue the anti-metabolite[116,117], to reduce them both simultaneously[118,119,120] or to switch to less potent drugs, such as cyclosporin A (if tacrolimus is used as first line) [83,113,121,122], azathioprine, sirolimus[123] or leflunomide. However, these protocol have never been compared head to head, thereby leaving clinicians rely on their experience and the clinical context. There are only four randomized-controlled trials on PVAN prevention or treatment (Table 1). Despite the lack of clinical evidence supporting a particular approach, many treatments are proposed for PVAN notably based on the demonstration of anti-viral activity *in vitro*. At our center, we first revised downwards the calcineurin inhibitor target and halved the anti-metabolite. If possible, we randomized PVAN patients in clinical trials.

Sirolimus

The Mammalian Target of Rapamycin (mTOR) complex-1 inhibitor Sirolimus is used as an immunosuppressive drug owing mostly to its capacity to inhibit IL-2 dependent T cell proliferation. It also has an impact on effector T cell metabolic programming and TReg generation and maintenance[124]. In addition, Sirolimus was shown *in vitro* to

reduce LT antigen replication but not BK virus DNA replication[125]. This could also occur *in vivo* and provide direct anti-viral effects[126]. However, Sirolimus is likely less potent as an immunosuppressive agent than calcineurin inhibitors[127,128]. Hence, it might be difficult to dissect the relative contribution of immunomodulation and anti-viral effects in human studies.

Leflunomide and cidofovir

Leflunomide has been increasingly used in PVAN patients. In its' active form, A771726, Leflunomide inhibits protein kinase activity and the synthesis of pyrimidines[129]. *In vitro*, it reduces LT antigen expression and BK DNA replication[130]. Cidofovir is a cytosine nucleoside analog which inhibits viral DNA polymerase in cytomegalovirus infections, but its antiviral effect in BK nephropathy is not known[131]. Although proposed as a potential therapeutic agent in PVAN, concerns remain related to Cidofovir's nephrotoxicity in patients with precarious renal function. Also, a pharmacology study concluded that Leflunomide and Cidofovir activity against BK virus is modest and that the selectivity index is low[132]. Finally, a systematic review on the treatment of PVAN concluded that there is no benefits of adding Cidofovir or Leflunomide to reduction of immunosuppression[112]. However, and as pointed by the authors, this conclusion is made from small cohorts and has not been addressed in a large randomized study.

Quinolone

Fluoroquinolones could also have an *in vitro* activity against polyomaviruses[133]. They inhibit the helicase activity of SV40 LT antigen[134], as well as DNA topoisomerase[135]. However, one month of levofloxacin wasn't superior to standard treatment in the treatment of BK viremia[136], and a 3-months course after transplant failed to prevent viruria and was associated with bacterial resistance in a randomized-control trial[137].

Immunoglobulin

Intravenous immunoglobulin (IVIG) were also proposed to treat BK nephropathy. As for other viral infections, the main effect of such treatment would be from neutralizing antibodies preventing cellular infection[138]. There is evidence supporting that this treatment might be useful in some refractory cases[139]. *In vitro*, co-incubation of BK virus with IVIG for 2 hours before WI-38 cells infection led to more than 90% diminution of viral DNA after 7 days in culture[138]. However, this effect was significantly diminished if IVIG treatment was given directly to cells before or 2 hours after the infection, suggesting direct neutralization of BK virus by BK-specific antibodies.

Cyclosporine A

The widely used calcineurin inhibitor Cyclosporine A was also shown to inhibit LT antigen and VP1 *in vitro*. However, its inhibitory effect on BK-specific T cells may override its benefits[24]. A randomized controlled trial comparing Cyclosporine A to Tacrolimus demonstrated a lower incidence of viruria in the Cyclosporine A group, but no decrease in viremia[116]. Whether these effects can be related to the anti-viral

effects or the relative reduced potency of Cyclosporine A[140] as an immunosuppressive drug is unknown.

In summary, very little evidence would support any strategy over the others. As such, clinical trials are required to define the best pharmacological approach to BK virus reactivation and PVAN. However, based on the available information, current clinical practices and existing recommendations, we can outline an algorithm (Figure 3) to guide clinical practice and summarize the areas of uncertainty.

5. Perspectives

To this day, reduction in immunosuppression remains the cornerstone of PVAN treatment, highlighting the role of the host's immune system in controlling viral reactivation and infection of the transplanted kidney. Unfortunately, reducing immunosuppression puts the patient at risk of rejection. Hence, providing specific anti-viral immunity without risking organ-threatening alloreactivity remains an unachieved goal. To overcome this hurdle, several approaches using immunosuppressive drugs with anti-viral properties are under evaluation, including the use of Everolimus (ClinicalTrials.gov NCT01624948, NCT01289301 and NCT01911546) and the association of Sirolimus and Leflunomide (controlled-trials.com ISRCTN40228609).

As cellular immunity is the key to control BK virus reactivation, measures to augment BK-specific T cell may become a form of next-generation PVAN treatment. There are two ongoing studies evaluating the presence of BK-specific T cells to predict risk of BK reactivation and nephropathy (ClinicalTrials.gov NCT02049827 and NCT01109186). These studies may provide important information about the degree of T cell immunity required to protect against the development of PVAN. Several approaches may be considered to boost BK-specific immunity, among them adoptive immunotherapy which seems particularly promising.

Adoptive T cell immunotherapy refers to the transfer of *ex vivo* manipulated T cells. The use of *ex vivo* "educated" T cells to prevent or treat viral reactivation in multiple

settings has been shown to be safe and efficacious. This approach was developed in the early 1990s to treat hematopoietic stem cell transplant patients suffering from EBV-related complications[141]. There is now evidence that several infectious agents can be treated with this approach in both HSCT and solid organ transplant patients. Although requiring expert cell-processing capabilities and clinical cell-therapy infrastructure, anti-viral adoptive immunotherapy has been shown to be cost-effective for the treatment of CMV and EBV-related complications[142,143]. The feasibility of producing autologous BK-specific T cells lines from viremic renal transplant patient was initially demonstrated by Comoli and colleagues[144]. Peripheral blood mononuclear cells were stimulated using autologous DC pulsed with BK virus antigen and exogenous IL-12, IL-7 and IL-2. In addition to the production of BK-reactive conventional T cells, the culture generated up to 66% γδ T cells which were found to be active against BK infected cells *in vitro*. A role for γδ T cells in the control of BK infection *in vivo* remains to be demonstrated, but innate lymphoid cells are increasingly recognized a key actors in viral infections[145]. A second group successfully expanded 15 BK-specific T cell lines, including one from a viremic kidney transplant recipient. However, cell expansion was limited and up to 20% NK cells were present in the final product[146]. Finally, the first demonstration that BK-specific T cell lines could be used clinically came from the Baylor College of Medicine group who treated HSCT patients with donor-derived multi-virus specific T cell lines[147]. The treatment cleared BK viremia in 5 out 7 patients and was not associated with significant side effects.

Conclusion

The occurrence of BK virus nephropathy almost exclusively in kidney transplant recipients but not in similarly immunosuppressed patients or in other settings of kidney injury indicates that a convergence of factors hinging around local injury and immunosuppression lead to PVAN. Additional factors may be the virulence of the donor-derived virus and HLA-mismatching. Despite these limitations, the central aspect of PVAN prevention and treatment remains a proficient host T cell immunity. In order to better prevent or treat BK-associated nephropathy, several variables will have to be defined, notably the relative contribution of virus-related and inflammation-related damage to renal dysfunction. Intervention trials designed to target the virus and/or fine tune BK-specific immunity will be required to ultimately define the best approaches to protect renal transplant recipients against PVAN.

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Table 1.
Randomized trials for prevention or treatment of BK virus infection in kidney transplant recipients

Study	Population	Protocol	follow-up	Primary Outcome	Results
Knoll, JAMA, 2014; 312 (20): 2006-14.	154 KTR	3 mo of Levofloxacin (500 mg daily) or placebo	46,5 wk (levofloxacin), 46,3 (placebo)	Occurence of BK viruria within the first year after transplantation	29% (levofloxacin) vs 33,3% (placebo); Hazard ratio, 0.91; 95% CI, 0.51-1.63; *p* =0.58
Lee, CJASN, 2014; 9(3): 583-389	39 Viremic KTR	1 mo Levofloxacin 500 mg daily or placebo	6 mo	Percentage reduction in plasma BK viral load at 3 months	70,3%(levofloxacin) vs 69,1% (placebo), p=0,93
Guasch, Transplantation 2010; 90(8): 891-897	46 Newly diagnosed or untreated PVAN	FK778 or reduction of immuno-suppression	6 mo	Change in urine BK viral load	-3,1 (FK778) vs -2,8 (control) p=0,586
Brennan, Am J T; 2005; 5(3): 582-594	200 KTR	FK506 or CyA	1 y	Incidence of BK virus infection with tacrolimus versus cyclosporine	Viruria: 46% (FK506) vs 13% (CyA), p=0,005. Viremia 12% (FK506) vs 11% (CyA) p=1

Study

KTR, Kidney transplant recipients; PVAN, polyomavirus associated nephropathy; CyA, cyclosporin A

Figures

Figure 1. BK polyoma virus cell entry and infection. Representation of mechanisms of viral cell entry, trafficking and infection highlighting action on the cell cycle machinery.

Figure 2. Physiopathology of PVAN. Depiction of PVAN development form latency in the uroepithelium (top) to the development of renal inflammation and fibrosis (bottom).

Figure 3. Algorithm to guide the screening, prevention and treatment of polyomavirus BK nephropathy after kidney transplant.

Figure 1

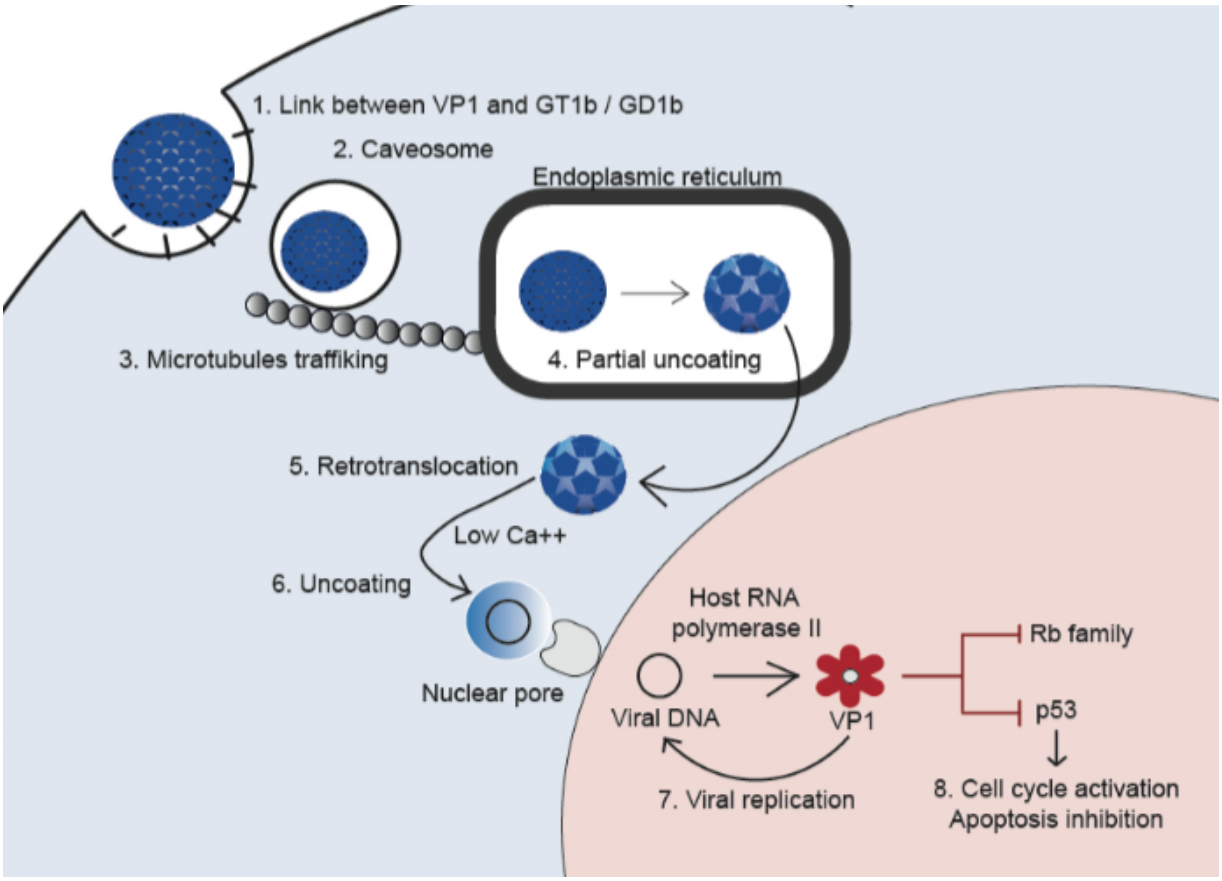

1. Link between VP1 and GT1b / GD1b
2. Caveosome
Endoplasmic reticulum
3. Microtubules traffiking
4. Partial uncoating
5. Retrotranslocation
Low Ca++
6. Uncoating
Nuclear pore
Host RNA polymerase II
Viral DNA
VP1
Rb family
p53
7. Viral replication
8. Cell cycle activation
Apoptosis inhibition

Figure 2

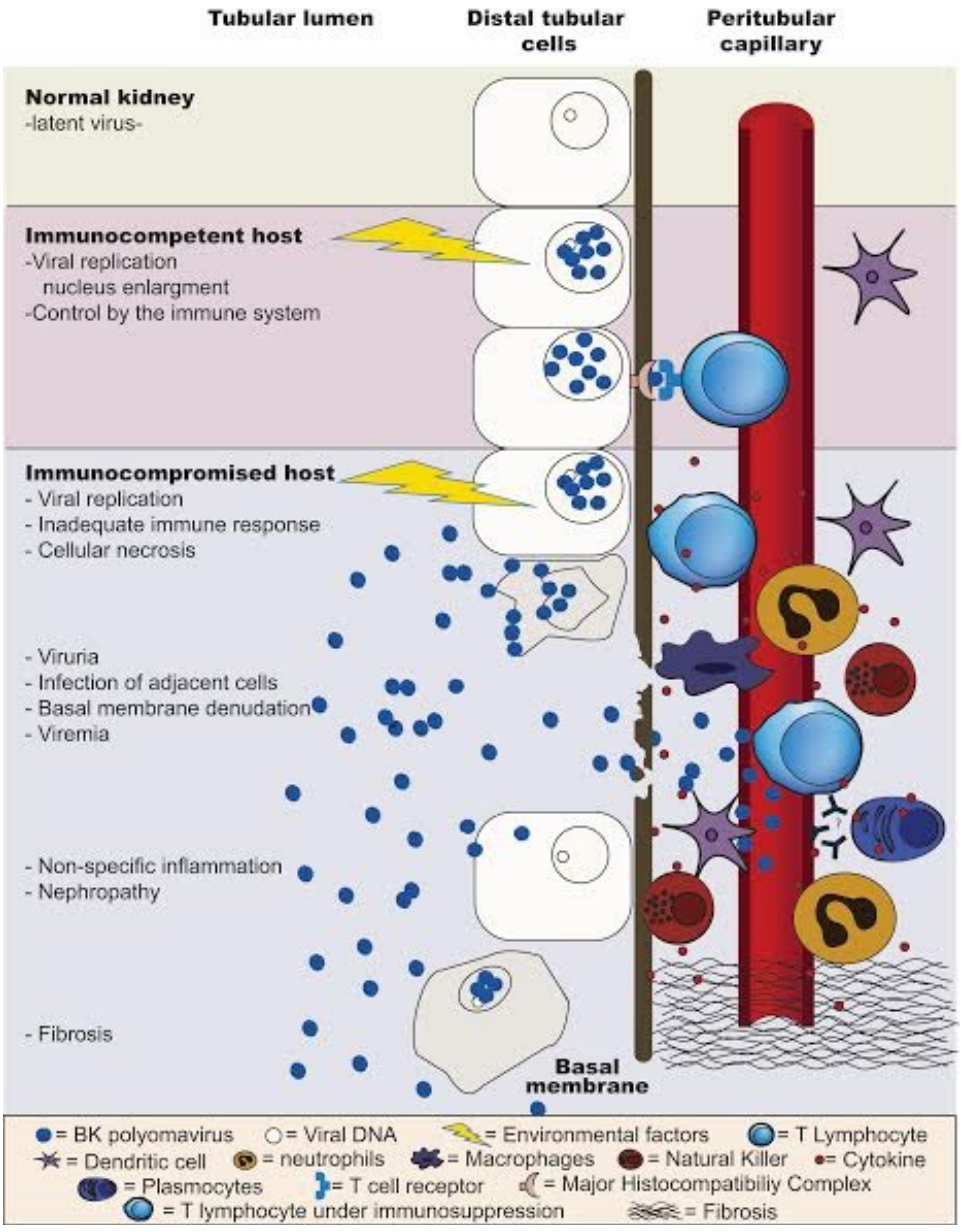
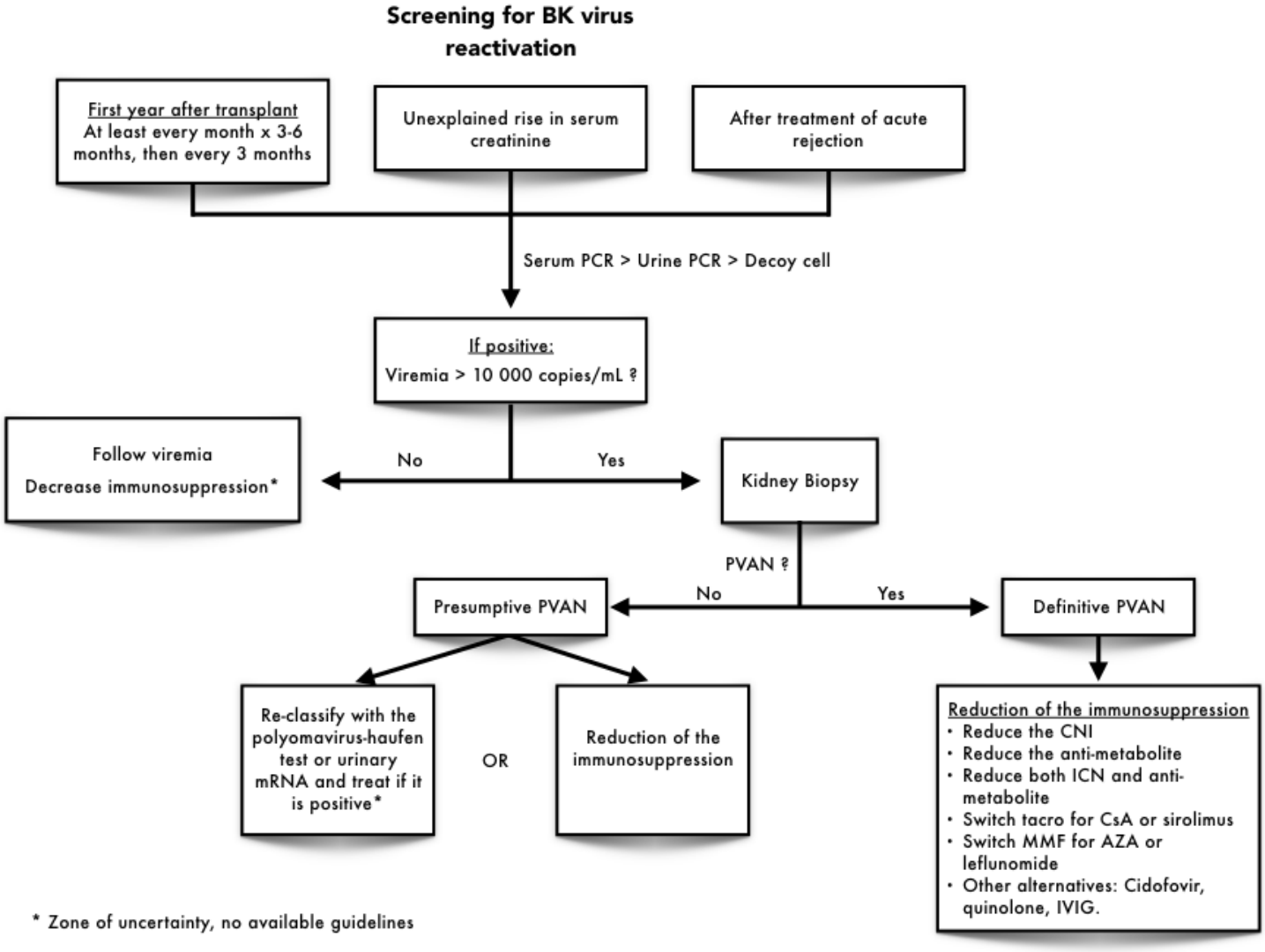

Tubular lumen
Distal tubular cells
Peritubular capillary
Normal kidney
-latent virus-
Immunocompetent host
-Viral replication
nucleus enlargment
-Control by the immune system
Immunocompromised host
- Viral replication
- Inadequate immune response
- Cellular necrosis
- Viruria
- Infection of adjacent cells
- Basal membrane denudation
- Viremia
- Non-specific inflammation
- Nephropathy
- Fibrosis
Basal membrane
= BK polyomavirus
= Viral DNA
= Environmental factors
= T Lymphocyte
= Dendritic cell
= neutrophils
= Macrophages
= Natural Killer
= Cytokine
= Plasmocytes
= T cell receptor
= Major Histocompatibiliy Complex
= T lymphocyte under immunosuppression
= Fibrosis

Figure 3


Screening for BK virus reactivation
First year after transplant
At least every month x 3-6 months, then every 3 months
Unexplained rise in serum creatinine
After treatment of acute rejection
Serum PCR > Urine PCR > Decoy cell
If positive:
Viremia > 10 000 copies/mL ?
No
Yes
Follow viremia
Decrease immunosuppression*
Kidney Biopsy
PVAN ?
No
Yes
Presumptive PVAN
Definitive PVAN
Re-classify with the polyomavirus-haufen test or urinary mRNA and treat if it is positive*
OR
Reduction of the immunosuppression
Reduction of the immunosuppression
• Reduce the CNI
• Reduce the anti-metabolite
• Reduce both ICN and anti-metabolite
• Switch tacro for CsA or sirolimus
• Switch MMF for AZA or leflunomide
• Other alternatives: Cidofovir, quinolone, IVIG.
* Zone of uncertainty, no available guidelines

3- Immunothérapie adoptive

3.1 Mise en contexte

L'immunothérapie adoptive consiste à injecter à un patient des cellules immunes après une expansion *ex vivo*. Les cellules sont soit autologues, donc provenant du receveur ou allogéniques, donc provenant d'un donneur, qu'il soit HLA-identique ou non (Figure 2). Cette thérapie est principalement utilisée dans le traitement de cancers réfractaires ou pour le traitement d'infections virales chez les patients fortement immunosupprimés (principalement après une greffe de cellules souches hématopoïétiques ou d'un organe solide). De plus, l'infusion de lymphocytes T régulateurs peut être utilisée pour favoriser la tolérance après une transplantation (23) ou dans le traitement de maladies auto-immunes (24).

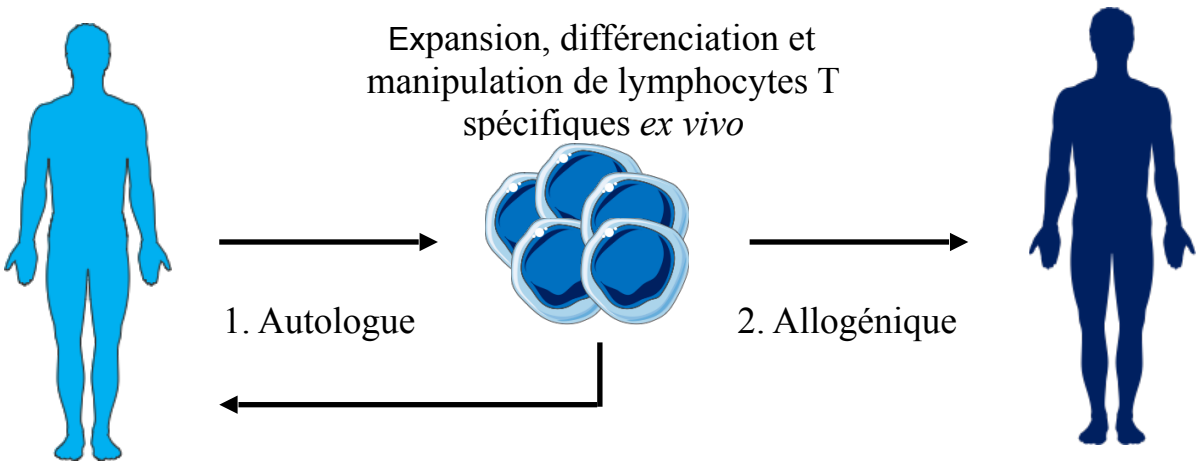


Figure 2. Immunothérapie adoptive

Les premiers transferts adoptifs de lymphocytes T mémoires virus-spécifiques se sont fait au début des années 90 afin de traiter les réactivations du cytomégalovirus (CMV) (25, 26) et d'Epstein–Barr virus (EBV) (27-29) chez les greffés de moelle. Les

premiers traitements ne consistaient qu'à infuser plusieurs leucocytes provenant du donneur, en espérant que plusieurs d'entre eux soient virus-spécifiques (infusion de lymphocytes du donneur/*donor lymphocyte infusions*). Cependant, cette approche entrainait plusieurs effets secondaires, tels qu'une réaction du greffon contre l'hôte (GVHD) (28, 30). Par la suite, les méthodes se sont raffinées afin de faire l'expansion que lymphocytes T virus spécifiques.

La source des lymphocytes doit être adaptée au contexte clinique. Pour traiter les patients ayant reçu une greffe de cellules souches hématopoïétiques, le donneur représente une bonne source de lymphocytes T parce que le système hémato-lymphoïde du patient est généralement de type donneur et particulièrement s'il est immun contre le virus pour lequel le receveur nécessite un traitement. C'est pourquoi le donneur a été utilisé dans la plupart des études visant à traiter l'EBV (29, 31-34), le CMV (26, 35-39), le polyomavirus JC (40) et même faire des lignées spécifiques pour plusieurs virus (41-44). La situation est plus complexe lorsque le répertoire du donneur est naïf (par opposition à un répertoire mémoire). Plusieurs ont réussi à manipuler les cellules *ex vivo* afin de leur conférer cette immunité mémoire, à l'aide de protocoles plus élaborés que ceux normalement utilisés (43, 45-48). Une autre solution est d'utiliser les cellules provenant d'un tiers (donc ni le donneur ni le receveur) étant le plus HLA-compatible que possible avec le receveur (30, 49-54). Cette compatibilité permet une plus grande réactivité anti-virale, cette réponse étant HLA-restreinte, ainsi qu'une réduction du risque de GVHD. Des banques de donneurs peuvent même être utilisées.

Dans les cas de transplantations d'organes solides, souvent HLA disparates, l'utilisation du donneur d'organe est souvent impossible et ne serait pas souhaitable puisque ses lymphocytes seraient rapidement rejetés par l'immunité du receveur et pourraient induire une GVHD. L'utilisation d'une tierce partie représente également un risque de réaction allogénique (rejet ou GVHD), surtout si elle n'est pas HLA identique. Il est donc préférable d'utiliser des lignées autologues ayant un plus grand potentiel de persistance (55-59). Le défi est alors de faire des lignées à partir du sang de patients immunosupprimés.

3.2 Efficacité / Innocuité

L'efficacité de l'immunothérapie adoptive antivirale est principalement démontrée pour la prévention et le traitement de syndromes lymphoprolifératifs post-transplantation (PTLD) qui sont majoritairement secondaires à une réactivation à EBV. À ce jour, plus de 100 patients ont reçu les lignées EBV-spécifiques en prophylaxie. De ces patients, aucun n'a développé de PTLD. De plus, les lignées antivirales ont été prouvées sécuritaires puisqu'aucun patient n'a développé de maladie du greffon contre l'hôte (GVHD) (30). Pour le traitement des PTLD, l'efficacité semble varier entre 50 à 70 % de rémission partielle ou complète (30, 52, 56) chez des patients qui étaient réfractaires au traitement médical standard. De plus, il faut considérer une grande hétérogénicité dans le mode de production des lignées (donneur autologue, allogénique ou tierce partie), dans la dose utilisée (allant de 2 doses de $10x10^6/m^2$ (29, 33) à 4 doses de $2x10^6$/kg (52)) ainsi que dans les critères

utilisés afin de parler de rémission. Bien que ces données ne peuvent pas directement prédire le fonctionnement de telles lignées dans d'autres situations cliniques, telle la néphropathie au polyomavirus BK, elles montrent néanmoins que l'immunothérapie adoptive est une thérapie ayant un réel potentiel thérapeutique et que cela semble très sécuritaire.

3.3 Différenciation des lymphocytes T

En plus des multiples différences identifiées ci-haut entre les différents protocoles d'immunothérapie adoptive, il semble que tous les lymphocytes T antigène-spécifiques ne soient pas égaux. En effet, il est de plus en plus accepté qu'il existe une corrélation entre le potentiel de renouvellement d'une cellule et son statut de différentiation (60).

L'activation d'un lymphocyte T naïfs se produira lors de la liaison de son TCR (et du CD3) avec un complexe CMH-antigène présent sur une cellule présentatrice d'antigène (notamment les cellules dendritiques) qui lui est spécifique, si la force de la stimulation est suffisante. Cette dernière sera proportionnelle à la concentration et l'affinité de l'antigène, aux stimuli de costimulation positifs et la durée de l'interaction (61). Une fois activé, il va proliférer et se différentier en lymphocyte T effecteur pouvant migrer au site inflammatoire ou en lymphocyte T mémoire, capable de produire une réponse encore plus grande lors d'un stimulus secondaire.

Sallusto et ses collègues ont été les premiers à suggérer deux sous-types de lymphocytes T mémoires, soit central (Tcm) ou effecteur (Tem), basé sur leur localisation, l'expression de marqueurs de surfaces et leur fonction effectrice (62). Les Tcm expriment CCR7, un récepteur de chimiokine, et CD62L, une molécule d'adhésion (63). Ils n'ont que peu de capacités effectrices immédiates et on les retrouve surtout dans les organes lymphoïdes secondaires. Les Tem, quant à eux, ont la capacité de migrer vers les tissus inflammés et sont donc retrouvés soit dans les organes lymphatiques secondaires ou en périphérie. Ils ont des fonctions effectrices immédiates et n'expriment pas CCR7 (62). Finalement, en aval de la chaine de différentiation on retrouve les lymphocytes T effecteurs, ayant un maximum de fonctions effectrices immédiates mais une durée de vie plus courte *(60)*. Selon le modèle de différentiation progressive, d'abord proposé par Lanzavecchia, c'est la force de stimulation qui dictera la différentiation (Figure 3) (64).

Ayant un plus grand potentiel prolifératif, les Tcm seraient donc préférables en immunothérapie adoptive. En effet, ils ont une plus grande capacité de persister *in vivo* et ont un plus grand potentiel prolifératif lors d'une re-stimulation (65). L'avantage des Tcm sur les Tem a été démontré dans un modèle vaccinal (66), des modèles tumoraux (63, 67) et avec de l'immunothérapie adoptive anti-cancer (68). De plus, dans un modèle d'immunothérapie chez des primates non humains, seulement les Tcm avaient la capacité de persister à long terme *in vivo* et avaient toutes les caractéristiques de cellules mémoires (69). Gattinoni et ses collègues ont également démontré la présence chez l'humain d'un sous-groupe de lymphocytes T mémoires

ayant des propriétés de cellules souches (Tscm) (70). Ils possèdent des marqueurs de surfaces semblables aux lymphocytes naifs (CD62L, CCR7, CD45RA), mais se distinguent principalement de ces derniers par une relâche rapide de cytokines après activation ainsi qu'une plus grande capacité de prolifération en réponse à une stimulation avec de l'IL-15 (70). Ils se distinguent des lymphocytes T naifs par la présence CD95 et de CD122 à leur surface. Étant en amont de la chaine de différentiation, ces lymphocytes pourraient être avantageux en immunothérapie adoptive (60). Cependant, ils ne représentent que 2 à 3 % des lymphocytes T (70).

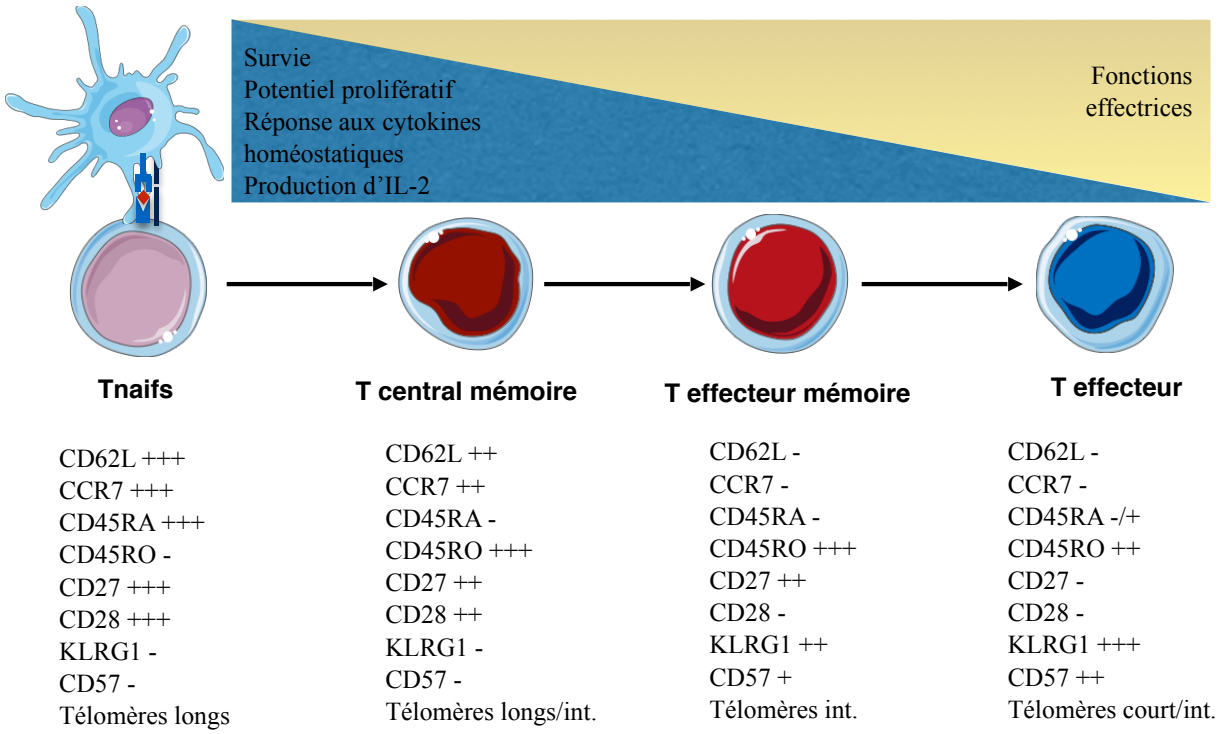


Figure 3. Différenciation des lymphocytes T, *adapté de Gattinoni L et al., 2012 (60)*

3.4 Résumé

L'Immunothérapie adoptive serait donc une thérapie idéale pour la prévention ou le traitement de la néphropathie associée au polyomavirus BK puisqu'elle permettrait d'augmenter l'immunité spécifique contre le virus sans augmenter le risque de rejet. Dans un contexte de transplantation d'organe solide comme la transplantation rénale, le protocole idéal comportant le moins de risque pour le patient, consisterait en une infusion autologue. Le défi est donc de produire des lignées à partir de sang de patients soumis aux immunosuppresseurs et à une exposition virale chronique. De plus, les patients étant chroniquement immunosupprimés, une maximisation de la différentiation centrale mémoire pourrait améliorer la survie de la lignée post réinfusion (65, 68, 69).

3.5 Hypothèse et objectifs

Notre hypothèse est que malgré l'exposition chronique au polyomavirus BK et la présence d'immunosuppresseurs, il serait possible d'obtenir des lignées de lymphocytes T BK-spécifiques à partir du sang de patients greffés rénaux réactivant le virus. Nos objectifs étaient donc de produire ces lignées à l'aide d'un protocole qui pourrait être utilisé en clinique, de démontrer leur spécificité pour le polyomavirus BK, d'évaluer leur risque de réaction allogénique et de les comparer à celles obtenues à partir du sang de donneurs sains.

RÉSULTATS

4- Résultats

4.1 Clinical-scale Rapid Autologous BK-virus Specific T cell Line generation from Kidney Transplant Recipients with Active Viremia for Adoptive Immunotherapy

L'utilisation d'immunosuppresseurs plus puissants en greffe rénale a permis d'améliorer la survie des greffons, au dépens d'une augmentation du risque néoplasique et infectieux. C'est ainsi que nous avons vu l'émergence de néphropathies associées au polyomavirus BK, pouvant évoluer jusqu'à la perte de greffon. Pour l'instant, le seul traitement démontré efficace de cette pathologie est une diminution de l'immunosuppression globale, augmentant de façon conséquente le risque de rejet. Notre objectif était donc de produire des lignées antivirales BK-spécifiques à partir du sang de patients greffés virémiques pour le BK, afin de prévenir et traiter la néphropathie associée au polyomavirus. Nous avons tout d'abord comparé les lignées obtenues à partir de greffés rénaux à celles obtenues à partir de donneurs sains. Bien que comparables au niveau de la spécificité et de la différentiation, l'expansion cellulaire chez les greffés n'était pas suffisante pour obtenir systématiquement une dose thérapeutique. L'ajout d'une stimulation à l'aide de cellules dendritiques nous a permis d'améliorer cette expansion cellulaire en plus de favoriser un phénotype central mémoire et d'améliorer la spécificité. Bien que polyclonales, les lignées cellulaires n'ont pas démontré d'alloréactivité *in vitro* et *in vivo.* De plus, nous avons démontré leur capacité de persister et de proliférer *in vivo*.

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Journal: Transplantation
Contribution des auteurs:

CL: Conception de l'étude, expérimentation et rédaction du manuscrit.
JO, MG, TP, AD, VGT, AB, LB: Aide à l'expérimentation et révision du manuscrit
CC et JSD: Conception de l'étude, supervision et aide à la rédaction du manuscrit

Clinical-scale Rapid Autologous BK-virus Specific T cell Line generation from Kidney Transplant Recipients with Active Viremia for Adoptive Immunotherapy

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Abbreviations:
BrdU, 5-Bromo-2′-deoxyuridine
CMV, cytomegalovirus
DC, dendritic cell
EBV, Epstein-Barr virus
VP1, Virus-encoded protein 1
LTA, Large T antigen
ELISpot, IFNγ enzyme-linked immunospot assay
GVHD, graft-versus-host disease
HC, healthy control
KTR, Kidney transplant recipient
NSG, NOD/SCID/IL2Rγ^{null}
PBMC, peripheral blood mononuclear cells
PVAN, Polyomavirus-associated nephropathy
Tcm, central memory T cell
Tem, effector memory T cell
Tn, naive T cell

Abstract

Background: Polyomavirus-associated nephropathy (PVAN) following BK virus reactivation in kidney transplant recipients (KTR) can compromise graft survival. Lowering immunosuppression is the only established approach to prevent or treat PVAN but non-specifically increasing host immune competence also augments rejection risk. *Ex vivo* T cell stimulation/expansion offers the possibility to generate BK-specific T cell lines for adoptive immunotherapy. The objective of this study was to develop and characterize a clinical scale protocol to generate BK-specific T cell lines from viremic KTR.

Methods: Peripheral blood mononuclear cells from healthy controls (HC) and viremic KTR were stimulated using BK-virus peptide libraries loaded or not on monocytes-derived dendritic cells. Cell counts, flow cytometry and next-generation sequencing were respectively used to assess T cell expansion, differentiation and clonal diversity. Enzyme-linked immunospots, cytotoxicity assays as well as adoptive transfer in NOD/SCID/IL2Rγ^{null} (NSG) mice were used to assess for pathogen-specificity and alloreactive potential of the generated T cell lines.

Results: T cell lines from KTR and HC showed similar characteristics, implying that ongoing immunosuppression and chronic virus exposure do not adversely affect the differentiation, specificity or clonal diversity of the T cell lines following *ex vivo* production. Using antigen-loaded dendritic cells improved T cell expansion, favored central memory T cell differentiation. The T cell lines were antigen-specific, with no evidence of alloreactive potential.

Conclusions: Using a rapid, clinically-compliant culture system, we show that autologous BK virus-specific T cell lines can be reliably generated from viremic KTR. Our results pave the way for the treatment or prevention of PVAN with adoptive immunotherapy.

Introduction

Latent BK polyomavirus infection in the urothelium is highly prevalent[1]. Reactivations are frequent in immunocompromised patients and pose a particular problem to kidney transplantation recipients (KTR). Polyomavirus-associated nephropathy (PVAN) can occur in up to 10% of KTR[2] and lead to graft loss in up to 50% of cases[3,4]. Reduction of immunosuppression is the most accepted strategy to treat BK-related complications[5] but has the disadvantage of increasing rejection risk by non-specifically increasing immune competence. Hence, KTR anti-viral immunity remains the cornerstone of PVAN prevention and treatment. The selective augmentation of host anti-BK immunity without a concomitant increase in the risk of graft rejection could solve the clinical conundrum facing transplant nephrologists treating PVAN. Adoptive immunotherapy using *ex vivo* expanded autologous BK-specific T cells from KTR may represent such ideal strategy.

It was recently shown in allogeneic hematopoietic cell transplantation that donor-derived T cell lines generated *ex vivo* and targeting several viruses, including BK virus, could control viremia and BK-related complications[6]. In solid organ recipients, adoptively transferred autologous or "third party" (neither derived from the donor nor the recipient) Epstein-Barr virus (EBV) or Cytomegalovirus (CMV)-specific T cell lines appear efficacious and safe despite ongoing immunosuppression[7-10]. Whether similar promising results could be obtained in refractory BK viremia or PVAN remain to be

established as no study using BK-specific T cell lines in the KTR population have been reported. As an essential stepping stone for the design of such trials is the development of reliable T cell manufacturing protocols that can be readily implemented in cell processing facilities.

In this work, a clinical-compliant system to rapidly generate BK virus-specific T cell lines was adapted and validated using both healthy control (HC) and KTR suffering from active or presumptive PVAN. Using as model a culture protocol capable of expanding virus-specific T cell lines from peripheral blood mononuclear cells (PBMC) in 9-14 days from healthy donors[6,11,12], we show that the reliable clinical-scale expansion of BK-specific T cell lines requires the addition of autologous BK virus antigen-pulsed monocyte-derived dendritic cells (DC). The use of DC not only improved T cell expansion but it also favored the generation of central memory T cells and conferred increased antigenic specificity. T cell lines derived from both HC and KTR were polyclonal, further expanded and persisted upon transfer into immunodeficient mice but did not show off-target alloreactivity *in vitro* and *in vivo*. Collectively, this work describes a readily translatable approach to manufacture autologous BK-specific T cell lines from KTR for adoptive immunotherapy.

Materials and Methods

Donors and T cell line generation

A total of 8 KTR from a single center and 5 volunteer HC were recruited on internal review board approved protocols (CÉR 13030 and 13125) for the various experiments conducted in this study. The KTR were all suffering from active viremia and 6 carried a diagnosis of definitive PVAN (Supplementary Table 1). PBMC were isolated from up to 200 ml of blood by gradient density separation (Ficoll-Paque, GE Healthcare, Baie d'Urfe, Canada). When indicated, cells were cryopreserved (20 to 50 x 10^6 cells per vial) in 10% dimethyl sulphoxide (DMSO, Sigma-Aldrich, Oakville, Canada), 20% human serum (Sigma-Aldrich) and 70% RPMI.

A total of 10 to 15 x 10^6 PBMC were directly pulsed with overlapping peptide libraries from virus-encoded protein 1 (VP1) and Large T antigen (LTA) as in [12, 11] (100 ng of each library in minimal volume for 30 minutes) (JPT, Berlin, Germany), or were co-cultured in a 1:10 ratio (stimulator: effector) with irradiated autologous DCs (40cGy) pulsed for 2 hours with the two peptide libraries (1µg/ml). T cell lines were generated in 14 days, using either peptide-pulsed PBMC or DC as previously described[12,13]. Specifically, all T cell cultures were performed using T cell media (45% Advanced RPMI 1640, 45% Click's medium, 10% human serum, 1X L-glutamine, IL-4 (1666U/mL-Feldan, Québec, Canada) and IL-7 (10ng/mL-Miltenyi, Auburn, CA) in a G-Rex10 vessel (Wilson Wolf Manufacturing, New Brighton, MN) and incubated at 37^0C and 5% CO_2. DC were prepared from circulating monocytes isolated by plastic

adherence and differentiated into DC as previously described[13]. Cells were counted by trypan blue exclusion with an automated cell counter (Countness, Invitrogen). At day 8 and 12, cultures were split if the cell concentration exceeded $45x10^6$ cells/mL.

IFNγ enzyme-linked immunospot assay (ELISpot), cytotoxicity assays and flow cytometry

ELISpot assays and analysis were performed by exposing 5×10^4 cells to the VPI or LTA antigenic peptide libraries, a non-targeted peptide library (negative control) or stimulated with an anti-CD3 antibody (positive control) overnight, according to the manufacturer's instructions (Mabtech Inc., Cincinnati, OH and vSpot Reader Spectrum, AID, Strassberg, Germany). Cytotoxicity was performed using a standard 4-hour chromium release assay using autologous or allogenic phytohemaglutinnin (PHA, Sigma-Aldrich) blasts pulsed with antigenic peptide libraries[12]. Cells were surface stained with monoclonal antibodies to: CD3, CD4, CD8, CD45RO, CD62L (BD Biosciences, Mississauga, ON), washed and fixed in PBS 2% FBS 1% PFA before acquisition on a LSRII instrument (BD Biosciences, Mississauga, ON). Data were subsequently analyzed using Flowlogic software (Inivai Technologies, Mentone, Australia) or Kaluza (Beckman Coulter, Indianapolis, IN).

Adoptive transfer in NOD/SCID/IL2Rγ^{null} mice

Seven- to 12-week-old NOD/SCID/IL2Rγ^{null} (NSG) mice were subjected to total body irradiation (250 cGy). The following day, the mice were injected

intravenously with 0.5×10^6 human T cells from either a 14 days culture (as described above) or a CD3+ positive selection of unstimulated T cells (Stemcell Technologies, Vancouver, BC). Intraperitoneal injections of rhIL-15 (1µg, 2000 U; Miltenyi) were administered twice a week for 3 weeks. Venipunctures were obtained weekly from week 2 or 3 until death or sacrifice (100-200 µL). Skin, liver, colon and short bowel samples were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin-eosin phloxine. When indicated, mice received 2 mg of 5-Bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) intraperitoneally every second day for 14 days starting on day 7 post transfer. The protocol was approved by the institutional authorities according to Canadian Council on Animal Care (CCAC) regulations. BrdU incorporation was assessed with flow cytometry using anti-BrdU monoclonal antibody (BD Biosciences) after a staining using BD Perm/WashTM Buffer, BD CytopermTM Permeabilization Buffer Plus and Deoxyribonuclease I (Sigma-Aldrich), according to the manufacturer's instructions.

Clonality analysis

Next-Generation-Sequencing (NGS) DNA-based TCR-gamma chain analysis was performed for clonality determination. DNA was extracted from T cell lines using DNAzol (Invitrogen) and quantified with the Qubit 2.0 system (ThermoFisher Scientific, Waltham, MA). TCR gamma-chain was amplified from 50ng of gDNA with LymphoTrack TRG assay (invivoscribe, San Diego, CA) and AmpliTaq Gold DNA polymerase (ThermoFisher Scientific) in the

SimpliAmp Thermal Cycler (ThermoFisher Scientific). Amplicons with sizes greater than 100 base pairs were positively selected with Agencourt AMpure XP beads (Beckman Coulter, Brea, CA). The libraries obtained were quantified on the ViiA 7 Real-Time PCR System with the Ion Library Taqman Quantitation kit (ThermoFisher Scientific). Next generation sequencing was completed on the Ion Proton semiconductor platform (ThermoFisher Scientific) using an Ion P1 v3 chip (ThermoFisher Scientific), prepared with the Ion Chef System (ThermoFisher Scientific). Fastq data were the analyzed with the LymphoTrack Bioinformatics Software (InvivoScribe). Up to 200 unique reads or 10% or total reads were obtained after the merging of duplicates using MAFFT, multiple sequence alignment software version 7 and determining the CDR3 region by IgBlast (www.ncbi.nlm.nih.gov/igblast/). Single nucleotide differences between 2 sequences outside the CDR3 region were considered sequencing errors. Clonality index was calculated using Shannon's Entropy formula, as described by Harden and colleagues[14].

Statistical analysis

All statistical analyses were performed using unpaired Student t-test using GraphPad Prism (version 5.0c; GraphPad Software) unless otherwise specified. P values < 0.05 were considered significant. Kaplan-Meier survival analysis was used for NSG mice.

Results

BK virus-reactive autologous T cell lines can be generated from viremic KTR

We initially used a protocol that has been shown to generate clinical-scale virus-specific T cell lines in just 9-14 days following the stimulation of PBMC with synthetic overlapping peptide libraries that cover the entire length of immunogenic viral proteins (overlapping 15-mers)[11,12]. Peptide libraries from two immunogenic BK virus proteins, VP1 and LTA, were used to stimulate T cells. This choice was based on previous studies highlighting the importance of these proteins in the generation of protective immune responses in the case of BK virus reactivation in KTR [4,15,16] and previous experience in allogeneic hematopoietic cell transplantation using T cell lines generated with these peptide libraries[6]. Direct stimulation of PBMC from HC (n=4) and KTR (n=5) revealed that VP1 and LTA-specific T cell lines are generated in both cases. (Figure 1). However, cellular expansion was highly variable (fold expansion mean of 12.1 ± 7.4 for HC and 4.6 ± 1.9 for KTR, p=0.155) (Figure 1A). Less than 50 million cells were obtained for 3/5 T cell lines generated from KTR PBMC, which may not be sufficient for treatment and ancillary testing at cell doses previously used in anti-viral adoptive immunotherapy trials[6,9,17]. Irrespective of donor type, the T cell lines contained more than 90% of CD4+ or CD8+CD3+ T cells (Figure 1B). Naive (Tn), central memory (Tcm) and effector memory (Tem) subsets were further defined based on CD45RO and CD62L expression. No differences were noted between T cell lines from HC

and KTR, which both contained a mixture of Tn, Tcm and Tem phenotype T cells (Figure 1C). Finally, T cell lines were assessed for reactivity against VP1 and LTA peptide libraries using the IFN-γ ELISpot assay (Figure 1D). Despite clear-cut reactivity to VP1 and LTA, the T cell lines from both HC and KTR displayed low-grade reactivity against control adenoviral peptides derived from the highly immunogenic Hexon protein.

Taken together, these results show that BK-reactive T cell lines can be rapidly generated from HC and KTR, but at levels that may not support clinical use. The use of PBMC previously exposed to BK-virus did not otherwise impact T cell differentiation or reactivity relative to PBMC from HC.

Peptide-loaded DC stimulation improves T cell expansion, specificity and differentiation

We next sought to determine whether improved expansion, differentiation and antigen-specificity of anti-BK virus T cell lines from viremic KTR could be achieved using DCs instead of PBMCs as antigen-presenting cells[13]. To this end, VP-1 and LTA peptide library pulsed-autologous monocytes-derived DC[13] were used at the beginning of the culture in 4 patients. PBMC containing responder T cells (cryopreserved at the time of blood collection) were added to mature DC pulsed with the antigenic libraries. This significantly increased KTR T cell lines expansion to clinical scale levels in all donors tested (Figure 2A)[6]. Although the proportion of CD4+ and CD8+ T cells were similar with both protocols (not shown), the addition of DC stimulation altered the differentiation

profile (Figure 2B). At the end of the 14-day culture, we noted an increase in the percentage of Tcm and a decrease in the proportion of Tn in the cultures stimulated with DC relative to the original condition. In HC, the addition of DC similarly increased expansion, but the variation in Tn or Tcm did not reach statistical significance (Supplementary Figure 1). The use of peptide-pulsed DC also led to more specific antigen reactivity as evaluated by ELISpot. Compared to the reference condition, T cell lines generated from DC-stimulated PBMC showed little to no reactivity towards adenoviral peptides resulting in a high ratio of VP1 to Hexon spot count (Figure 2C-D). The LTA/ Hexon spot count ratio also improved but the difference was not statistically significant. These results show that the use of a single round of peptide-pulsed autologous DC improves BK-specific T cell generation in KTR in terms of expansion, differentiation and antigen specificity.

T cell lines obtained from viremic KTR and HC are similarly polyclonal

Chronic infections such as HIV[18], EBV[19] and CMV[20] and end-stage renal disease[21] are proposed to narrow TCR repertoires. *In vitro* expansion of antigen-specific T cells can also restrict the TCR repertoire[22,23]. Rapidly generated T cell lines from both HC and actively BK viremic KTR were polyclonal (Figure 3) as assessed by a robust TCRγ chain next-generation sequencing approach to identify clonal populations from all mature T cell subtype populations[24]. The absolute number of unique reads was not statistically different between both groups, with a mean of 724794 ± 113824

(HC) and 547837 ± 44741 (KTR), p=0.099 (Figure 3A). Polyclonality was further estimated using the clonality index as proposed by Harden et al. and applied to our results[14]. Both groups had similar indices, with means of 0.240 ± 0.036 (HC) and 0.246 ± 0.044 (KTR), p= 0.448 (Figure 3B). An index toward 0 is indicative of a polyclonal repertoire while an index closer to 1 infers oligoclonality. No difference were found between the two groups in terms of number of clones required to reach 10 percent of the total reads (Figure 3C) or the percentage of total reads represented by the 20 most prevalent clones in each T cell line (Figure 3D). Thus, rapidly generated T cell lines remained polyclonal in both HC and KTR despite BK-virus antigen restriction, suggesting that KTR maintain a large repertoire that can be mobilized for adoptive immunotherapy.

BK-specific T cell lines are not alloreactive in vitro and in vivo

The prospect of infusing *ex vivo* activated polyclonal autologous T cells to solid organ recipients raises the concern of inducing cellular rejection, despite previous evidence that T cell lines targeting other viruses were safe in this population[7,9,17]. Hence, we tested whether VP-1/LTA-specific T cell lines displayed evidence of alloreactivity *in vitro* and *in vivo*. The BK-specific T cell lines generated following peptide-loaded DC exposure did not lyse allogeneic or autologous targets (loaded or not with a control peptide library) and, as expected from the ELISpot data, we noted a dose-related specific cell cytotoxicity of autologous targets loaded with the VP1 and LTA peptide libraries

(repeated measures ANOVA p=0.057 for 10:1, p=0.048 for 20:1 and p=0.027 for 40:1) (Figure 4A). To further ascertain that the T cell lines lacked alloreactive potential, we performed adoptive transfer into NOD/SCID/IL2Rγ^{null} (NSG) immunodeficient mice. Mice were given hIL-15 to support engraftment in the first three weeks post transfer[25]. While NSG mice cannot reject the xenogeneic human cells, they express murine histocompatibility antigens that are recognized with high avidity by human T cells resulting in severe xenogeneic graft-versus-host disease (GVHD). As such, it represent a stringent system to test for alloreactive potential. As anticipated, T cells directly isolated from PBMC caused lethal GVHD (decreased activity, skin changes, prostration and weight loss) 36 to 63 days post adoptive transfer. In contrast, the infusion of an equal number of cells from the *ex vivo* generated T cell lines was very well tolerated (Figure 4B-C). Histological evidence of GVHD was present in the skin and liver[26] of unmanipulated T cell recipients, but not in mice injected with T cell lines (Figure 4D). Neither group had significant evidence of intestinal GVHD (not shown). Collectively, these data support the notion that rapidly generated anti-BK T cell lines do not retain alloreactive potential.

BK-specific T cell lines can expand and persist in vivo.

The success of adoptive immunotherapy hinges on the capacity of the infused T cells to expand *in vivo* after transfer and persist. We used the NSG mouse model to assess whether the adoptively transferred BK-specific T cells lines

were capable of expanding in response to a homeostatic stimulus and persist after adoptive transfer. Labeling experiments with BrdU showed that a fraction of adoptively transferred T cells proliferated in the first three weeks, while human IL-15 was supplemented. When compared to T cells from mice not exposed to BrdU, spleen CD4+ (mean of 11.48% ± 2.57 versus 4.20% ± 1.03) and CD8+ (mean of 13.44 %± 1.45) from BrdU-exposed mice showed evidence of BrdU incorporation (Figure 5A). Weekly peripheral blood sampling further revealed that the percentage of human T cells continued to increase after IL-15 supplementation. We noted that compared to week 3, both CD4+ and CD8+ had expanded at week 6 (paired T-test p=0.028 for CD4+ and p=0.034 for CD8+) (Figure 5B). In all animals followed until 11-12 weeks post-transfer (n=11), human T cells persisted without clinical evidence of GVHD (Figure 5C). Collectively these data indicate that *ex vivo* generated BK-reactive T cell lines maintain their capacity to proliferate and persist *in vivo* without triggering xenogeneic GVHD.

Discussion

We report herein a rapid, clinically compliant protocol to generate autologous BK-specific T cell lines from PVAN or actively BK viremic KTR from a single venipuncture. This process required stimulation with antigen-pulsed DC to achieve clinical-scale expansion in contrast to a similar culture system already used in clinical trials, which has been shown to generate EBV-specific or multi-virus (including BK) specific T cell lines from PBMC alone[6,11,12]. The reasons

why stimulation with BK antigenic peptides alone resulted in low T cell expansion might be related to the immunogenicity of the VP1 and LTA peptide pools or the nature of the BK-specific repertoire in HC and immunocompromised KTR. Two other studies have similarly reported limited growth of BK-specific T cells[22,27]. Blyth et al. generated T cell lines from 12 hemodialysis patients, one healthy control and one KTR. The T cell line from the KTR expanded 0.9 fold and contained 75% CD3+ and 21% CD56+CD3- cells, suggesting the presence of NK cells. Likewise, Comoli et al. generated T cell lines using inactivated virus pulsed on DC and IL-2 from 6 KTR, which expanded poorly (1.5 fold). Expansion was improved using IL-12 and IL-7 early in the culture process but this led to the presence of a large proportion of γδ T cells (up to 69%), whose potential relevance to adoptive immunotherapy of refractory viral infections is unclear. This contrasts with our results showing the reliable expansion of CD4+ and CD8+ T cells after the use of dendritic cells for antigen presentation. Recently, Dasari and colleagues proposed and elegant method to obtain multivirus-specific T cell lines from solid organ recipients using an adenoviral vector coding for several HLA-restricted antigens as a platform for antigen presentation. Two cell lines were obtained from PVAN patients but it is unclear whether this system expands BK-virus specific T cells to clinical scale levels[28].

The use of peptide-pulsed autologous DC decreased the proportion of Tn in the T cell lines and reciprocally increased Tcm but not Tem. This was to be expected using a system where T cell stimulation is increased[29]. The improved

antigen specificity of the T cell lines may be linked to the decrease in Tn representation. Moreover, the generation of a Tcm-rich T cell product may be associated with greater clinical efficacy owing to the intrinsic features of this subset (long-term persistence, capacity to self-renew, *in vivo* proliferative potential) relative to Tem or more differentiated effectors[30]. Therefore, the use of a single DC-based stimulation along with IL-4 and IL-7 generates T cell lines with favorable features for immunotherapy after two weeks of culture.

The T cell lines generated with our approach did not show evidence of alloreactive potential both *in vitro* and *in vivo* despite their polyclonality and capacity to expand and persist in NSG mice. Previous experiments with autologous anti-EBV T cell line generation from solid organ transplant recipients did not show donor-specific alloreactivity[7,9,17]. Unfortunately, donor cells were unavailable to us to confirm these findings (6 KTR out of 8 received a transplant from a deceased donor and live donor were unreachable- Supplementary Table 1). Also, we could not evaluate the *in vivo* therapeutic efficacy of our T cell lines given that no mouse model of human BK-virus infection exists. This will have to be addressed in clinical trials. Our work paves the way for such trials by establishing a clinical-compliant protocol to generate autologous BK-specific T cell lines from KTR. In addition, our system is amenable to modifications in order to refine the preparation of BK-specific T cell lines. Among these, genetic engineering of virus specific T cell lines appears promising[31]. For instance, the expression of resistance genes allowing the virus-specific T cells to elude pharmacological immunosuppression may

further improve T cell function and persistence[31]. Moreover, our system could be used to generate third party BK-specific T cell lines given our results with healthy controls (Figure 1 and supplementary Figure 1). These third party T cell lines would require more extensive characterization to define anti-viral responses restricted to specific HLA alleles before they could be used and would be unlikely to persist long term after transfer [32-34]. However, once characterized, they could be cryopreserved and banked for rapid distribution and use.

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Disclosure

The authors declare no conflicts of interest.

Figures

Figure 1. BK-specific T cell lines obtained from viremic kidney transplant recipients (KTR) are similar to those from healthy controls (HC)

Comparison of T cell lines from healthy control (HC) (n=4) to KTR (n=5) after 14-days in culture. A) Fold expansion (left), (horizontal bar in the middle represents the mean, top and bottom horizontal bars represent SEM), p=0.155 and absolute cell number (right) p=0.291. B) Percentage of CD3 + T cells and their distribution as CD4+ or CD8+ T cells. C) CD4+ and CD8+ T cell differentiation profile, as defined by CD45RO and CD62L, one representative dot plot (left) and compiled results (right, mean and SEM). D) One representative ELISpot result (for $5x10^4$ cells plated) (top) and compiled results (bottom, mean and SEM). Hexon (adenovirus) peptide library was used as an irrelevant control peptide library (Irr). SEM, standard error of the mean, Tn, naive T cell (CD45RO-CD62L+); Tcm, central memory T cell (CD45RO+CD62L+); Tem, effector memory T cell (CD45RO+CD62L-).

Figure 2. DC stimulation improves T cell expansion, specificity and differentiation

Comparison of T cell lines from kidney transplant recipients (KTR) without (DC-, n=5) and with dendritic cell stimulation (DC+, n=4) at the end of the 14-days culture. A) Fold expansion (horizontal bar in the middle represents the mean, top and bottom horizontal bars represent SEM) p=0.042 (left) and absolute cell number at the end of the 14-days culture, p=0.073 (right). B)

CD4+ and CD8+ T cell differentiation profile, as defined by CD45RO and CD62L expression (representative dot plots on the left) and compiled results (right) represented as mean with SEM, p=0.038 for Tn CD4+ T cells and p=0.008 for Tcm CD8+ T cells. C) Comparison of the number of spots on ELISpot assay with and without DC stimulation and D) ratio of spots produced in response to targeted (VP1 or LTA) or control (Hexon) peptide libraries. Boxes are to represent the distribution of results with horizontal bars indicating the mean and whiskers representing SEM. p=0.002 for VP1/Hexon. SEM, standard error of the mean, Tn, naive T cell; Tcm, central memory T cell; Tem, effector memory T cell.

Figure 3. T cell line clonality from viremic kidney transplant recipients (KTR) and healthy controls (HC) are similar

At the end of the 14-days culture, DNA was extracted from HC and KTR T cell lines and TCR-gamma chain analysis was performed for clonality determination. A) Absolute number of unique TCRγ reads in the two groups (n=4 per group) and B) Clonality index between the two groups (mean with SEM). C) Schematic representation of the most abundant clones that represent 10% of total reads in all samples tested. D) Percentage of total reads represented by the 20 most prevalent sequences for both groups (mean with SEM). SEM, standard error of the mean.

Figure 4. BK-specific T cell lines do not induce alloreactivity *in vitro* and *in vivo*

At the end of the 14-days culture, alloreactivite potential was determined *in vitro* in a chromium-51 release assay. T cell lines were also infused in NSG mice to evaluate the *in vivo* alloreactive risk. A) Specific lysis (%) of four targets at three different effector:target ratios in a 4-hour chromium-51 release assay (n=4 T cell lines from HC and KTR, each value was obtained in triplicates, mean with SEM), repeated measures ANOVA for effector:target ratio of 10:1 (p=0.057), 20:1 (p=0.048) and 40:1 (p=0.027). Allogenic or autologous phytohemagglutinin (PHA) blasts were pulsed or not with peptide libraries, VP1/LTA or control pp65 (CMV) peptide libraries. B) Kaplan-Meier survival analysis after T cell lines (n=14) or freshly isolated T cells from PBMC (n=7) injections, from 4 different T cell lines, log-rank test, **p=0.001. C) Percent weight variation between the weight on the day of death (or sacrifice) and the day of infusion. Mean (SEM), ***p<0.001. D) One representative hematoxylin-eosin phloxine staining of liver and skin of a non-infused NSG and recipients of a BK-specific T cell line or freshly isolated unmanipulated T cells (10X). Arrows point out graft-versus-host disease manifestations (portal space infiltration and epidermal thickening), white bar in images=100µm. HC, healthy control, KTR; kidney transplant recipient; SEM, standard error of the mean.

Figure 5. BK-specific T cell lines can persist and proliferate *in vivo.*

Humain BK-specific T cell lines were also injected in NSG mice to assess their persistance and proliferative potentiel *in vivo*. A) One representative histogram showing BrdU staining of CD4+ and CD8+ from mouse spleens expressed as the % of BrdU positive cells by flow cytometry and compiled results from 6 mice compared to mice not receiving BrdU (control) from 2 independent experiments for both CD4+ ($p=0.087$) CD8+ ($p=0.004$) T cells. Means are represented with SEM. B) CD4+ and CD8+ T cells expansion in the peripheral blood at week 6 compared to week 3, as measured by flow cytometry (number of events counted per standard volume of blood, time and speed of acquisition), n=14 from 4 independent experiments. C) One representative dot plot of CD4+ and CD8+ T cell in mouse's blood and spleen, 12 weeks after T cell line injection. SEM, standard error of the mean.

Description of Supporting Information

Supplementary Table 1. Patient characteristics

Supplementary Figure 1. Effect of DC stimulation on T cell expansion, differentiation and specificity in healthy donors

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Figure 1

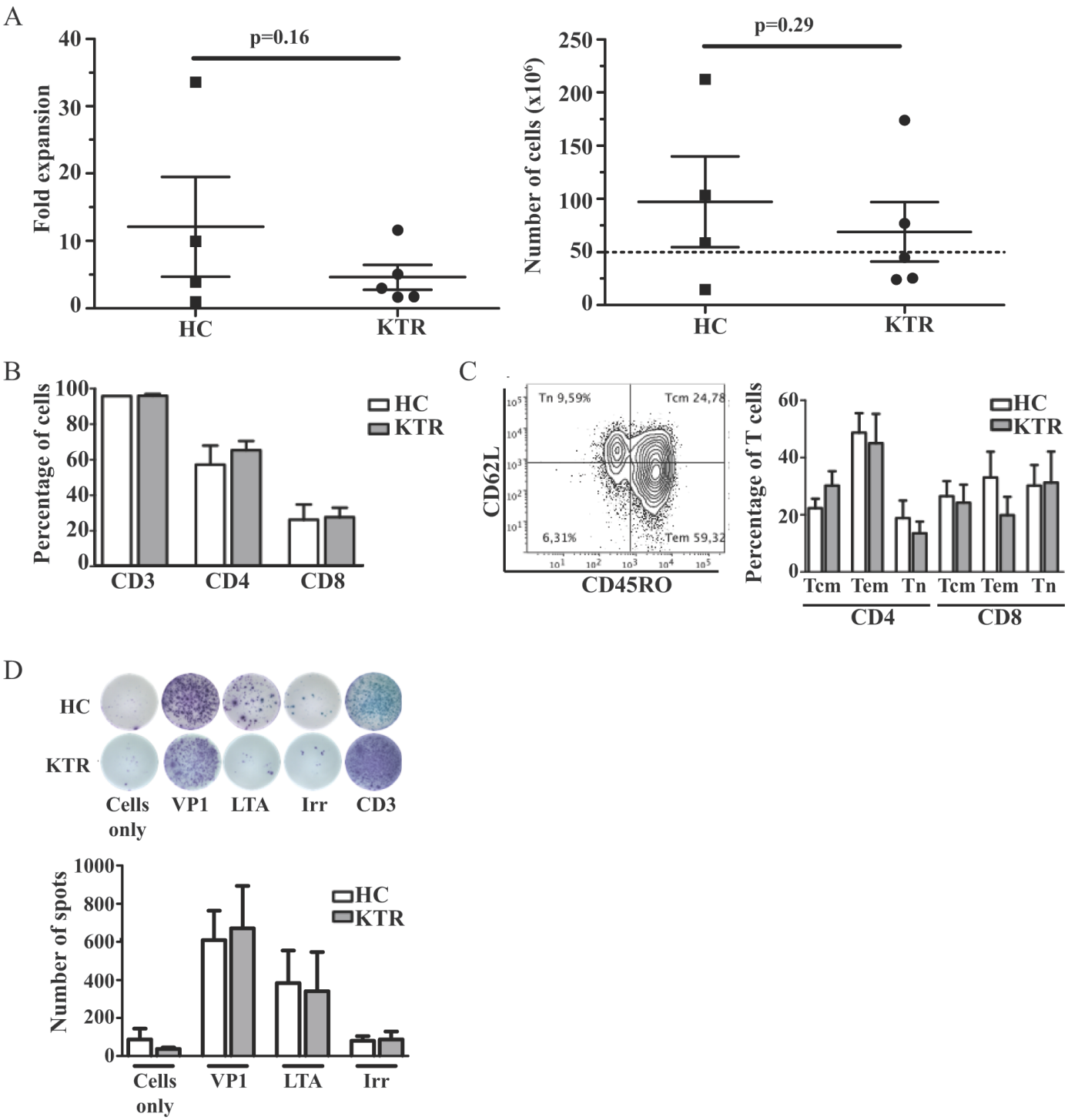

A
p=0.16
Fold expansion
40
30
20
10
0
HC
KTR
p=0.29
Number of cells (x10⁶)
250
200
150
100
50
0
HC
KTR
B
Percentage of cells
100
80
60
40
20
0
HC
KTR
CD3
CD4
CD8
C
Tn 9,59%
Tcm 24,78
6,31%
Tem 59,32
CD62L
CD45RO
Percentage of T cells
60
40
20
0
HC
KTR
Tcm
Tem
Tn
Tcm
Tem
Tn
CD4
CD8
D
HC
KTR
Cells only
VP1
LTA
Irr
CD3
Number of spots
1000
800
600
400
200
0
HC
KTR
Cells only
VP1
LTA
Irr

Figure 2

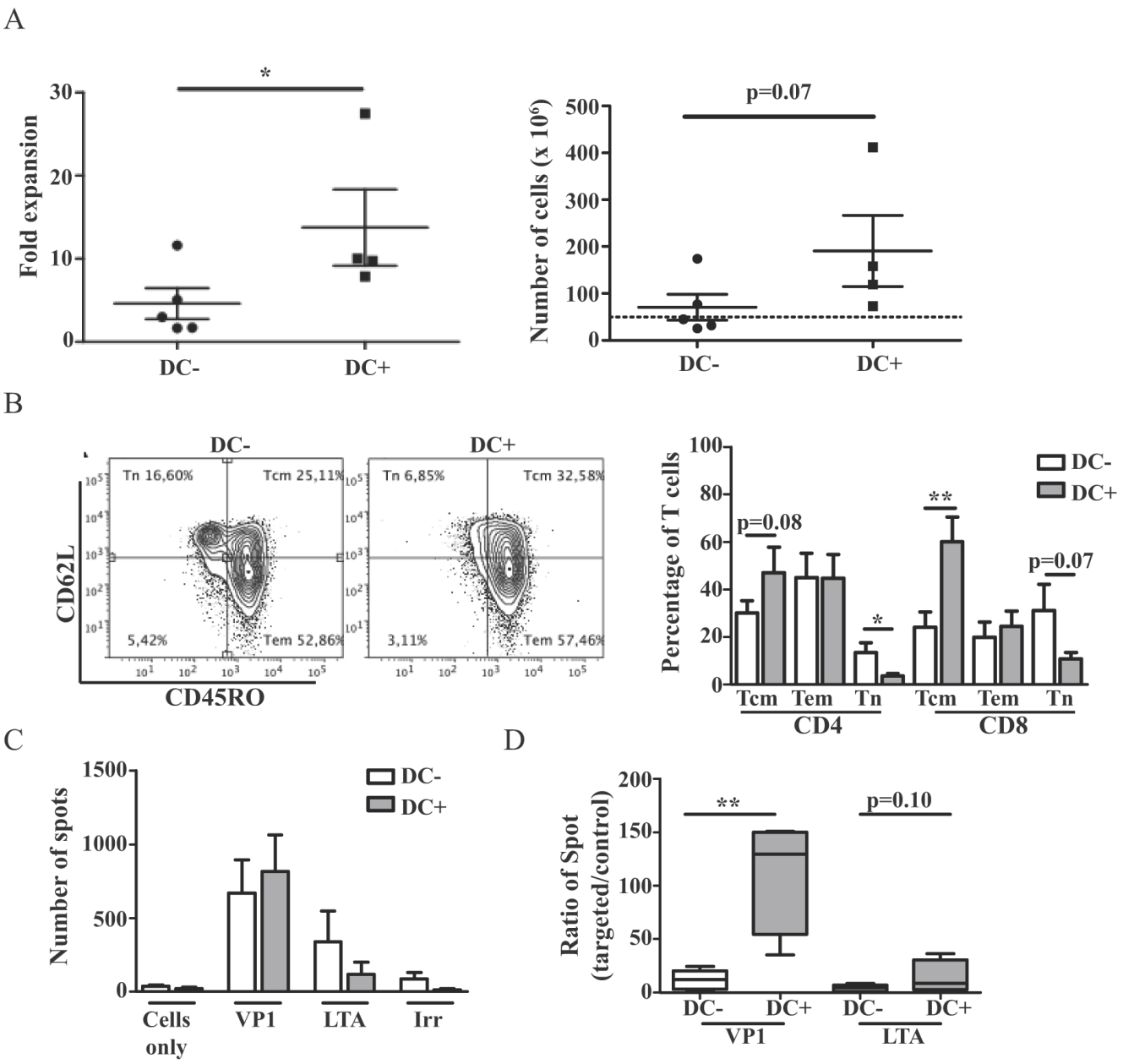

A
Fold expansion
*
DC-
DC+
Number of cells (x 10^6)
p=0.07
B
DC-
DC+
Tn 16,60%
Tcm 25,11%
5,42%
Tem 52,86%
Tn 6,85%
Tcm 32,58%
3,11%
Tem 57,46%
CD62L
CD45RO
Percentage of T cells
p=0.08
**
*
p=0.07
Tcm Tem Tn Tcm Tem Tn
CD4
CD8
C
Number of spots
Cells only
VP1
LTA
Irr
D
Ratio of Spot (targeted/control)
**
p=0.10
VP1
LTA

Figure 3

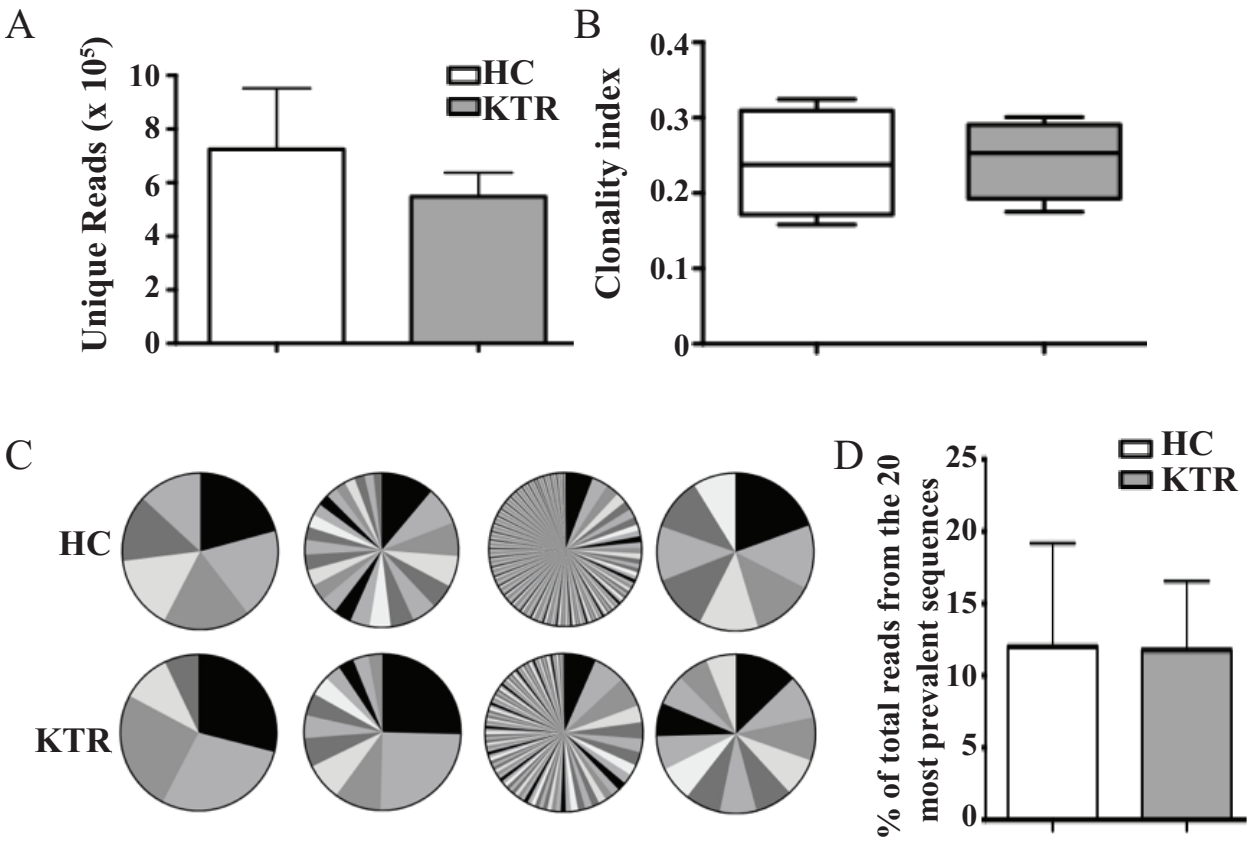
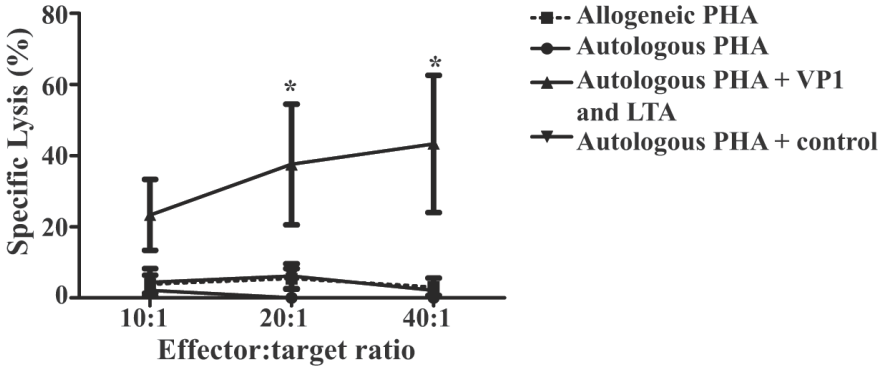
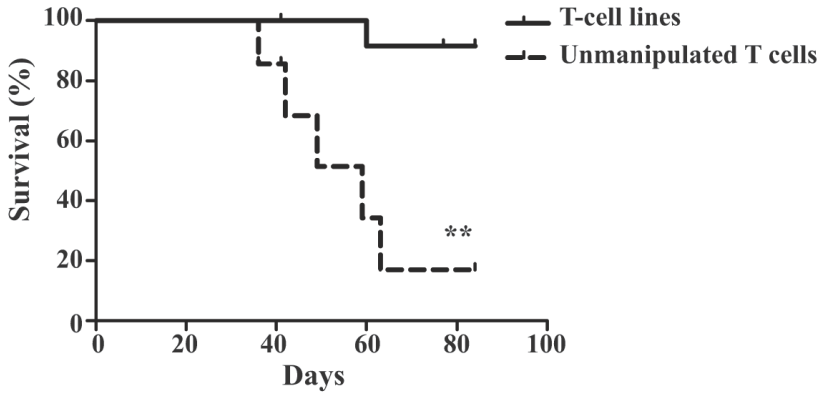

A
Unique Reads (x 10^5)
10
8
6
4
2
0
HC
KTR
B
Clonality index
0.4
0.3
0.2
0.1
0
C
HC
KTR
D
% of total reads from the 20 most prevalent sequences
25
20
15
10
5
0
HC
KTR

Figure 4

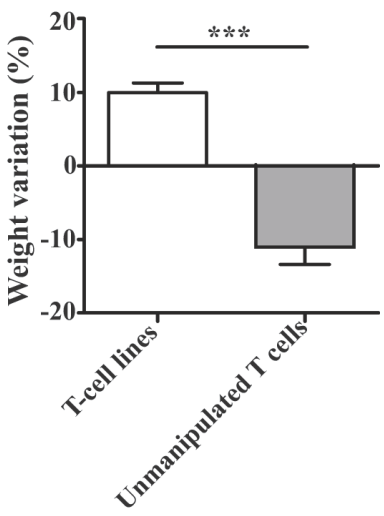
A



B



C



D

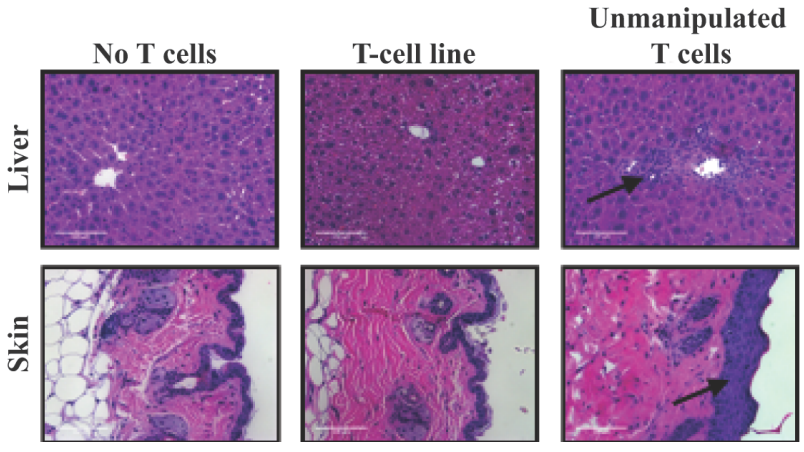
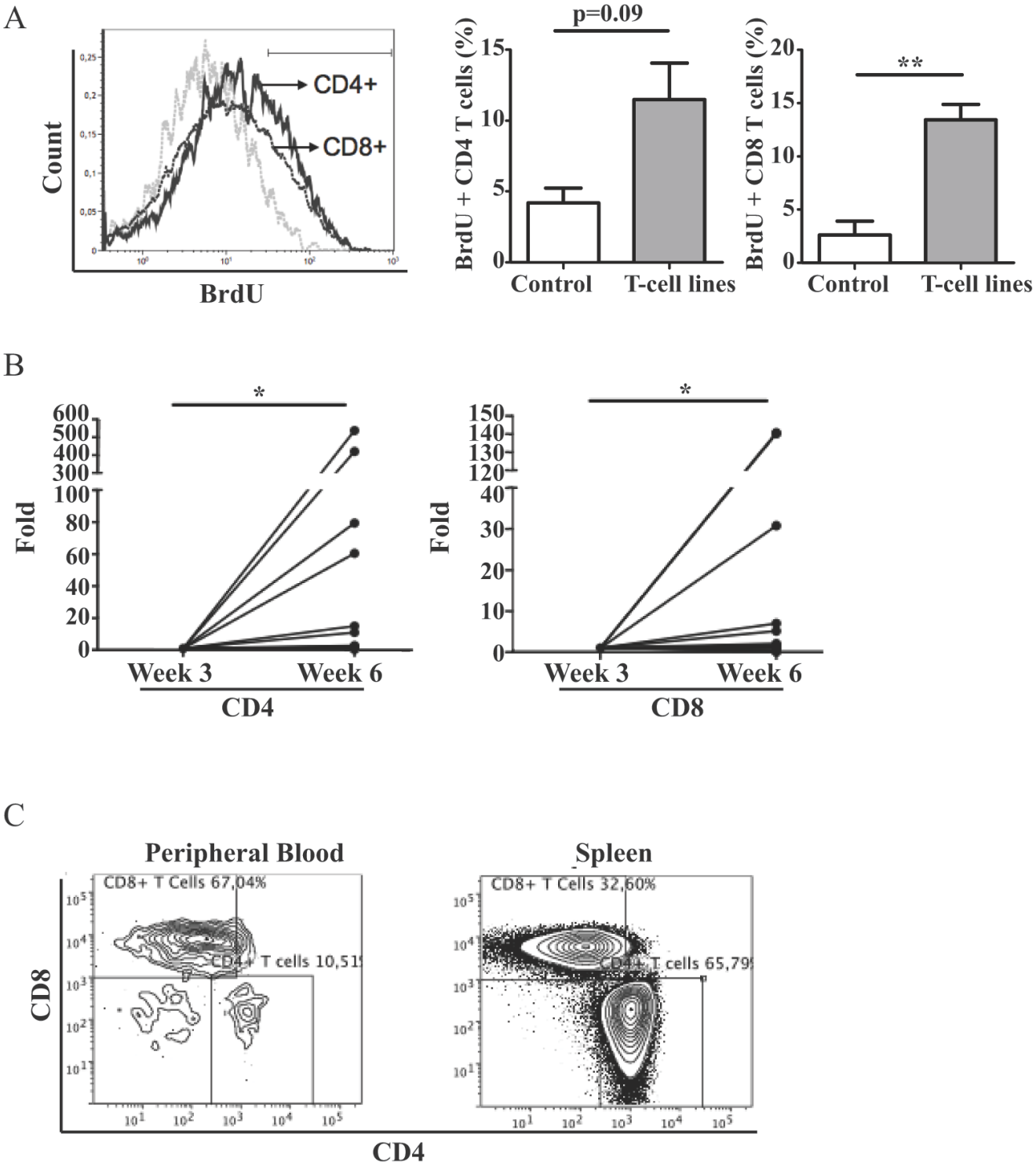


Figure 5

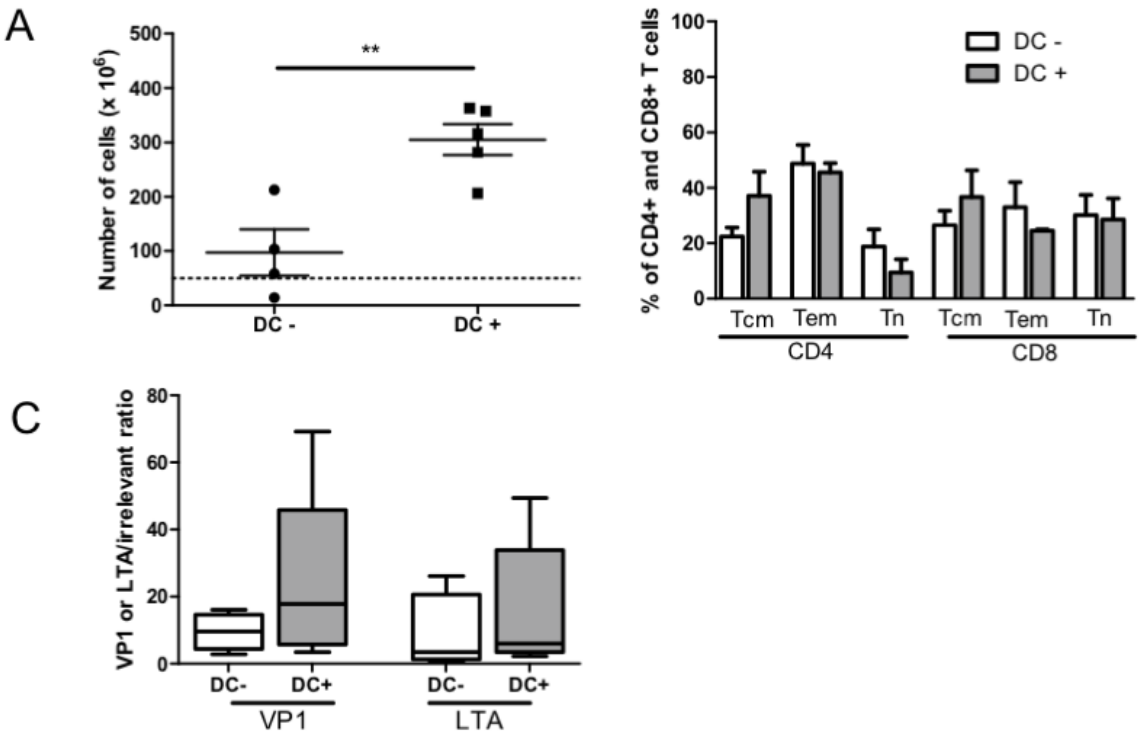

A
Count
CD4+
CD8+
BrdU
p=0.09
BrdU + CD4 T cells (%)
Control
T-cell lines
**
BrdU + CD8 T cells (%)
Control
T-cell lines
B
*
Fold
Week 3
Week 6
CD4
*
Fold
Week 3
Week 6
CD8
C
Peripheral Blood
CD8+ T Cells 67,04%
CD4+ T cells 10,51
Spleen
CD8+ T Cells 32,60%
CD4+ T cells 65,79
CD8
CD4

Supplementary Table 1.

Number of patients (n)	8
Age at the time of the transplant, mean (SD)	48.9 (8.3)
Sex, male (%)	100
Donor type; Deceased/ Living donor	6/2
Original disease (n) - Polycystic kidney disease - Diabetic nephropathy - Membrano-proliferative glomerulonephritis - Reflux nephropathy - Focal segmental glomerunosclerosis - IgA nephropathy	 3 1 1 1 1 1
Induction (n) - Basiliximab - Anti-thymocyte globulin	 6 2
Maintenance immunosuppression (n) - Prednisone, tacrolimus and mycophenolate mofetil	 8
Immunosuppression (n) at the time of blood sampling - Prednisone, tacrolimus and mycophenolate mofetil - Prednisone and tacrolimus - Prednisone, cyclosporine, azathioprin - Prednisone and cyclosporine	 3 3 1 1
HLA mismatches (A, B and DR), mean (SD)	3.9 (1.2)
CPRA at the time of transplant, mean (SD)	22.9 (37.0)
Renal function at the time of blood sampling - Creatinine (µmol/L), mean (SD) - Estimated GFR (CKD-EPI), mean (SD)	 153 (51) 52 (20)
History of acute rejection (n) - None - Borderline - Acute cellular rejection	 6 1 1
Transplant number: (n) - First transplant - Second transplant	 6 2
Time between transplant and first positive viremia, months, mean (SD)	3.8 (2.8)
Pic BK viremia, mean (SD)	1.6×10^5 (1.4×10^5)
Definitive PVAN (n) Presumptive PVAN (n)	6 2
Viremia duration at the first blood sampling, months, mean (SD)	19.3 (24.1)

SD, Standard Deviation; CPRA, Calculated Panel Reactive Antibodies; PVAN, polyomavirus-associated nephropathy

Supplementary Figure 1.



Supplementary Figure 1. Effect of DC stimulation on T cell expansion, differentiation and specificity in healthy donors

Comparison of T-cell lines from healthy donors without (DC-, n=4) and with dendritic cell stimulation (DC+, n=5) at the end of the 14-days culture. A) Absolute cell number at the end of the 14-days culture, p=0.002. B) CD4+ and CD8+ T cell differentiation profile, as defined by CD45RO and CD62L expression. C) Comparison of ELISpot ratios of the number of spots after VP1 and LTA stimulation on the number after an irrelevant peptide library stimulation (hexon from adenovirus) between both groups, mean (SEM), p=0.157 for VP1 and p=0.258 for LTA.

DISCUSSION ET PERSPECTIVES

Nous avons donc réussi à adapter et valider un protocole se conformant aux exigences cliniques et nous permettant de générer rapidement des lignées cellulaires de lymphocytes T BK-spécifiques à partir du sang de patients greffés virémiques et immunosupprimés. Bien que comparables aux lignées cellulaires obtenues à partir de donneurs sains, ces lignées avaient une tendance vers une moins bonne expansion cellulaire. L'ajout d'une stimulation à l'aide de cellules dendritiques comme cellules présentatrices d'antigènes nous a permis d'améliorer l'expansion cellulaire, le statut de différentiation et la spécificité. De plus, les lignées cellulaires obtenues à partir de sang de donneurs sains et de greffés se sont avérées polyclonales et aucune différence n'a pu être détectée entre les deux groupes. Malgré cette polyclonalité, les lignées n'ont pas démontré d'alloréactivité significative *in vivo* et *in vitro*. Finalement, nous avons démontré que les cellules injectées peuvent persister et proliférer *in vivo* avec et même sans stimulation cytokinienne. Le protocole que nous avons adapté est donc prêt à être transféré en clinique. Cependant, plusieurs éléments seront à prendre en considération lors du transfert clinique.

Disparité des HLA

En transplantation rénale, les HLA ne sont que rarement jumelés. En effet, selon les règles d'attribution des greffons de Transplant Québec, seulement une petite partie des points est accordée au jumelage des HLA. En effet, un maximum de 4 points est accordé pour un jumelage en HLA-DR et un maximum de 8 points est donné pour un jumelage parfait en HLA-A, B et DR, comparativement à un maximum de 18 points pour un patient étant en dialyse depuis 10 ans (71).

Une plus grande disparité entre le donneur et le receveur est un facteur de risque assez bien établi de de néphropathie associée au polyomavirus (22, 72). Cela peut s'expliquer par une discordance entre les HLA du receveur et ceux des cellules rénales présentant les antigènes viraux, mais également par une plus grande immunosuppression de ces patients, soit en prévention ou en traitement d'un rejet. Cela pourrait donc également influencer l'efficacité des lignées cellulaires. En contrepartie, l'immunité du receveur demeure la pierre angulaire du traitement de la néphropathie, même chez ces patients, puisque la réduction de l'immunosuppression est le meilleur traitement. De plus, l'apparition de lymphocytes T spécifiques pour le BK virus coincide normalement avec une amélioration de la fonction du greffon et une résolution de la virémie (73-75). Dans un cas de disparité totale entre les HLA du donneur et du receveur, les lymphocytes T spécifiques pourraient tout de même empêcher la propagation virale à d'autres organes HLA concordants, tels que la vessie. De plus, les CD4+ activés pourraient aider à l'activation de cellules comme les macrophages et les lymphocytes B pouvant agir de façon HLA-indépendantes.

Plusieurs épitopes immunogènes ont été décrits pour le polyomavirus BK, principalement pour les protéines VP1 et LTA. Bien qu'ils peuvent parfois être présentés par plus d'un HLA, ils sont généralement HLA-restreints (76). Considérant la disparité des HLA en transplantation rénale, la stimulation de lymphocytes T à l'aide de peptides uniques (ou la combinaison de quelques-uns) devrait être individualisée à chaque patient selon les concordances de HLA entre le donneur et le

receveur. Bien que faisable, cette méthode est beaucoup plus complexe que l'utilisation de librairies peptidiques, permettant d'appliquer un protocole unique à un ensemble de patients.

De base, Comoli *et al*. ont démontré que les patients transplantés rénaux semblent avoir une plus faible prévalence de lymphocyte T BK-spécifiques comparativement à des donneurs sains (73), pouvant être une conséquence de l'immunosuppression. De plus, les infections chroniques telles que le HIV (77), l'EBV (78) et le CMV (79) ainsi que l'IRT (80) pourraient entrainer une restriction du répertoire TCR. Malgré ces différences avec les donneurs sains, notre analyse du TCR gamma a démontré une polyclonalité similaire entre les deux groupes. Cela suggère que notre processus ne favorise pas l'émergence de clone unique et que le répertoire de base du donneur BK n'est pas déficient comparativement à celui des donneurs sains. Finalement, cela évoque la possibilité que nos lignées pourraient être réactives à des épitopes présentés par plusieurs HLA, y compris les HLA concordants, favorisant ainsi un plus grand potentiel thérapeutique.

Support technique

Une des grande barrière à l'utilisation à large échelle de cette technologie est la nécessité de centre de thérapie cellulaire spécialisé. Au Canada, seulement deux centres ont présentement l'accréditation FACT (Foundation for the accreditation of cellular therapy) leur permettant d'utiliser des cellules plus que minimalement manipulées, soit le Centre d'Excellence en Thérapie Cellulaire de l'Hôpital

Maisonneuve-Rosemont et le Manitoba Blood & marrow Transplant Program à Winnipeg (81). Devant cette barrière logistique, les deux solutions possible pouvant permettre l'application de cette thérapie à large échelle est le développement de réseaux de collaborations, pouvant être envisagés à court terme, et le développement de nouveaux centres de thérapie cellulaire, qui est l'objectif à long terme. De plus, l'expansion de l'immunothérapie adoptive, telle que vécue en oncologie dans les dernières années, stimulera les innovations permettant de simplifier et de rendre plus accessibles ces thérapies.

Survie des lignées

En transplantation d'organe solide, où l'immunosuppression doit être continuée, la survie des lignées cellulaires sera diminuée. Dans une étude utilisant des lignées EBV-spécifiques en prévention de PTLD chez les patients à risque, les ELISpot sont retournés au niveau préinfusion en 2 à 6 mois, ce qui est inférieur aux patients après greffe de moelle (59). Cependant, le traitement semble avoir été efficace cliniquement, puisqu'aucun patient n'a développé de PTLD et les deux patients avec PTLD ont eu une réponse au moins partielle (59).

De plus, la différentiation mémoire de ces lignées n'avaient pas été étudiée (59) et une maximisation du nombre de Tcm ou de Tscm pourrait améliorer la survie des lignées virus-spécifiques. La modification génétique de ces lignées pourrait également être une avenue intéressante afin d'améliorer la survie des lignées. Si ces méthodes ne sont pas suffisantes, les stratégies alternatives comprennent d'infuser

plus d'une dose au patient ou de combiner les stratégies comme une diminution de l'immunosuppression ou l'utilisation d'agents anti-viraux à l'immunothérapie adoptive.

Modification génétique des lignées cellulaires

L'immunothérapie adoptive s'inscrit dans une tangente vers une médecine plus personnalisée. Afin d'améliorer le produit cellulaire, il est possible de modifier les conditions de cultures ou encore de modifier génétiquement les lignées cellulaires. Cette dernière approche est très intéressante puisqu'elle ouvre la porte à des possibilités quasi infinies.

Tout d'abord, l'expression transgénique d'IL-2 (82, 83), d'IL15 (83, 84) et du récepteur alpha de l'IL-7 (85, 86) pourraient permettre d'améliorer l'expansion et la persistance des cellules, sans en altérer la spécificité. Une surexpression transgénique d'IL-12 augmente l'efficacité des cellules, mais en diminue la survie (87). Cette approche est donc à écarter dans un modèle d'infection latente et pourrait même être toxique à haute dose (88).

Afin d'utiliser les molécules de co-stimulation à notre avantage (signal 2), il est possible de modifier certains régulateurs inhibiteurs de l'activation du lymphocyte T. Par exemple, nous pouvons jumeler le domaine extracellulaire et transmembranaire du CTLA4 à un domaine cytoplasmique CD38 (89), abroger l'expression de Cbl-b ou de SHP-1 (90, 91) ou encore convertir PD-1 en un récepteur activateur en échangeant sa queue cytoplasmique pour celle de CD28 (92).

Afin d'optimiser la survie des lignées de lymphocytes T utilisées dans le traitement de patients soumis à une immunosuppression chronique, il est également possible de leur conférer une résistance aux immunosuppresseurs. Cela est maintenant possible pour les inhibiteurs de la calcineurine (93-95), l'acide mycophénolique (96, 97) et les inhibiteurs de mTor (98). Avec le même objectif, nous pouvons également moduler certains acteurs de l'apoptose tels que Bcl-2 (99), Bcl-X(L) (100), Bid (101) et Fas (102).

Le plus grand risque associé aux différentes approches mentionnées précédemment est l'immortalisation et la prolifération clonale de ces lignées. Cela a été décrit dans les lymphocytes exprimant IL-15 (103), IL-2 et son récepteur (104) et chez des souris transgéniques exprimant l'IL-15 (105). Afin de prévenir ou traiter ces complications, l'ajout d'un gène ¨suicide¨ inductible semble une solution prometteuse. Par exemple, il est possible d'induire une caspase 9 humaine modifiée inductible avec un dimérisateur chimique spécifique (83, 106-109), d'induire l'expression du CD20 afin de sensibiliser la cellule au rituximab (110, 111) ou d'utiliser un gène de thymidine kinase viral provenant de l'herpes simplex sensibilisant la cellule à l'effet du ganciclovir (112-115). Cependant, cette dernière protéine est immunogénique et pourrait diminuer la survie de la cellule (116). Il est également possible de transduire les cellules avec une variante de la thymidylate kinase humaine (tmpk) afin que les cellules soient tuées par une dose d'azidothymidine (AZT) (117) ou d'induire

l'expression d'un polypeptide EGFR tronqué sensibilisant la cellule au cetuximab (118).

Dans le contexte précis d'immunothérapie adoptive antivirale chez les greffés rénaux soumis à une infection chronique comme le polyomavirus BK, une des combinaisons de modifications génétiques avec le plus grand potentiel serait donc de conférer une résistance à un immunosuppresseur comme le tacrolimus en association avec un gène suicide comme la caspase 9 modifiée.

CONCLUSION

Pour l'instant, la néphropathie au polyomavirus BK est le reflet d'une immunosuppression globale et non spécifique. Le futur de la transplantation d'organe solide réside probablement en l'accomplissement d'un état de tolérance immune avec le greffon, ce qui permettrait d'éviter tous les effets secondaires non désirés des immunosuppresseurs, tels les infections et les néoplasies. En attendant, l'immunothérapie adoptive antivirale pour le polyomavirus BK est probablement la meilleure solution puisqu'elle ne met pas en péril la survie du greffon et elle utilise l'immunité cellulaire du patient que l'on sait être la clé du traitement. Nous avons développé un protocole de thérapie cellulaire très intéressant qui est prêt à être testé en clinique dans une étude de phase I/II qui pourrait résoudre cette problématique.

Finalement, en plus de transférer ce protocole en clinique, il serait très intéressant de l'améliorer grâce à une combinaison de modifications génétiques qui pourrait être de conférer un gène de résistance aux inhibiteurs de la calcineurine et un gène suicide afin de compenser en sécurité.

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