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# ÉTUDE DE LA RÉGULATION HOMÉOSTATIQUE DES POPULATIONS LYMPHOCYTAIRES T PÉRIPHÉRIQUES

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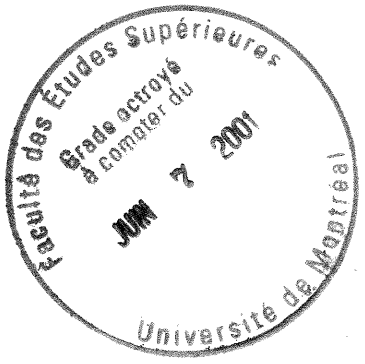

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Cette thèse intitulée:

ÉTUDE DE LA RÉGULATION HOMÉOSTATIQUE DES POPULATIONS LYMPHOCYTAIRES T PÉRIPHÉRIQUES

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**Sommaire**

Cette étude de la régulation homéostatique des populations lymphocytaires T périphériques cherche à comprendre les mécanismes par lesquels sont régulées la maturation, la différenciation et l'expansion des cellules T, afin d'élucider les causes de l'hypoplasie lymphoïde associée à la réaction de greffon contre l'hôte (GVH). De plus, la compréhension des voies de différenciation ontogénique utilisées post-greffe médullaire devrait s'avérer essentielle à l'élaboration d'approches thérapeutiques visant à stimuler la production de cellules T chez des individus lymphopéniques.

La plupart des patients qui subissent une transplantation médullaire présentent une atrophie thymique secondaire à l'âge, la maladie et les traitements. Notre première étude a permis d'évaluer l'impact de l'hypoplasie thymique sur l'ontogénie des lymphocytes T post-greffe, en absence de réaction de GVH. Chez des souris euthymiques, les cellules T proviennent d'une différenciation thymique des progéniteurs hématopoïétiques. Par contre, chez des receveuses thymectomisées, l'origine des cellules T dépend de la composition du greffon. Si ce dernier contient des cellules T, celles-ci prolifèrent et repeuplent le pool T périphérique de la receveuse. Par contre, si le greffon ne contient pas de cellules T, les lymphocytes T proviennent exclusivement d'une maturation extrathymique des progéniteurs hématopoïétiques greffés. La voie de différenciation ontogénique utilisée post-greffe médullaire dépend donc de deux facteurs, la présence/absence du thymus et la composition cellulaire du greffon. Ces facteurs ont une

influence déterminante sur le niveau d'immunocompétence du patient et possiblement sur l'apparition de la réaction du greffon contre l'hôte.

Notre seconde étude visait à déterminer l'effet de la GVH sur les différentes voies de différenciation ontogénique utilisées post-greffe médullaire. L'étude de la production thymique et du niveau de prolifération des cellules T périphériques a permis de démontrer que l'hypoplasie lymphoïde associée à la GVH provient en partie d'une diminution de la production thymique. Par contre, le transfert de cellules T matures à des receveurs atteints de GVH a permis de mettre en évidence une perturbation de l'environnement nécessaire au maintien des populations cellulaires T périphériques. Ce désordre est compatible avec une diminution du nombre de niches périphériques.

Finalement, l'étude d'une souris transgénique nous a permis de démontrer qu'une exposition chronique à l'oncostatin-M permet aux ganglions lymphatiques de soutenir le développement extrathymique des cellules T et d'induire une augmentation du nombre de niches T périphériques disponibles. La compréhension des mécanismes par lesquels l'oncostatin-M induit ces changements permettrait d'entrevoir la possibilité de contrer les effets délétères de la GVH et ainsi d'améliorer la reconstitution immune des patients greffés médullaires. De plus, un tel traitement permettrait de compenser l'atrophie graduelle du thymus et ainsi de maintenir, chez des individus âgés ou lymphopéniques, une production constante de cellules T naïves ainsi qu'un répertoire de cellules T diversifié. Ce traitement permettrait peut-être de réduire l'incidence de certaines maladies auto-immunitaires, d'infections

récurrentes et de cancers, et ainsi d'améliorer la survie et les conditions de vie de ces personnes.

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**AICD**: activation-induced cell death

**BrdU**: 5'-bromodésoxyuridine

**CMH**: complexe majeur d'histocompatibilité

**CNTF**: ciliary neurotropic factor

**CPA**: cellule présentatrice d'antigènes

**CT-1**: cardiotrophine

**FACS**: fluorescence-activated cell sorter

**GVH**: réaction du greffon contre l'hôte (graft-versus-host desease)

**GVL**: réaction anti-leucémique (graft-versus-leukemia)

**HEV**: high endotheliale venule

**LCMV**: lymphocytic choriomeningitis virus

**IL**: interleukine

**LIF**: leukemia inhibitory factor

**OM**: oncostatin M

**TCR**: récepteur des cellules T (T cell receptor)

**TME**: cellules T mémoires effectrices

**TMC**: cellules T mémoires centrales

**VSV**: vesicular stomatitis virus

**VV**: vaccina virus

## Introduction

### 1. Le développement des cellules T

#### 1.1. Les différentes populations de cellules T

Les populations lymphocytaires T périphériques se composent de deux populations distinctes: les cellules T naïves et mémoires (1). Ces populations occupent différentes niches homéostatiques et possèdent des rôles différents mais complémentaires (2). Les cellules T naïves, qui composent plus de 99% de la diversité totale du répertoire des cellules T, permettent au système immunitaire d'être en mesure de reconnaître et de répondre à la majorité des antigènes rencontrés lors d'une première immunisation (3). Par contre, les cellules T mémoires permettent l'élaboration d'une réponse immune rapide et efficace lors d'une seconde rencontre avec l'antigène (4;5). L'expression du récepteur de chémokines CCR7, qui contrôle la localisation des cellules T et des cellules dendritiques au niveau des ganglions lymphatiques (6), a permis la classification des lymphocytes T mémoires en deux catégories. Les cellules T mémoires $CCR7^-$ expriment les récepteurs nécessaires à leur localisation au niveau des sites d'inflammation et présentent une fonction effectrice immédiate. Par contre, les cellules T $CCR7^+$ expriment les récepteurs nécessaires à leur localisation au niveau des ganglions lymphatiques, ne présentent pas de fonction effectrice immédiate mais stimulent efficacement les cellules dendritiques. Ces lymphocytes peuvent se différencier en cellules effectrices $CCR7^-$ lors d'une seconde stimulation. Ces deux types cellulaires ont respectivement été

dénommés cellules T mémoires effectrices (TME, $CCR7^-$) et cellules T mémoires centrales (TMC, $CCR7^+$). Ces cellules peuvent persister des années suite à l'immunisation (6). La persistance des cellules T mémoires nécessite la présence de l'antigène dans le cas des TME (7-9) alors que la persistance des TMC est totalement antigène indépendante (10-12).

Les lymphocytes T naïfs possèdent une durée de vie limitée d'environ 6 mois chez la souris (13) et doivent donc être continuellement remplacés afin de conserver un répertoire immunologique adéquat. La survie de ces cellules est dépendante de signaux transmis via le récepteur des cellules T (TCR) (2). Tout comme lors de la sélection thymique, ces signaux proviennent d'interactions de faible affinité entre le TCR et certains complexes peptides du soi et complexe majeur d'histocompatibilité (CMH) (14-17). De plus, l'expression de molécules du CMH sur les cellules dendritiques périphériques est nécessaire à induire cette sélection positive périphérique des cellules T naïves (14;18). Finalement, différentes études ont également démontré que des cellules naïves injectées à des receveurs lymphopéniques se divisent, colonisent l'espace périphérique et acquièrent un phénotype de type mémoire/activé (19-21). Ces résultats ont donné naissance au concept de prolifération homéostatique des cellules T naïves.

### 1.2. Le développement thymique des cellules T

Le développement des lymphocytes T débute au niveau du thymus où la sélection positive et négative des thymocytes immatures permet la production d'un répertoire T extrêmement diversifié et apte a réagir contre pratiquement toutes les substances étrangères et ceci tout au long de

l'existence d'un individu. La durée de vie des cellules T naïves étant limitée, le maintien du répertoire T périphérique dépend d'une production thymique constante (22;23). Cependant, le thymus s'atrophie progressivement avec l'âge et le niveau de production de cellules T par le thymus diminue graduellement (24-29). Cette diminution de la production de lymphocytes T entraîne une perte de la diversité du répertoire T, une augmentation de la susceptibilité a de nouveaux pathogènes ainsi qu'une accumulation de cellules T autoréactives en périphérie (22;30). L'atrophie thymique est donc responsable du déclin important de la compétence immune observée chez les personnes âgées (31). De plus, de nombreuses observations suggèrent que la compétence immunitaire a une influence majeure sur la durée de vie et que des perturbations de la réponse T seraient impliquées au niveau de l'augmentation de la fréquence des infections, des cancers et des maladies auto-immunes avec l'âge (32-37).

Les causes de la sénescence thymique sont présentement inconnues (24;38). Certaines études ont démontré que ce ne sont pas les progéniteurs hématopoïétiques qui sont directement impliqués car l'administration de moelle osseuse provenant de souris jeunes ne permet pas de limiter la sénescence thymique (39). Le développement de cette dégénérescence peut par contre s'expliquer par le fait que la thymopoïèse peut être vue comme un procédé énergétiquement coûteux et qu'aucune pression sélective ne tend à maintenir le répertoire T au même niveau chez des individus âgés que chez des individus plus jeunes (24).

Les raisons pour lesquelles le développement des cellules T se déroule dans le thymus demeurent énigmatiques. L'hypothèse actuelle explique la localisation thymique du développement des lymphocytes T par l'expression de certaines chémokines et récepteurs de chémokines. Certaines études soulèvent la possibilité que les chémokines TECK et SDF-1$\alpha$ soient impliquées au niveau de cette localisation thymique (40-43). Par contre, aucune autre molécule d'adhésion exprimée sur les précurseurs des cellules T ou sur les cellules stromales du thymus n'a encore été caractérisée, ni son rôle démontré au niveau de l'entrée sélective ou de la survie préférentielle des précurseurs des cellules T à l'intérieur de l'environnement thymique (43-46). De plus, différents modèles murins ont permis de démontrer la présence d'une maturation extrathymique des progéniteurs hématopoïétiques au niveau de la moelle osseuse (47;48), de l'intestin (49;50) et du foie (51;52). La capacité de cette maturation extrathymique à produire et maintenir les différentes populations de lymphocytes T périphériques au niveau des ganglions lymphatiques et de la rate est extrêmement limitée et ne peut en aucun cas compenser pour une fonction thymique déficiente (53).

### 1.3. L'oncostatin-M et le développement extrathymique des cellules T

L'oncostatin (OM) est une cytokine de 28 kD qui induit la différenciation et la prolifération de nombreuses lignées cellulaires (54). Le cDNA de l'OM code pour une protéine de 227 acides aminés qui est structurellement et fonctionnellement très similaire aux molécules de la

famille de l'IL-6 (55). Les cytokines membres de la famille de l'IL-6 (IL-11, LIF, CNTF, CT-1) sont caractérisées par l'utilisation d'un récepteur commun, la gp-130. Ce récepteur a une expression ubiquitaire et se retrouve dans de nombreux organes incluant le cœur, les reins, les poumons, le foie le cerveau, le placenta et les organes lymphoïdes. Le récepteur de l'OM humaine est un hétérodimère composé de la gp-130 associée au récepteur du LIF ou au récepteur spécifique de l'OM (56;57). Par contre, chez la souris, l'OM n'utilise que son récepteur spécifique (58). Les effets de l'OM sont multiples et sont très souvent semblables à ceux des cytokines appartenant à cette famille (55). Les principaux effets biologiques attribués aux cytokines de la famille de l'IL-6 sont reliés à la régulation de diverses réponses immunitaires, de l'hématopoïèse et des réactions inflammatoires. Certains modèles animaux, ont permis de démontrer que l'OM possède une activité anti-inflammatoire et qu'elle permet de diminuer les dommages tissulaires reliés à l'inflammation (59;60). Chez la souris, cette cytokine aux effets pléïotropiques est produite tardivement lors du cycle d'activation des cellules T et des macrophages. De plus, des études chez le singe ont démontré que ceux-ci sont très sensibles aux effets pharmacologiques et toxicologiques de l'OM : la fièvre et l'induction de réaction inflammatoires aiguës (60).

En 1996, des chercheurs de l'Institut Pharmaceutique Bristol Myers Squibb à Seattle, ont démontré que l'expression d'un transgène codant pour l'OM bovin, murin ou humain, et dont la régulation de l'expression était sous le contrôle du promoteur proximal de Lck (LckOM) ou de celui du CD34, stimulait l'accumulation de lymphocytes T immatures et matures au niveau

des ganglions lymphatiques (61;62). De plus, cette accumulation de cellules T d'origine extrathymique était observée chez des souris exprimant le LIF, une autre cytokine membre de la famille de l'IL-6, mais pas chez des souris exprimant le transgène de l'IL-6 ou de son récepteur (63;64). Les cellules T matures retrouvées chez la souris LckOM étaient fonctionnelles car la reconstitution de souris nu/nu à l'aide de moelle osseuse provenant de souris LckOM stimulait le développement de cellules T au niveau des ganglions lymphatiques et permettait le développement d'une réponse immune contre des cellules de mélanomes murins. Finalement, des expériences de croisements à l'aide de souris déficientes en IL-6 ou en récepteur de l'IL-7 ont permis de démontrer que le développement des cellules T au niveau des ganglions lymphatiques n'était pas dépendant de l'IL-6 mais était dépendant du signal transmis par le récepteur de l'IL-7 (62).

L'intérêt que nous portons à ce modèle provient du fait que 1) aucun autre modèle montrant un développement extrathymique des cellules T aussi important n'a jusqu'ici été décrit et que 2) ce modèle démontre qu'une maturation des cellules T peut se dérouler hors du thymus. Dans le cadre de la greffe médullaire et de pathologies responsables d'une déplétion des cellules T, ce modèle permet d'espérer pouvoir compenser l'atrophie thymique reliée à l'âge, la maladie ou les traitements. Les effets pharmacologiques/toxicologiques sévères de l'OM préviennent toutefois son utilisation clinique. La finalité de l'étude de ces souris est d'arriver à isoler les composantes responsables de l'effet de l'OM sur le développement des

lymphocytes T afin de développer une approche thérapeutique applicable à l'humain.

## 2. Contrôle homéostatique des différentes populations de cellules T

### 2.1. Les niches périphériques

Les conséquences d'une réduction progressive de la fonction thymique sont dramatiques chez des individus présentant une hypoplasie lymphoïde importante provenant de traitements de chimiothérapie ou de certaines infections comme celle par le HIV-1 (65-69). Le problème provient de ce que le thymus n'augmente pas sa production de cellules T suite à une déplétion des lymphocytes T périphériques. Chez un adulte, la production thymique et la taille du compartiment T périphérique sont régulées de façon indépendante. La fonction thymique n'est pas affectée par le nombre ou le ratio CD4/CD8 des cellules T périphériques (23;70). De plus, la taille du compartiment lymphocytaire T périphérique demeure constante (71) malgré la présence d'une exportation thymique soutenue ou augmentée (suite à la greffe de lobes thymiques) et d'une prolifération périphérique constante des cellules T matures (23;72). Donc, ces résultats suggèrent la présence d'une régulation homéostatique périphérique permettant que la perte cellulaire soit équivalente à la production.

De plus, l'existence d'une régulation homéostatique périphérique indépendante des cellules T naïves et mémoires (73) et de leurs ratios CD4/CD8 (23;74) démontre que ces différentes populations cellulaires interagissent différemment avec leur environnement. Ce type de régulation suggère la présence d'environnements spécialisés, ou niches, procurant les conditions requises à la survie et au développement de ces populations de cellules T périphériques spécifiques (75;76). La présence d'une compétition

entre les membres d'une même population implique que les ressources d'un tel environnement sont limitées (2). Ce type d'organisation favorise la survie de cellules dominantes à l'intérieur d'une même population mais limite la compétition cellulaire entre les membres de populations distinctes. Le contrôle homéostatique des populations lymphoïdes T périphériques serait le résultat d'une telle compétition (2).

Différentes études ont démontré la présence d'une compétition entre membres d'une même population. Freitas *et al.* (75) ont observé une telle compétition au niveau des cellules T $CD8^+$ périphériques et démontré la présence d'une sélection préférentielle des cellules activées et en division. Cette étude mentionne également que la destinée d'une population T $CD8^+$ est influencée par la présence ou de l'absence de cellules T $CD8^+$ compétitrices. Tanchot *et al.* (22) ont démontré, à l'aide d'un modèle transgénique pour le TCR $\alpha/\beta$, que les lymphocytes naïfs nouvellement produits par le thymus n'influencent pas la population de cellules T mémoires mais qu'ils remplacent graduellement les cellules T naïves et autoréactives périphériques. Le système immunitaire semble donc être régulé de façon à exclure toute compétition entre les cellules T naïves et mémoires/activées. Cette organisation permettrait la conservation du large répertoire T produit par le thymus et d'une mémoire immunologique durable résultant de l'expansion et de la persistance de certains clones de cellules T périphériques.

### 2.2. Localisation et structure des niches périphériques

La nature ainsi que la localisation des signaux impliqués dans le processus de régulation homéostatique des populations de lymphocytes T périphériques demeurent largement inconnues. Certaines études ont premièrement démontré l'importance du complexe majeur d'histocompatibilité CMH sur la survie des cellules T périphériques. Les résultats présentés par Tanchot *et al.* (15) montrent que la survie des cellules T $CD8^+$ naïves périphériques est dépendante d'une interaction avec la molécule du CMH I par laquelle ces cellules sont restreintes. Leur prolifération exige également la présence de leur peptide spécifique. Par contre, la survie des cellules T $CD8^+$ mémoires ne requière pas de molécule du CMH I spécifique mais leur prolifération est cependant dépendante de la présence du CMH I par laquelle ces cellules sont restreintes. Les lymphocytes T CD4 semblent posséder le même type d'exigences (77). De plus, l'implication des cellules dendritiques semble déterminante car la seule expression du CMH II sur ces cellules est suffisante à permettre la survie des cellules T $CD4^+$ (18). Les cellules dendritiques semblent constituer un élément fondamental au niveau des niches périphériques. De par leur expression abondante de molécules du CMH de classe I et de classe II et leur patron d'expression de chémokines et cytokines spécifiques, les cellules dendritiques semblent posséder toutes les caractéristiques requises afin de contrôler la localisation tissulaire des lymphocytes T périphériques ainsi que l'interaction TCR-CMH nécessaire à leur survie et leur prolifération (18).

Finalement, des résultats récents de l'équipe de Marrack (78;79) suggèrent qu'un élément important du phénomène d'immunodominance est la présence d'une compétition pour des ressources limitées au niveau des cellules présentatrices d'antigènes (CPA): facteurs solubles, molécules du CMH ou facteurs de co-stimulation. Les cellules dont l'affinité pour un antigène est supérieure excluraient les cellules T de faible affinité ce qui donnerait lieu à ce phénomène. Ces résultats démontrent que 1) les CPA sont en quantité limitée et que 2) seules les cellules dominantes à l'intérieur d'une population accèdent à certaines ressources. Les deux facteurs impliqués dans ce modèle de l'immunodominance sont les mêmes que ceux nécessaires à la présence d'une régulation homéostatique des cellules T. Il est donc possible que non seulement la régulation homéostatique des cellules T périphériques mais aussi diverses étapes du développement des lymphocytes T soient régulés par un processus de compétition cellulaire.

### 2.3. Les chémokines et la régulation homéostatique des cellules T

Les chémokines sont de petites protéines sécrétées que l'on associe généralement à leur capacité à recruter les leucocytes. Elles sont regroupées en quatre sous-familles caractérisées par le nombre et la localisation de leurs résidus cystéines en N-terminal. Les récepteurs de chémokines possèdent sept domaines transmembranaires et sont couplés aux protéines G. L'expression tissulaire des chémokines et de leurs récepteurs est très large et selon le cas constitutive ou inductible. Leur expression est généralement dépendante du niveau d'activation ou de différenciation cellulaire. La régulation de leur expression ainsi que leur participation au recrutement

cellulaire suggèrent que les chémokines et leurs récepteurs participent aux processus de différenciation, de maturation ainsi qu'à la fonction effectrice des différents leucocytes (80-87).

Il est intéressant de mentionner qu'une chémokine peut lier plusieurs récepteurs et qu'un récepteur peut lier plusieurs chémokines (81). Ainsi, une chémokine peut recruter plusieurs types cellulaires exprimant différents récepteurs et un type cellulaire peut répondre à plusieurs chémokines même s'il n'exprime qu'un seul récepteur. L'importance d'une telle organisation est particulièrement évidente lors d'une réponse inflammatoire car de nombreux types de leucocytes doivent être recrutés rapidement à des sites d'infection divers. De plus, nous pensons que l'orchestration d'événements aussi complexes que la maturation et la régulation homéostatique des cellules T ainsi que l'induction d'une réponse immune requiert la participation des chémokines et de leurs récepteurs.

Les chémokines et leurs récepteurs orchestrent la migration et la localisation tissulaire des différentes cellules impliquées lors du développement des lymphocytes T. Ces molécules sont impliquées au niveau de la migration des cellules dendritiques des tissus périphériques vers les organes lymphoïdes secondaires (88-90). Elles participent aussi au passage des cellules T du milieu intravasculaire vers le milieu extravasculaire aux sites d'inflammation et au niveau des organes lymphoïdes secondaires, à leur migration intra-tissulaire et finalement au niveau du tropisme entre cellules T et cellules dendritiques (91-94). De plus, l'expression sélective de chémokines et de leurs récepteurs sur certaines populations lymphocytaires

telles que les cellules T naïves et activées (91;94;95) et de type Th1 et Th2 (81;82;96-98) suggère un recrutement sélectif compatible avec 1) les rôles biologiques de chacune des ces populations et 2) le concept des niches périphériques. Nombre d'études ont de plus démontré que certaines cytokines régulent l'expression de chémokines et de leurs récepteurs (81;99;100). Finalement, la disparition de certains ganglions lymphatiques suite à l'inactivation du gène du récepteur aux chémokines CXCR5 (101) démontre l'implication de ces molécules au niveau du développement des organes lymphoïdes secondaires. Ces résultats démontrent clairement l'importance des chémokines et de leurs récepteurs au niveau de la localisation tissulaire des différentes populations cellulaires et donc de la communication et du développement cellulaire.

Le modèle le plus étudié d'interaction entre les chémokines et les différentes cellules participant au développement des cellules T concerne les chémokines SLC, ELC et leur récepteur CCR7. Ces chémokines recrutent les cellules dendritiques et les cellules T via le récepteur CCR7 (95;102;103) exprimé par les cellules dendritiques matures et les cellules T $CD4^+$ et $CD8^+$ (104). SLC est exprimé par les "high endothelial venules" (HEV) (91;105), permet l'induction d'une adhésion cellulaire ferme via certaines intégrines et leurs récepteurs (92;106) et participe au processus de migration à travers l'endothélium vasculaire. De plus, la sécrétion de SLC (95) et ELC (94) par les cellules dendritiques des ganglions lymphatiques suggère que ces chémokines sont également impliquées au niveau de la migration intra-tissulaire et donc de la rencontre entre les lymphocytes T et les cellules

dendritiques. Le modèle dominant expliquant cette migration suppose la formation d'un gradient de chémokines, sous forme de dépôts au niveau de la matrice extracellulaire, permettant de diriger les lymphocytes (107). C'est lors de leur interaction avec les cellules dendritiques que les lymphocytes T peuvent être activés, tolérisés ou éliminés. De plus, le contrôle de l'équilibre homéostatique des cellules T périphériques requiert possiblement un contact cellulaire entre les cellules dendritiques des ganglions lymphatiques et les cellules T. L'implication possible des chémokines SLC, ELC et de leur récepteur CCR7 lors de ces interactions cellulaires illustre bien le rôle déterminant que les chémokines semblent jouer au niveau de la biologie des cellules T. Ces molécules pourraient donc s'avérer importantes à l'élaboration de traitements visant 1) à stimuler la reconstitution immune post-greffe médullaire et 2) prévenir les effets délétères de la GVH sur cette reconstitution immune.

Finalement, *in vivo*, l'entrée et la migration des thymocytes à travers la jonction corticomédullaire, ainsi que la sortie des thymocytes du thymus requièrent des signaux provenant de un ou plusieurs récepteurs couplés aux protéines G, probablement des récepteurs de chémokines (42).(108;109) De plus, l'expression thymique de certaines chémokines produites par les cellules dendritiques du thymus et qui recrutent les macrophages, les cellules dendritiques et les thymocytes (81;97) porte à croire que les chémokines et leurs récepteurs sont impliqués tout au long du développement des lymphocytes T. Ces molécules participent au recrutement et à la localisation tissulaire favorisant ainsi la communication intercellulaire et le développement

des lymphocytes T. Leur implication possible au niveau de la sélection thymique (110) ainsi que leur rôle déterminant lors de l'activation, de la migration et de la phase effectrice des diverses populations de lymphocytes T démontre l'importance de ces molécules sur le développement des cellules T. Finalement, l'importance de leur rôle au niveau du contrôle homéostatique périphérique des populations lymphoïdes T découle directement de leur participation au recrutement et à la localisation tissulaire des lymphocytes T. Un contrôle suppose une interaction et une interaction suppose une localisation, les chémokines y ont certainement un rôle à jouer.

### 2.4. Développement de la réponse immune

Des études réalisées chez certaines souris possédant des organes lymphoïdes secondaires dont l'architecture est déficiente ont démontré que l'élaboration d'une réponse immune dépend non seulement de la présence de cellules T, cellules B et de cellules présentatrices d'antigènes (CPA), mais également d'interactions spécifiques à l'intérieur même de l'environnement très structuré des organes lymphoïdes secondaires. L'absence de rate ($Hox11^{-/-}$) (111), de ganglions lymphatiques ou de plaques de Peyer (aly/aly) (112) ainsi que l'altération de la structure de la pulpe blanche de la rate résulte en un déséquilibre entre la réplication virale et la capacité du sujet à développer une réponse immune. Les conséquences d'un tel dérèglement sont illustrées par une incapacité à éliminer certains virus comme le LCMV, le VSV et le VV (113).

La théorie actuelle suggère qu'un lymphocyte T naïf ne rencontre habituellement un antigène et ne peut être stimulé qu'à l'intérieur des organes

lymphoïdes secondaires. Le transport d'antigènes des tissus périphériques jusqu'aux organes lymphoïdes secondaires est donc un élément déterminant pour l'induction d'une réponse immune (114). De nombreuses études démontrent que la production et présentation de chémokines et de récepteurs de chémokines de type constitutif ou inflammatoire régulent la localisation tissulaire des cellules T et des cellules dendritiques. Ces molécules sont sécrétées par les cellules dendritiques ou certaines cellules endothéliales comme les HEV des ganglions lymphatiques. Ce recrutement se fait tant au niveau des organes lymphoïdes secondaires qu'aux sites d'infection et d'inflammation (43;82;88;94;97;115-120). Le rôle déterminant des chémokines au niveau de la formation des organes lymphoïdes et du tropisme entre les cellules T et dendritiques est clairement observable chez les souris dont l'expression de la chémokine SLC est déficiente ou chez celles présentant une mutation au niveau de son récepteur CCR7 (115;119). Ces souris présentent des malformations au niveau de l'architecture des organes lymphoïdes secondaires, une déficience de la migration des cellules T et des cellules dendritiques vers les ganglions lymphatiques, une réponse T et B retardée ainsi qu'une grande sensibilité aux infections. La rencontre entre les cellules T et dendritiques au niveau des organes lymphoïdes secondaires, l'activation des cellules T et leur migration aux sites d'infection sont toutes sous le contrôle des chémokines et de leurs récepteurs.

### 2.5. L'hypoplasie lymphoïde associée à la réaction du greffon contre l'hôte (GVH)

Des études chez la souris et l'humain ont démontré que la réaction

anti-leucémique (GVL) observée lors d'une immunothérapie adoptive représente un traitement efficace de la leucémie. Par contre, la réaction de GVH associée à cette thérapie engendre des dommages importants à de nombreux organes du receveur. De plus, une des conséquences les plus remarquables de la GVH est une déficience profonde et durable du développement des cellules T qui est responsable de l'état d'immunodéficience grave observée chez les individus greffés (121-124). Les cellules du greffon sont donc les cellules effectrices de la GVH mais en sont également les victimes (125). Différentes études ont démontré à l'aide de souris transgéniques et de chimères hématopoïétiques que la production de cellules T post-greffe médullaire provient principalement d'une maturation thymique des progéniteurs hématopoïétiques mais également d'une expansion périphérique des cellules T matures du greffon (30;126-128).

L'expansion des cellules T mature du greffon permet le développement d'un répertoire T diversifié et le transfert d'une mémoire immunologique au receveur. Des études de notre laboratoire ont démontré que, suite à leur transfert, les lymphocytes T alloréactifs du greffon prolifèraient mais que très rapidement ces cellules entraient en phase d'apoptose (AICD) (129). Nous avons également démontré que la voie apoptotique dépendante de la molécule Fas était impliquée au niveau de l'AICD des lymphocytes T $CD4^+$ et $CD8^+$. De plus, les lymphocytes T non-alloréactifs ne montrent pas de signe d'apoptose et n'expriment pas la molécule FAS lorsque transplantés seuls. Par contre, lorsque injectés parallèlement à des cellules alloréactives, ces lymphocytes montrent un niveau d'expression de FAS et un taux d'apoptose

augmentés. L'expansion des cellules T matures alloréactives et non-alloréactives du greffon est donc compromise par la GVH car ces cellules entrent rapidement en apoptose et disparaissent, compromettant ainsi la reconstitution immunitaire du receveur. La production de lymphocytes T post-greffe médullaire semble donc provenir principalement d'une maturation thymique des progéniteurs hématopoïétiques du greffon. Il est donc urgent de déterminer les effets de la réaction de GVH sur le développement thymique et le devenir des cellules T nouvellement produites par le thymus afin d'élucider les causes de l'hypoplasie lymphoïde associée à la réaction de GVH.

## Article 1

# Thymic and extrathymic differentiation and expansion of T lymphocytes following bone marrow transplantation in irradiated recipients

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## Abstract

Thymic function is severily impaired in most marrow transplant recipients. In order to evaluate the impact of thymic hypoplasia on T cell reconstitution following marrow transplantation, we compared the phenotype and function of T lymphocytes in thymectomized recipients to those of euthymic hosts. Irradiated C57BL/6 mice ($Thy1.2^+$, $Ly5.1^+$) received $10^7$ T cell-depleted B6.Ly5.2 bone marrow cells ($Thy1.2^+$, $Ly5.2^+$), with or without $3x10^5$ B6.PL lymph node cells ($Thy1.1^+$, $Ly5.1^+$) as a source of T lymphocytes. Multiparameter flow cytometric analysis showed that in euthymic mice (group 1), T cell reconstitution was carried out by donor hematopoietic stem cells that differentiated in host's thymus. In contrast, the origin of chimeras' T cells in athymic recipients depended on the presence/absence of T cells in the graft. When T lymphocytes were present in the graft (group 2), their progeny constituted the vast majority of splenic T cells on day 100 posttransplant. When the graft did not contain T lymphocytes (group 3), T cell reconstitution resulted from extrathymic maturation of donor hematopoietic progenitors; T cells differentiating along this pathway expressed lower levels of T cell receptor and a large proportion of the $CD8^+$ subset expressed $CD8\alpha\alpha$ homodimers. The T cell receptor $V\beta$ profile of all chimeras was similar to that of normal C57BL/6 mice. As compared to T cells found in euthymic recipients, those of mice from groups 2 and 3 were less abundant, particularly with regard to the $CD4^+$ subset, had the CD44/CD45 phenotype of activated/memory cells, and expressed high levels of IL-2 receptor $\beta$ chain. Thus, these results show that both the presence/absence of the thymus and the composition of the grafted inoculum determine the source and extent of posttransplant T cell reconstitution. Because they determine the nature of the differentiation pathway taken during T cell development in the host, these two factors can have a critical influence on the appearance of graft versus host disease and the level of host immunocompetence.

The frequency and severity of infections in bone marrow transplant (BMT) recipients are of such magnitude that numerous studies have been carried out to decipher the mechanisms of posttransplant immunodeficiency [1, 2]. They have shown that T cells represent the Achille's heel of immune recovery following BMT. While reconstitution of monocytes/macrophages, B lymphocytes, and NK cells is a question of weeks or months, T lymphocytes remain numerically and functionally deficient for years. [3-5] Thus, even at 5 years after transplant, i) mean $CD4^+$ T cell counts are subnormal and the proportion of $CD4^+$ cells with a naive phenotype ($CD45RA^{high}/CD45RO^{low}$, L-selectin$^+$, $CD29^{low}$, $CD11a^{low}$) is decreased, particularly in older recipients [4], and ii) frequencies of both cytotoxic and helper T lymphocyte precursors remain greatly depressed [5]. T cell defects are more severe in patients with chronic graft versus host disease (GVHD) and those receiving a T-cell depleted marrow graft [2, 6, 7]. Furthermore, out of forty eight thymi studied at autopsy 4 to 1472 days posttransplant, only two showed significant thymopoiesis; the remainder showed a profound atrophy, and contained only mature T lymphocytes without immature cortical thymocytes [8, 9]. Obviously, these autopsy data were skewed to reflect histology of sick patients, often with severe GVHD, and it is possible that thymus function is better preserved in clinically well patients. Nevertheless, phenotypic analyses combined with studies of anti-HSV and anti-CMV T cell responses performed in 22 patients by de Gast et al., provided evidence that T cell recovery during the first 6 months after autologous BMT was mainly due to proliferation of mature T cells present in the marrow graft, and not to generation of new T cells from T cell precursors [10]. Collectively, these studies provide convincing evidence that thymic function is severely deficient in BMT recipients, particularly in adults.

Recipient age, chemotherapy, endogenous and exogenous corticosteroids, and GVHD are among the numerous factors that can cause thymic atrophy and impair T cell development following BMT [11-16]. In this context, two alternative pathways could contribute to host T cell reconstitution: expansion of mature T lymphocytes present in the

marrow inoculum, and extrathymic maturation of donor stem cells. Although the contribution of these pathways is overshadowed by the production of new thymic emigrants in young euthymic recipient mice [17, 18], recent studies suggest that the situation is different in athymic hosts [17, 19, 20]. Thus, mature T lymphocytes have a considerable expansion potential (up to $8x10^{15}$-fold) and have been shown to proliferate and survive long-term in athymic mice [17, 19, 20]. Alternatively, T cell repopulation in recipients with poor thymic function, particularly those whose graft has been T-cell depleted, could involve extrathymic maturation of T cell progenitors. This alternative maturation pathway, first described in old or athymic mice, has been found to take place in a number of sites, particularly the gut and the liver whose epithelium, as in the case of the thymus, is of endodermic origin [21, 22]. T cells of extrathymic origin are phenotypically and functionally different from thymic T lymphocytes, and are rare in peripheral organs but abundant in sites such as the intestinal epithelium, where they may have an important role in local immune responses [21-23]. While T lymphocytes generated in the intestine (intraepithelial lymphocytes) constitute a sessile population, others, which probably mature in the liver or the bone marrow, have the potential to disseminate widely and to repopulate secondary lymphoid organs [24-26]. In view of these findings, the goal of the present study was to evaluate the potential contribution of extrathymic pathways in T cell reconstitution following BMT in athymic recipients. More specifically, our aim was to compare T cell ontogeny in euthymic and athymic BMT recipients, to determine the respective contribution of the two thymus independent pathways mentioned above, and to evaluate the phenotype, T cell receptor repertoire, and function of T lymphocytes that have matured in the thymus vs the periphery.

## Materials and methods

*Mice.*

The following strains of mice were used throughout these studies: C57BL/6J (H-$2^b$) (Thy-$1.2^+$, Ly-$5.1^+$), B6.PL-Thy-1A/Cy (B6.PL; Thy-$1.1^+$, Ly-$5.1^+$), B6.SJL-Ptprc$^a$Pep$3^b$/BoyJ (Ly$5^a$) (B6.Ly5.2; Thy-$1.2^+$, Ly-$5.2^+$), and B10.BR/SgSnJ (H-$2^k$). All mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in a conventional facility. All mice used as cell donor or irradiated recipients were between 8 and 20 weeks of age.

*Monoclonal antibodies. (MoAb).*

Cytotoxic anti-Thy-1.2 (5a-8; mouse IgG) MoAb was obtained from Cedarlane (Hornby, Ontario, Canada); PE-conjugated anti-Thy-1.1 (MRC OX-7; mouse IgG1) MoAb and his isotypic control were from Caltag Laboratories (Hornby, Ontario, Canada). The following MoAbs were obtained from PharMingen (San Diego, CA): FITC-conjugated anti-TCR-α/β (H57-597; hamster IgG), anti-Vβ3 (KJ25; hamster IgG), anti-Vβ5.1,2 (MR9-4; mouse IgG1), anti-Vβ6 (RR4-7; rat IgG2b), anti-Vβ7 (TR310; rat IgG2b), anti-Vβ8.1,2 (MR5-2; mouse IgG2a), anti-Vβ9 (MR10-2; mouse IgG1), anti-Vβ$10^b$ (B21.5; rat IgG2a), anti-Vβ11 (RR3-15; rat IgG2b), anti-Vβ13 (MR12-3; mouse IgG1), anti-Vβ14 (14-2; rat IgM), anti-Vβ$17^a$ (KJ23; mouse IgG2a), anti-CD45.2 (Ly-5.1; 104; mouse IgG2a), anti-CD45.1 (Ly5.2; A20; mouse IgG2a); PE-conjugated anti-Thy-1.2 (30-H12; rat IgG2b), anti-NK-1.1 (PK136; mouse IgG2a), anti-TCR γ/δ (GL3; hamster IgG), anti-CD44 (Pgp-1; IM7; rat IgG2b), anti-CD45RB (23G2; rat IgG2a), anti-IL-2Rβ (TM-b1; rat IgG2b), anti-CD8β (Ly-3.2; 53-5.8, rat IgG1); and specific Cy-chrome™-conjugated anti-CD4 (RM4-5; rat IgG2a), anti-CD8α (53-6.7; rat IgG2a) MoAbs, and their isotypic controls.

*Thymectomy.*

At 4 to 8 weeks of age, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (Somnotol; MTC Pharmaceuticals, Cambrige, Ontario, Canada) 75 mg/kg. Thymectomy was performed with a suction cannula introduced over the suprasternal notch. Completeness of thymectomy was verified in each animal by visual inspection and histologic examination at the time of each experiment. Cell transplantation was performed 2 to 4 weeks after thymectomy.

*Cell transplantation.*

Mice were transplanted as described previously [18]. Briefly, recipient mice received 9.5 Gy total body irradiation from a $^{60}$Co source at a dose rate of 128 cGy/min on day 0, the day of the transplant. Bone marrow cells were obtained from the tibiae and femurs from donor mice and T cell-depleted with specific anti-Thy-1.2 MoAb. Axillary and cervical LN were collected, teased apart, and washed. Bone marrow cells ($10^7$) ± LN cells ($3 \times 10^5$) were given as a single intravenous injection, via the tail vein, in a volume of 0.5 ml of serum-free RPMI 1640 media (Life Technologies, Burlington, Canada).

*Depletion of Thy-1.2$^+$ cells.*

Bone marrow cells were resuspended at a concentration of $1 \times 10^7$ cells/ml in RPMI 1640 supplemented with 5% FCS (HyClone Laboratories, Logan, UT), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies), and incubated with anti-Thy1.2 MoAb (Cedarlane) at 4$^{o}$C for 1h. They were then pelleted by centrifugation, resuspended in rabbit serum (Low-Tox-M rabbit complement; Cederlane) as a source of complement, and incubated at 37$^{o}$C for 1 h. Cell suspensions were washed three times, analyzed for efficacy of depletion by flow cytometry, and then adjusted for injection. Marrow cells with Thy1.2 staining below the flow cytometry threshold have no functionally rearranged Vβ genes [27].

*Fluorescence-activated cell sorter analysis and sorting.*

Direct immunofluorescence staining was performed with FITC-, PE-, and Cy-chrome™-conjugated MoAbs. One million splenocytes per sample were incubated for 25 min on ice with the appropriate dilution of MoAbs, in a final volume of 125 µl PBS/0.1% BSA/0.1% $NaN_3$. After three washes, cells were analyzed for surface fluorescence on a FACScan® flow cytometer (Becton Dickinson, San Jose, CA) or sorted with a FACStar$^{Plus}$ apparatus (Becton Dickinson). Nonspecific binding of MoAbs was assessed by labeling cells with appropriate fluorochrome-conjugated isotype-matched controls.

For single or double immunofluorescence staining, $10^4$ (group 1) or $3 \times 10^4$ (groups 2 and 3) cells were collected in list mode using the Lysis II program (Becton Dickinson) and analyzed with the CellQuest program (Becton Dickinson). For triple immunofluorescence staining, acquisition was performed to collect $1.5 \times 10^4$ double stained cells, in order to increase the number of cells bearing the third marker. $CD4^+$ or $CD8^+$ cells, double stained with anti-Thy1.1/1.2, anti-Ly5.1/5.2, or anti-TCR α/β, were gated and then analyzed for expression of Vβ elements, CD44, CD45RB, NK1.1, IL-2Rβ, or CD8β. Sorting was performed at an analysis rate of 3500 cells/s in normal-R mode. The sorted samples were reanalyzed by flow cytometry to check the purity of each cell suspension ($\geq$ 99%)

***Proliferation assays*** .

Spleen cells were placed in 96-well flat bottomed microtiter plates at $1.5 \times 10^5$ cells/well in 200 µl of culture medium (RPMI 1640, 10% FCS, 4 mM l-glutamine, 10 mM Hepes buffer, 50 µM 2-mercaptoethanol, 100 U/ml penicillin G, 100 µg/ml streptomycin) in triplicates. The following final concentrations of mitogens were used: Phytohemagglutinin (PHA;Wellcome Reagents, NC) 2 µg /ml, Concanavalin A (Con A; Sigma Chemicals Co., St. Louis, MO) 2 µg/ml, Pokeweed (Sigma Chemicals Co.) 5 µg/ml. For stimulation with interleukin-2 (IL-2; Genzyme Corp., Cambridge, MA), cells were adjusted to a concentration of $5 \times 10^5$ cells/well in 200 µl of medium before addition of 100 U/ml of IL-2. Plates were incubated for 72 h at

37°C in a humidified atmosphere of 5% $CO_2$. For mixed leukocyte reaction (MLR), responding spleen cells (5 x $10^6$ cells per ml) were stimulated for 5 days in culture medium with an equal number of irradiated (25 Gy) B10.BR stimulator cells (H-2 incompatible with effector cells). During the final 18 h of the incubation, 1 μCi of [$^3$H]TdR (Dupont NEN, Markham, Ontario, Canada) was added in each well. After the incubation, cells were transferred onto glass fiber filters by an automated cell harvester and incorporation of [$^3$H]TdR was measured with a scintillation counter. The stimulation index was calculated as: SI = (mean cpm of stimulated culture)/(mean cpm of control culture).

*Cytotoxic T lymphocyte (CTL) assays.*

Cytotoxic activity was assessed in a standard $^{51}$Cr-release assay following a 5-day MLR with B10.BR stimulator cells. Target blast cells were prepared by culturing B10.BR splenic cells with 2 μg/ml Con A for 48 hours. Target cells were then labeled with 100 μCi of $Na_2{}^{51}Cr$ (DuPont Co., Wilmington, DE) for 90 min and then washed three times with RPMI 1640 supplemented with 5% FCS. Different ratios of effector cells were added to a fixed number of target cells (5 x $10^3$ cells) in a final volume of 200 μl per well culture medium. The plates were centrifuged, and then incubated for 3 h at 37°C in a humidified atmosphere of 5% $CO_2$. At the end of the incubation period, plates were recentrifuged once more and 100 μl of the supernatant was harvested from each well and counted in a gamma counter. All test were done in triplicates. Spontaneous release was < 15%. Results were expressed as a percentage of specific lysis calculated as follows: % specific lysis = 100 x (experimental release - spontaneous release)/(maximum release - spontaneous release).

*NK assays.*

Following depletion of red blood cells by hypotonic lysis, effector spleen cells were resuspended in culture medium and adjusted to different cell concentrations (2 x $10^7$ and 1 x $10^7$ cells per ml); these steps were performed at 4°C to preserve NK activity. YAC cells were

labelled with $Na_2{}^{51}Cr$ as described above and used as target cells at a concentration of $1 \times 10^5$ cells per ml. Using microtiter plates with U-shaped wells, 100 µl each of target cells and effector cells were added per well in triplicates. Plates were centrifuged and incubated for 4 h at 37°C in a humidified atmosphere of 5% $CO_2$. Afterwards, plates were recentrifuged and 100 µl of each well was harvested and counted in a gamma counter. Specific lysis was calculated as described earlier.

## Results

*Origin and numbers of T lymphocytes in the spleen of recipient mice.*

Following BMT, T cell populations can reconstitute from three different sources: donor hematopoietic stem cells, mature T lymphocytes present in the graft, and residual host cells. In order to discriminate for T cell origin, we transplanted C57BL/6 recipients ($Thy1.2^+$, $Ly5.1^+$) with T-depleted B6.Ly5.2 bone marrow cells ($Thy1.2^+$, $Ly5.2^+$), with or without B6.PL lymph node cells ($Thy1.1^+$, $Ly5.1^+$) as a source of mature T lymphocytes. In order to evaluate the influence of the thymus on posttransplant T cell ontogeny, both euthymic and thymectomized hosts were studied. We analysed spleen cells from three experimental groups (Table 1), 95 to 105 days posttransplant: in group 1 (euthymic, T-cell replete graft), C57BL/6 mice received a mixture of bone marrow and lymph node cells, group 2 (athymic, T-cell replete graft) recipients received the same type of graft but were thymectomized two weeks before BMT, and group 3 (athymic, T-cell depleted graft) thymectomized hosts were grafted with only bone marrow cells.

Both the numbers and origin of splenic T cells were strikingly different in the three experimental groups (Fig. 1A). Group 1 chimeras had normal numbers of T lymphocytes ($31.9 \times 10^6 \pm 2.9$), most of which were $Thy1.2^+$, $Ly5.2^+$ and thus, originated from donor hematopoietic stem cells . In group 2, the majority of T lymphocytes derived from grafted mature T cells ($Thy1.1^+$, $Ly5.1^+$), while in group 3 practically all T cells originated from donor hematopoietic stem cells. In all recipients, the contribution of residual host cells was negligible. Of particular significance, the number of $Thy1^+$ cells was much lower in mice from group 2 ($8.8 \times 10^6 \pm 2.8$) and group 3 ($7.6 \times 10^6 \pm 1.9$). In contrast, athymic mice showed no deficit of $NK1.1^+$ cells, which in fact were slightly increased in group 3 (Fig. 1A). Thus, athymic chimeras presented significant T lymphocytopenia as compared to their euthymic counterparts. In the former, the origin of T cell reconstitution depended on the content of the

grafted inoculum. When mature T cells were present in the graft, they expanded in the host and constituted the dominant population. In their absence, restoration of peripheral T cell compartments depended on extrathymic maturation of donor hematopoietic stem cells.

*CD4/CD8 phenotype and T cell receptor (TCR) repertoire of chimeras' T lymphocyte populations.*

In mice from group 1, cells bearing T cell receptor (TCR) αβ chains showed a normal CD4/CD8 ratio (1.7 ± 0.4) (Fig. 1B). However, this ratio was decreased in group 3 (0.96 ± 0.3), and even more in group 2 (0.5 ± 0.08). This is consistent with observations suggesting that $CD4^+$ T cells are more dependent on normal thymic function than $CD8^+$ lymphocytes [28, 29]. In the three experimental groups, i) almost all $CD4^-CD8^+$ and $CD4^+CD8^-$ T lymphocytes expressed αβ TCR ii) low numbers of $TCR\alpha\beta^+$ cells were $CD4^-8^-$, and iii) γδ TCR was only found on a few $CD4^-CD8^+$ T cells (Fig.1B). In normal euthymic animals, the production of γδ cells by the thymus is low, and these cells represent only a small proportion of T lymphocytes found in the spleen and lymph nodes [30]. Similarly, our results show that, at least for the splenic compartment, extrathymic maturation of T lymphocytes following BMT is skewed toward production of $TCR\alpha\beta^+$ lymphocytes.

The TCR repertoire of classical $TCR\alpha\beta^+$ T lymphocytes is imparted through thymic education and is influenced by both MHC and non-MHC genes [31, 32]. Thus, TCR Vβ chain usage is quite constant between mice of a given inbred strain but shows marked inter-strain variability [28, 33]. We wanted to determine if the Vβ profile of $TCR\alpha\beta^+$ cells that arose through expansion of grafted mature T cells (group 2), or from extrathymic maturation of donor stem cells (group 3), would be different from that of the progeny of donor stem cells that were educated and selected in host's thymus (group 1). This was not the case. Indeed, the Vβ profile of $CD4^+8^-$ and $CD4^-8^+$ T lymphocytes was very similar in the three experimental

groups (Fig. 2). In each of them,Vβ chain usage was similar to what we previously reported in normal C57BL/6 mice [18].

*CD44 density and CD45RB expression.*

CD44/CD45 phenotyping is a practical and widely used approach to distinguish "naive" from "activated/memory" T cell subsets [34]. Although it has been reported that some activated/memory cells display a naive phenotype [35], there is a general consensus that CD44/CD45 phenotype can be used to define two cell population with different mitotic history: one of putatively pure activated/memory cells, and one which is highly enriched in naive T lymphocytes [30, 34, 36]. For both $CD4^-8^+$ and $CD4^+8^-$ T lymphocytes, CD44 expression is low in naive, and high in activated/memory cells. Furthermore, $CD4^+8^-$ lymphocytes differ by their expression of CD45 isoforms; thus, expression of CD45RB is high on naive but low on activated/memory cells [37]. We observed markedly different expression of both CD44 and CD45RB in athymic versus euthymic recipients. Indeed, in mice from groups 2 and 3, CD44 expression was upregulated in $CD4^+$ and $CD8^+$ subsets, while the proportion of $CD4^+$ lymphocytes expressing the CD45RB isoform was strikingly decreased (Fig. 3). Mackall et al. previously observed similar results for $CD4^+$ cells in mice treated like our group 2 [17]. Therefore, in athymic hosts, the CD44/CD45 phenotype of T lymphocytes derived either from grafted mature T cells or from extrathymic differentiation of donor stem cells was typical of activated/memory cells.

*Markers acquired following thymic vs extrathymic maturation.*

Extrathymic T lymphocytes found in normal, nude, or thymectomized mice have atypical phenotypes. Depending on the organ studied and the experimental model, four characteristics have been reported in various combinations: decreased intensity of TCR/CD3 complex expression, increased proportion of $NK1.1^+$ cells, presence of $CD8^+$ cells expressing CD8αα homodimers instead of classical αβ heterodimers, and higher proportion of IL-2

receptor β chain (IL-2Rβ)$^+$ elements [21-23, 38, 39]. These parameters were analyzed in our three experimental groups (Fig. 4). Results in group 1 were similar to those of C57BL/6 controls (data not shown), and can therefore be considered as representative of typical thymus-derived lymphocytes. In all experimental groups, only a very small proportion of CD4$^+$ or CD8$^+$ cells expressed the NK1.1 molecule (Fig. 4A). In groups 2 and 3, the percentage of cells expressing the IL-2Rβ chain was significantly increased among the CD8$^+$ ($p < 0.05$, t test) but not the CD4$^+$ subset (Fig. 4B). While in groups 1 and 2 practically all CD8$^+$ cells were CD8α$^+$β$^+$, in group 3 half the CD8$^+$ elements expressed CD8αα homodimers (Fig. 4C). To compare the level of TCRαβ expression, the proportion of TCR$^{high}$/TCR$^{low}$ was analyzed for both CD4$^+$ and CD8$^+$ subsets (Fig. 4D). The distinction between high and low levels of TCR expression was arbitrarily defined by taking as a reference the median intensity of TCR expression by CD4$^+$ cells in C57BL/6 mice (n=3, data not shown). It should be noted that the intensity of TCRαβ labeling is lower in CD8$^+$ than in CD4$^+$ lymphocytes. While the TCR intensity in group 2 was similar to that of group 1, it was markedly decreased in group 3 for both the CD4$^+$ and CD8$^+$ subsets. Thus, the phenotype of group 3 was typical of extrathymic T lymphocytes: the level of expression of TCRαβ was decreased, and the CD8 subset was characterized by i) an increased proportion of IL-2Rβ$^+$ elements, and ii) the expression of CD8αα homodimers by ~50% of the cells. Extrathymic T cells can be characterized as NK1.1$^+$ or NK1.1$^-$ [21, 39]; practically all CD4$^+$ and CD8$^+$ cells found in group 3 animals belonged to the NK1.1$^-$ subset. The phenotype of group 2, in which practically all T cells derived from grafted mature T lymphocytes, was similar to that of group 1 except for the increased proportion of (Ly5.1$^+$) CD8$^+$ cells that expressed the IL-2Rβ chain. Because these T cells clearly received a thymic education (in the donor), expression of IL-2Rβ on a larger proportion of CD8$^+$ elements should be considered a sign of "activation" in this group, as this molecule is found not only on extrathymic T cells but also on activated T lymphocytes [40, 41].

*Proliferative responses and cytotoxic activity.*

Mice from groups 2 and 3 responded very poorly to Con A, PHA, and Pokeweed mitogens, as well as to alloantigens (mixed leukocyte reaction) (Fig. 5). In contrast, proliferative responses of group 1 mice were similar to those of normal C57BL/6 mice, except for slightly lower responses to Pokeweed and alloantigens (Fig. 5). The small differences between group 1 and controls were not statistically significant and were possibly age-related because, when tested on day 100 posttranplant (at 23 weeks of age), mice from group 1 were older than control mice (aged 6-8 weeks). T lymphocytes represented 30-32% of spleen cells in mice from group 1, but only 8-12% in mice from groups 2 and 3 (data not shown). Therefore, low proliferative responses in groups 2 and 3 could depend either on qualitative or quantitative T cell defects. To evaluate these possibilities, we tested C57BL/6 spleen cell suspensions containing only 10% T lymphocytes. These cell suspensions were obtained by mixing unseparated C57BL/6 spleen cells with adequate numbers of C57BL/6 non-T cells, and will be referred to as B6M. Non-T cells were Thy1.2$^-$ cells sorted out (with a FACStar$^{Plus}$ flow cytometer) from spleen cell suspensions labeled with an anti-Thy1.2 MoAb. Thus, B6M cell preparations did not contain antibody-coated cells, a parameter that could have interfered with functional assays. Proliferative responses of B6M cells in the presence of mitogens or alloantigens were lower than those of standard C57BL/6 cells and very similar to those of groups 2 and 3 (Fig. 5). This suggest that proliferative defects observed in groups 2 and 3 likely depend on the decreased proportion of T lymphocytes in recipients' spleen.

As chimeras in groups 2 and 3 had high numbers of IL-2R$\beta^+$ T cells, we evaluated the effect of IL-2 on these cell populations. When compared to group 1, the proliferative response of group 2 mice was increased 4-5 fold, while that of group 3 was normal. As the number of IL-2R$\beta^+$ cells was similar in groups 2 and 3, this suggests that not all IL-2R$\beta^+$ cells have the same proliferative potential and that activated/memory T cells of thymic origin are more responsive to IL-2 than extrathymic T cells.

Analysis of cytotoxic effectors yielded a different profile (Fig. 6). The three experimental groups showed normal NK activity against YAC targets. CTL responses against MHC-incompatible B10.BR cells were slightly to moderately decreased in the three experimental groups when compared to C57BL/6 controls, and showed the following hierarchy: group 2 > group 1 > group 3. The small impairment of CTL activity in group 1 vs C57BL/6 controls was possibly age-related (see above). Comparison with B6M controls suggest that the low CTL activity in group 3 probably depend on the decreased T cell numbers. The relatively high cytotoxic activity observed in group 2 may be related to the very high proportion of $CD8^+/IL\text{-}2R\beta^+$ T cells in this group (Fig. 4B). These $CD8^+/IL\text{-}2R\beta^+$ T cells seem to be very sensitive to IL-2. Indeed, when compared to group 1, the proliferative response of splenocytes from group 2 to IL-2 was increased 4-5 fold (Fig. 5).

## Discussion

The results presented herein demonstrate that three pathways can contribute to T cell ontogeny following BMT. The source and extent of T cell reconstitution in our chimeras was determined by two factors: the presence or absence of the thymus, and the composition of the grafted inoculum. In the presence of a thymus, T cell reconstitution was carried out by donor hematopoietic stem cells that received thymus education in the host and generated phenotypically and functionally normal peripheral T cell pools. As reported previously, transplanted mature T cells did not contribute to long-term T cell restoration in this context [17, 18]. In athymic recipients, however, the origin of chimeras' T cells depended on the cell content of the graft. When T cells were present in the graft (group 2), they expanded considerably in the host and represented the large majority of spleen T cells on day 100 posttransplant. In their absence (group 3), T cell pool restoration depended upon extrathymic maturation of donor hematopoietic stem cells. In groups 2 and 3, T cells were less abundant and presented atypical phenotypes when compared to group 1. A number of evidences, presented in the introduction section, suggest that most BMT patients present a severe thymic hypoplasia. Thus, although this remains to be tested, it seems likely that, in these individuals, T cell ontogeny is similar to what we observed in groups 2 and 3.

As mature T cells expand considerably and persist long-term in athymic hosts (group 2), why do they disappear in euthymic recipients (group 1)? We think that this reflects a growth advantage for new thymic emigrants over old activated/memory cells. Indeed, in normal individuals, Weng et al. found a 1.4 kb difference in telomeric length between naive and activated/memory cells [36]. Such a difference corresponds to 14 more previous cell divisions per memory cell than per naive cell. When assessed in vitro, the replicative potential of activated/memory cells was 128 times lower than that of naive cells [36]. Thus, it appears likely that, because of its lower replicative potential, the progeny of grafted mature T cells

cannot compete with new thymic emigrants for occupation of peripheral T cell niches. As a corollary, a normal host thymus should thus protect from GVHD by leading to disappearance of grafted mature T cells, which are required for initiation of GVHD. This is consistent with the observation that acute GVHD is more severe in recipients whose thymic function is impaired because of age or thymectomy [42-44]. Using the same line of reasoning, we would hypothesize that the replicative potential of grafted mature donor T cells is superior to that of T cells that arose by extrathymic maturation of donor hematopoietic stem cells, because the latter were relatively abundant in group 3 but rare in group 2. Therefore, in the context of BMT, the ability to repopulate host's (splenic) T cell pool would decrease according to following hierarchy: new thymic emigrants (found in group 1) > grafted mature T cells (found in group 2) > extrathymically differentiated T cells (found in group 3). We intend to test this assumption by studying the kinetics of in vivo BrdU labeling of these cell populations.

Acute and chronic GVHD are intimately linked, and the former is usually the harbinger of the latter [45]. However, Parkman reported in a seminal observation that, while T lymphocytes found in mice suffering from acute GVHD are alloreactive, those found at the time of chronic GVHD are autoreactive [46]. Furthermore, numerous reports have shown that chronic GVHD shares many clinical, histologic and immunologic features with autoimmune diseases [47]. How could an alloimmune reaction generate an autoimmune disorder? It is clear that the thymus is a direct target of acute GVHD and that this process makes the host functionally athymic [11, 14, 18, 48]. We have shown herein that in athymic, but not in euthymic hosts, thymus-independent T lymphocyte populations emerge and repopulate peripheral lymphoid organs. A number of studies have demonstrated that extrathymic maturation differs from thymic education in that the former entails positive selection, but no negative selection [49-52]. Unlike classical T cells [53, 54], some extrathymic T lymphocytes cannot be deleted in the periphery, possibly because of differences in expression of costimulatory molecules and/or in TCR-mediated signaling [22, 55]. Thus, extrathymic T

lymphocytes contain potentially autoreactive T cells. Therefore, we would like to suggest that thymus damage could provide the link between acute and chronic GVHD. According to this mechanistic explanation, destruction of the thymus by alloreactive T cells at the time of acute GVHD, would favor the production of extrathymic T cells which, by virtue of their autoreactivity, would have the potential to initiate chronic GVHD. In the nude mice, the presence of extrathymically derived autoreactive clones does not cause disease under steady state conditions, but injection of IL-2 causes the appearance of autoimmune manifestations similar to those found in chronic GVHD [50]. As the appearance of chronic GVHD often closely follows an infectious process, it is tempting to speculate that this situation is caused by IL-2-mediated recruitment of "innocent bystander" autoreactive T cell clones.

It was shown very recently that T cells differentiating in the liver, and probably also in the bone marrow, can repopulate secondary lymphoid organs of thymectomized BMT recipients [25,26]. T cell populations found in the liver [25] or the spleen (this study) of thymectomized mice are similar to those of nude mice [29, 56] except for one parameter, the ratio of $TCR\alpha\beta^+$ /$TCR\gamma\delta^+$ cells. Indeed, whereas 50-65% of T cells in nude mice express $\gamma\delta$ TCR, almost all T cells found in thymectomized mice express $\alpha\beta$ TCR. These observations support the concept that *extrathymic* differentiation of $TCR\alpha\beta^+$ cells is impaired in congenitally nude mice when compared to thymectomized mice [57]. In this regard, it should be remembered that the nude mutation has pleiotropic effects that are not limited to the thymus, and involve, for instance, the skin and the endocrine system [58, 59].

It is interesting to see that the V$\beta$ profile of mice from group 3 was identical to that of euthymic recipients. Although this observation is not sufficient to conclude that the repertoire of T lymphocytes which differentiate in the thymic vs extrathymic environment are identical, it shows that T cells generated extrathymically are polyclonal and display significant TCR diversity. This concept is consistent with experiments showing that extrathymic cells of

diverse origins can contribute to T cell selection and differentiation [60, 61]. Nevertheless, thymic epithelial cells have an unmatched ability to provide costimulatory signal(s) necessary for positive selection [62]. Studies on the relations between expression of endogenous superantigens and the TCR repertoire suggest that negative selection has a more important influence than positive selection on the Vβ profile [32, 63]. Superantigens which associate with I-E molecules are most effective in deleting specific Vβ elements [32, 33]. Our finding that extrathymic T cells have the same Vβ profile as T cells of thymic origin, coupled with the notion that the former are not subjected to negative selection [49, 52], suggest that, at least in C57BL/6 mice (H-$2^b$), positive selection plays a major role in shaping the Vβ profile. This role may possibly be more evident in H-$2^b$ mice, because they lack I-E molecules.

Athymic recipients (groups 2 and 3) presented significant lymphocytopenia, affecting particularly the $CD4^+$ subset, and their spleen cells displayed deficient *in vitro* proliferative responses to mitogens and alloantigens probably because of the quantitative T cell defect. These abnormalities are commonly reported in long-term human BMT recipients [2-7]. Furthermore, their $CD4^+$ and $CD8^+$ subsets showed a CD44/CD45 phenotype characteristic of activated/memory lymphocytes. In contrast, these recipients showed normal NK activity, and CTL mediated cytotoxicity was markedly decreased only in group 3. Further studies on isolated T cell subsets will be required to evaluate the functional integrity of the signal transduction pathways in these extrathymic T cells. It is interesting to note that mice from Group 2 differed from those from group 3 in two points: response of the former to IL-2 was greatly enhanced, and their capacity to mount cytotoxic T cell responses was superior. Thus, the presence of mature T cells in the graft may affect immunocompetence of athymic hosts. This observation may explain why recipients of T cell-depleted marrow grafts have a higher incidence of infections [7, 64]. The phenotypic and functional characteristics of T cells in group 3 animals were similar to those of nude or senescent mice [28, 65, 66]. The fact that recipients with poor thymic function grafted with T cell-depleted BMT may resemble nude

mice suggests that treatment with some cytokines could be beneficial for these patients. Indeed, it has recently been shown that transgenic expression of interleukin-7 restored T cell numbers and proliferative responses in nude mice [67]. This observation supports the concept that production of interleukin-7 is a most important role of the thymus [68, 69], and shows that extrathymic T lymphocytes are responsive to this molecule. Other cytokines, such as c-*kit* ligand, TNF-α, and IL-1α, are normally involved in expansion of immature thymocytes [70, 71], and it will be critical to determine whether they could have a positive influence on maturation and function of extrathymic T cells. It will be important to investigate these possibilities, not only to improve the immune competence of marrow transplant recipients, but also that of aging individuals in which the role of extrathymic T cell maturation increases progressively to compensate for the "normal" thymic involution associated with senescence [72].

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**Table 1. Experimental design.**

**1.1 Experimental groups**

**1.2 Origin of T lymphocytes**

## Figure Legends

**Figure 1.** Origin and numbers of T lymphocytes in the spleen of recipient mice from the three experimental groups (day 100 ± 5 posttransplant). A) The origin of Thy1.2$^+$ Ly5.2$^+$, Thy1.1$^+$ Ly5.1$^+$, and Thy1.2$^+$ Ly5.1$^+$ cells is presented in table 1. Absolute counts were obtained by two-color staining, except for NK1.1$^+$ cell counts which were based on one-color staining. B) CD4/CD8 ratio and TCR usage. Absolute counts (of all T cells, regardless of origin) were obtained by two-color immunofluorescence. Results are presented as the mean ± SD (3-4 mice per group).

**Figure 2.** Vβ repertoire of CD4$^+$ and CD8$^+$ chimeras' T cells (day 100 ± 5 posttransplant). Three-color staining was performed with FITC-labeled anti-Vβ, Cy-chrome$^{TM}$-labeled anti-CD4 or anti-CD8, and PE-labeled anti-Thy1.2 (group 1 and 3) or anti-Thy1.1 (group 2). In each panel, the percentage of cells bearing a particular Vβ element was calculated by dividing the number of cells bearing this element by the total number of TCRαβ$^+$ cells in this CD4$^+$ or CD8$^+$ subset. Results are presented as the mean ± SD (three mice per group). * = not done.

**Figure 3.** Differential expression of CD44 and CD45RB in the three experimental groups (day 100 ± 5 posttransplant). Three-color staining with FITC-labeled anti-Ly5.1 or anti-Ly5.2, Cy-chrome$^{TM}$-labeled anti-CD4 or anti-CD8, and PE-labeled anti CD44 or anti-CD45RB. A) Expression of CD44 on CD4$^+$ and CD8$^+$ lymphocytes. B) Expression of CD45RB on CD4$^+$ cells. Results are from one representative experiment out of three.

**Figure 4.** Phenotype acquired following thymic vs extrathymic differentiation (day 100 ± 5 posttransplant). Results are from one representative experiment out of three, and are presented separately for CD4$^+$ and CD8$^+$ subsets in group 1, 2, and 3. A) Expression of NK1.1. Three-color staining with FITC-labeled anti-Ly5.1 or anti-Ly5.2, Cy-chrome$^{TM}$-labeled

anti-CD4 or anti-CD8, and PE-labeled anti-NK1.1. Numbers represent the percentage of $NK1.1^+$ cells in the gated cell population expressing both CD4 or CD8 and Ly5.1 or Ly5.2. B) Expression of IL-2Rβ. Three-color staining with FITC-labeled anti-TCRα/β, Cy-chrome™-labeled anti-CD4 or anti-CD8, and PE-labeled anti-IL-2Rβ. Numbers represent the percentage of IL-$2R\beta^+$ cells in the gated cell population expressing both TCR α/β and CD4 or CD8. C) Expression of CD8 αβ heterodimers. Three-color staining with FITC-labeled anti-Ly5.1 or anti-Ly5.2, Cy-chrome™-labeled anti-CD8α, and PE-labeled anti-CD8β. Numbers represent the percentage of $CD8\beta^+$ cells in the gated cell population expressing both $CD8\alpha^+$ and Ly5.1 or Ly5.2. D) Intensity of TCR αβ expression. Two-color staining with FITC-labeled anti-TCR αβ and Cy-chrome™-labeled anti-CD4 or anti-CD8. Numbers represent the percentage of $TCR^{low}$ and $TCR^{high}$ among $CD4^+$ or $CD8^+$ cells.

**Figure 5.** Proliferative responses of splenocytes from control C57BL/6 mice and chimeras from the three experimental groups (day 100 ± 5 posttransplant). B6M are C57BL/6 cell suspensions containing 10% T cells; they were obtained by mixing unseparated C57BL/6 spleen cells with $Thy1.2^-$ cells isolated by flow cytometry from spleen cell suspensions labeled with an anti-Thy1.2 MoAb. The proportion of T lymphocytes in spleen cell suspensions was 30-32% in C57BL/6 controls and mice from group 1, and 8-12% in mice from groups 2 and 3. Mixed leukocyte reaction (MLR) was carried out with irradiated B10.BR stimulator cells. Results from four independent experiments are expressed as the mean ± SD; 3-4 mice per group. * $p < 0.05$ vs C57BL/6 controls (Mann-Whitney *U* test and Fischer exact test).

**Figure 6.** Cytotoxic activity of splenocytes from control C57BL/6 mice and chimeras from the three experimental groups (day 100 ± 5 posttransplant). B6M cells were prepared as in Fig. 5. Results from three independent experiments are expressed as the mean ± SD; 3 mice per group. A) CTL cytotoxicity was tested on B10.BR Con A blast targets. B) NK lysis was tested on YAC targets.

**Table 1-1**

**Table 1.** Experimental design.

1.1 Experimental groups

| | Graft | | Host Thymectomy |
|---|---|---|---|
| | $10^7$ Thy1.2-depleted B6.Ly5.2 BM cells (Thy1.2$^+$, Ly5.2$^+$) | $3x10^5$ B6.PL LN cells (Thy1.1$^+$, Ly5.1$^+$) | C57BL/6 recipients (Thy1.2$^+$, Ly5.1$^+$) |
| Group 1 | + | + | - |
| Group 2 | + | + | + |
| Group 3 | + | - | + |

1.2 Origin of T lymphocytes

| Phenotype | Source |
|---|---|
| Thy1.2$^+$, Ly5.2$^+$ | Grafted BM-derived hematopoietic stem cells |
| Thy1.2$^+$, Ly5.1$^+$ | Residual host cells |
| Thy1.1$^+$, Ly5.1$^+$ | Grafted LN-derived mature T cells |

Figure 1-1

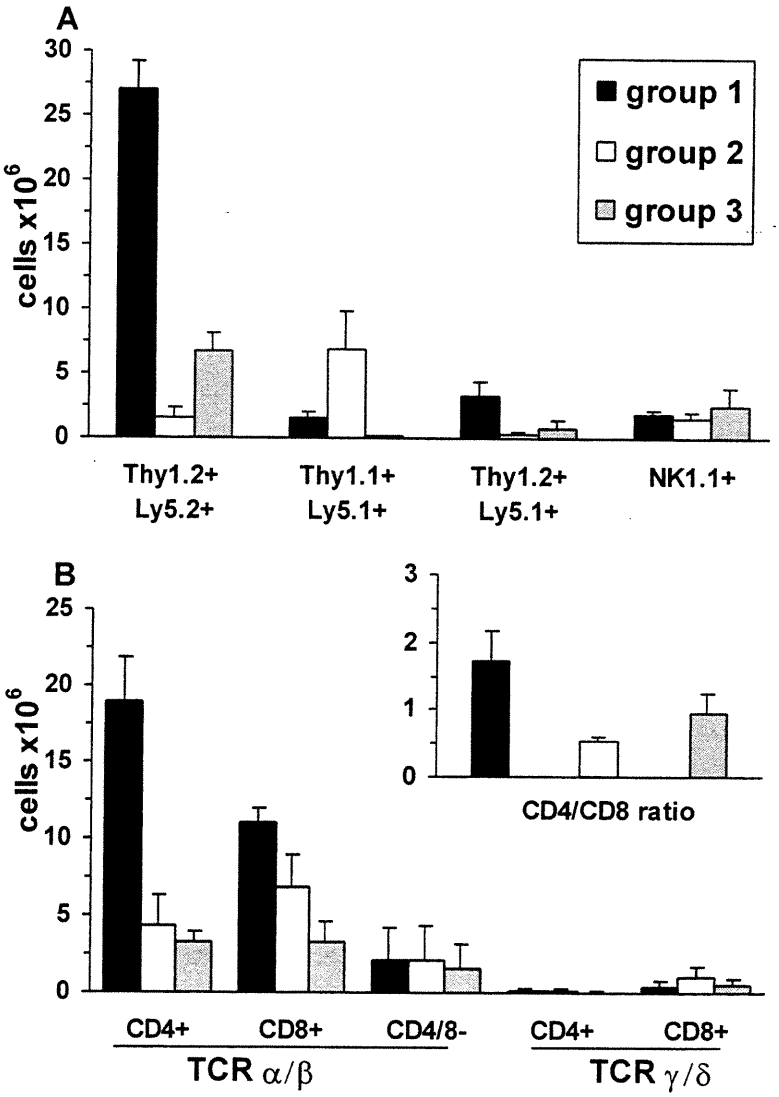

A
cells x10^6
30
25
20
15
10
5
0
group 1
group 2
group 3
Thy1.2+ Ly5.2+
Thy1.1+ Ly5.1+
Thy1.2+ Ly5.1+
NK1.1+
B
cells x10^6
25
20
15
10
5
0
3
2
1
0
CD4/CD8 ratio
CD4+
CD8+
CD4/8-
CD4+
CD8+
TCR α/β
TCR γ/δ

**Figure 1-2**

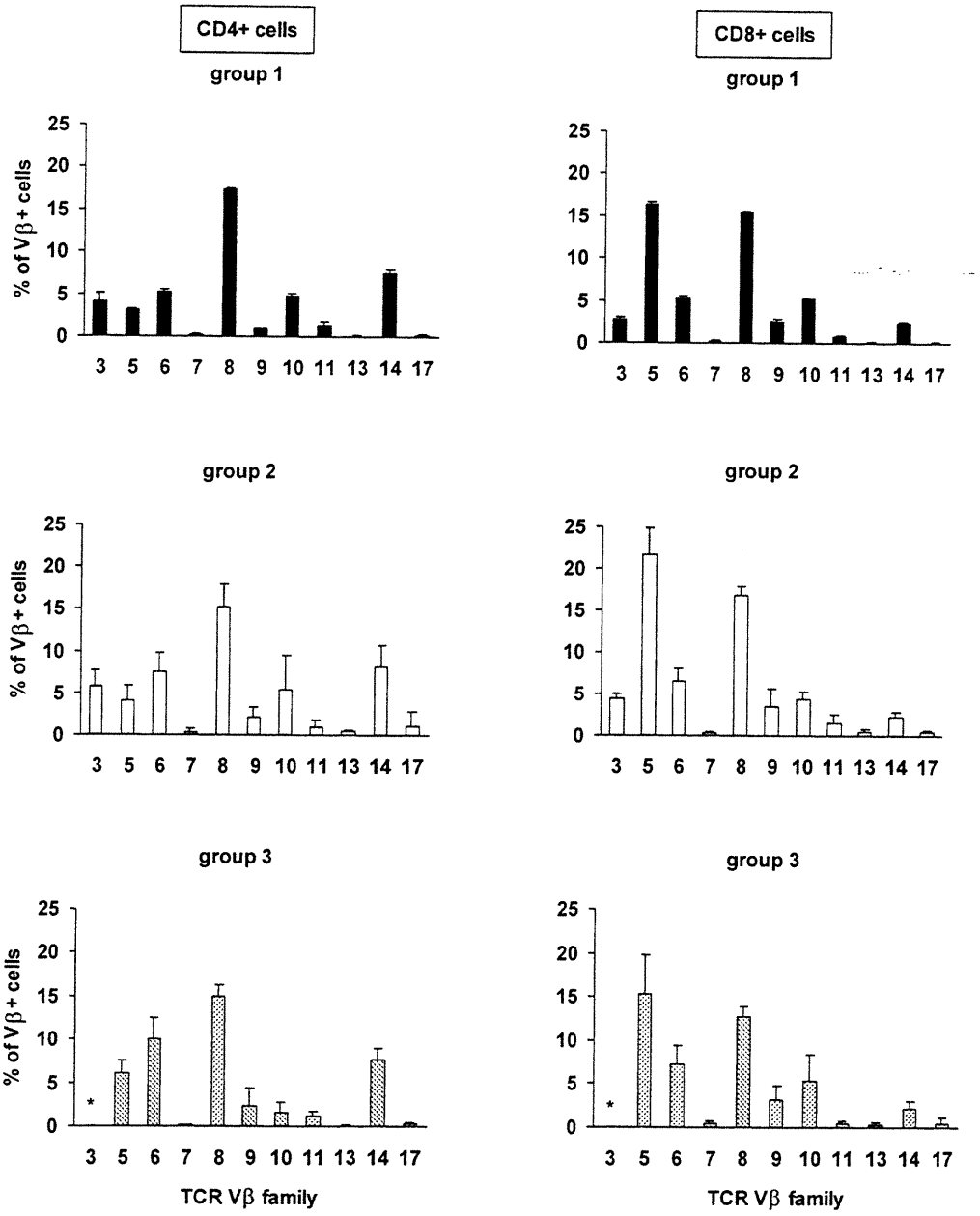

CD4+ cells
group 1
CD8+ cells
group 1
% of Vβ+ cells
25
20
15
10
5
0
3 5 6 7 8 9 10 11 13 14 17
group 2
group 2
group 3
group 3
*
TCR Vβ family
TCR Vβ family

Figure 1-3

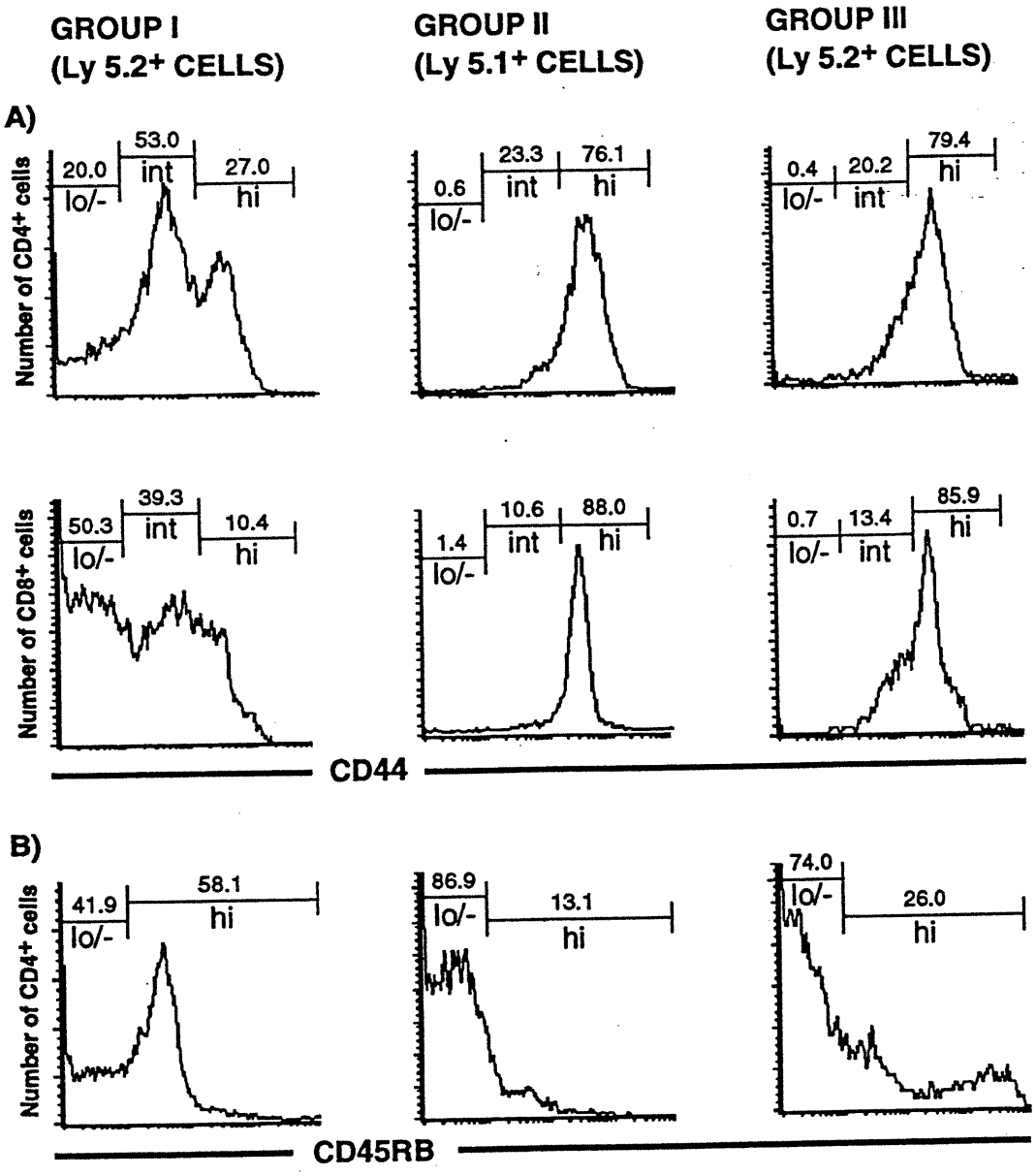

GROUP I
(Ly 5.2+ CELLS)
GROUP II
(Ly 5.1+ CELLS)
GROUP III
(Ly 5.2+ CELLS)
A)
Number of CD4+ cells
20.0
lo/-
53.0
int
27.0
hi
0.6
lo/-
23.3
int
76.1
hi
0.4
lo/-
20.2
int
79.4
hi
Number of CD8+ cells
50.3
lo/-
39.3
int
10.4
hi
1.4
lo/-
10.6
int
88.0
hi
0.7
lo/-
13.4
int
85.9
hi
CD44
B)
Number of CD4+ cells
41.9
lo/-
58.1
hi
86.9
lo/-
13.1
hi
74.0
lo/-
26.0
hi
CD45RB

Figure 1-4

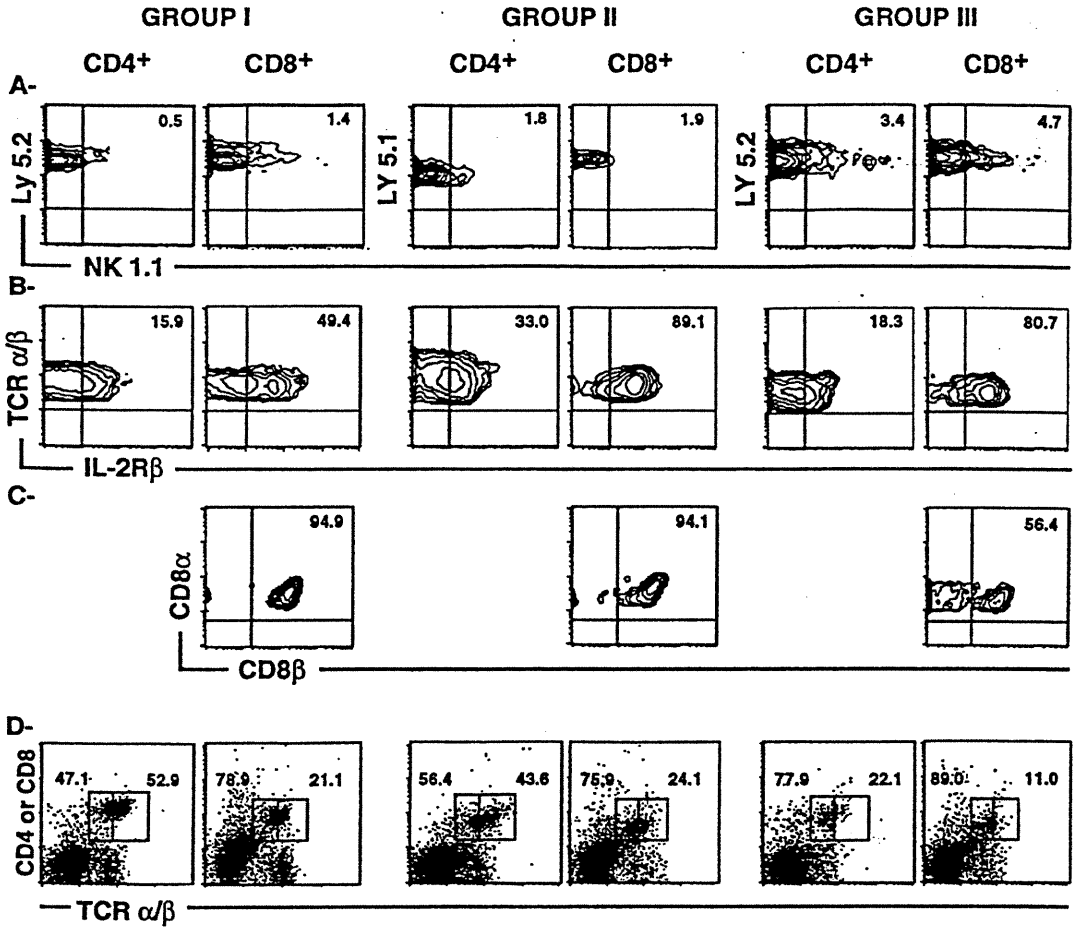

GROUP I
GROUP II
GROUP III
CD4+
CD8+
CD4+
CD8+
CD4+
CD8+
A-
Ly 5.2
0.5
1.4
LY 5.1
1.8
1.9
LY 5.2
3.4
4.7
NK 1.1
B-
TCR α/β
15.9
49.4
33.0
89.1
18.3
80.7
IL-2Rβ
C-
CD8α
94.9
94.1
56.4
CD8β
D-
CD4 or CD8
47.1
52.9
78.9
21.1
56.4
43.6
75.9
24.1
77.9
22.1
89.0
11.0
TCR α/β

**Figure 1-5**

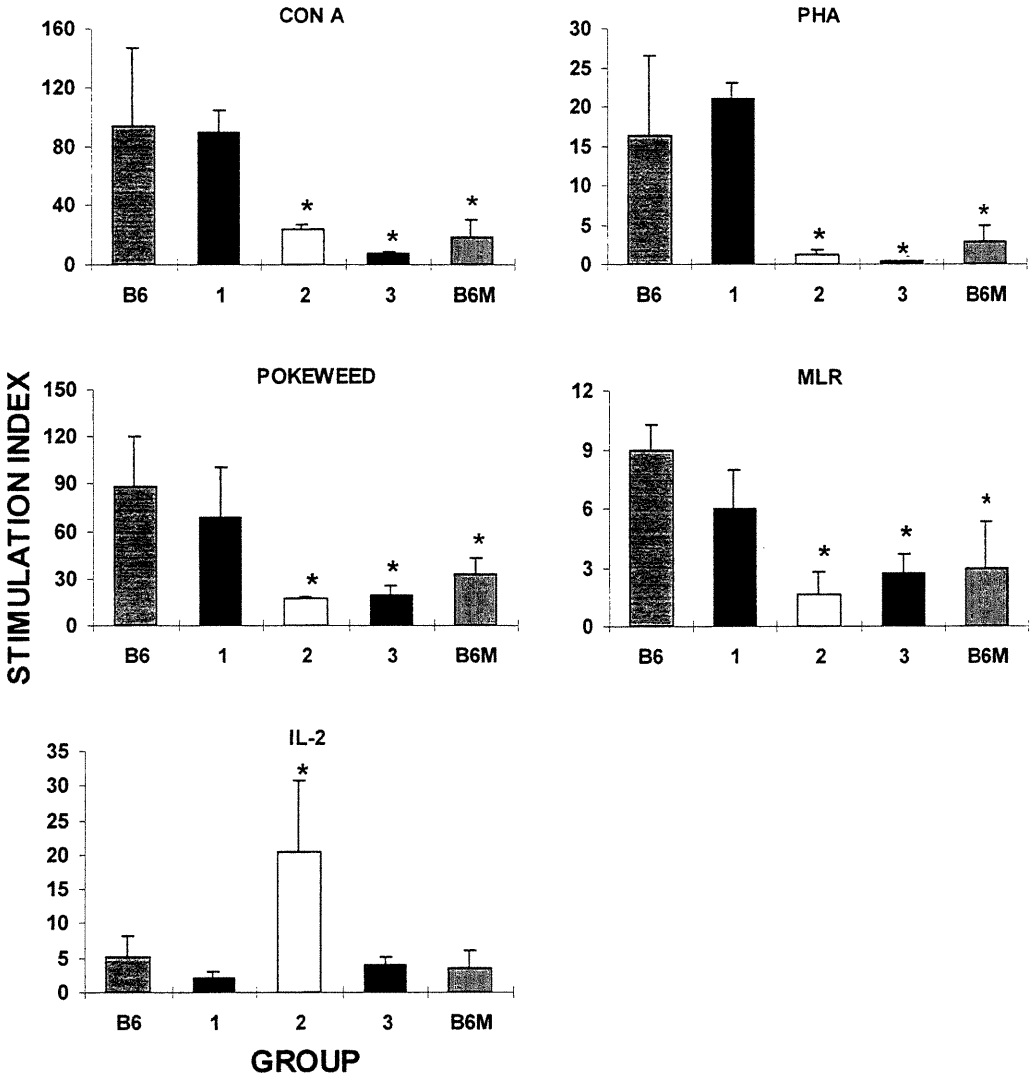
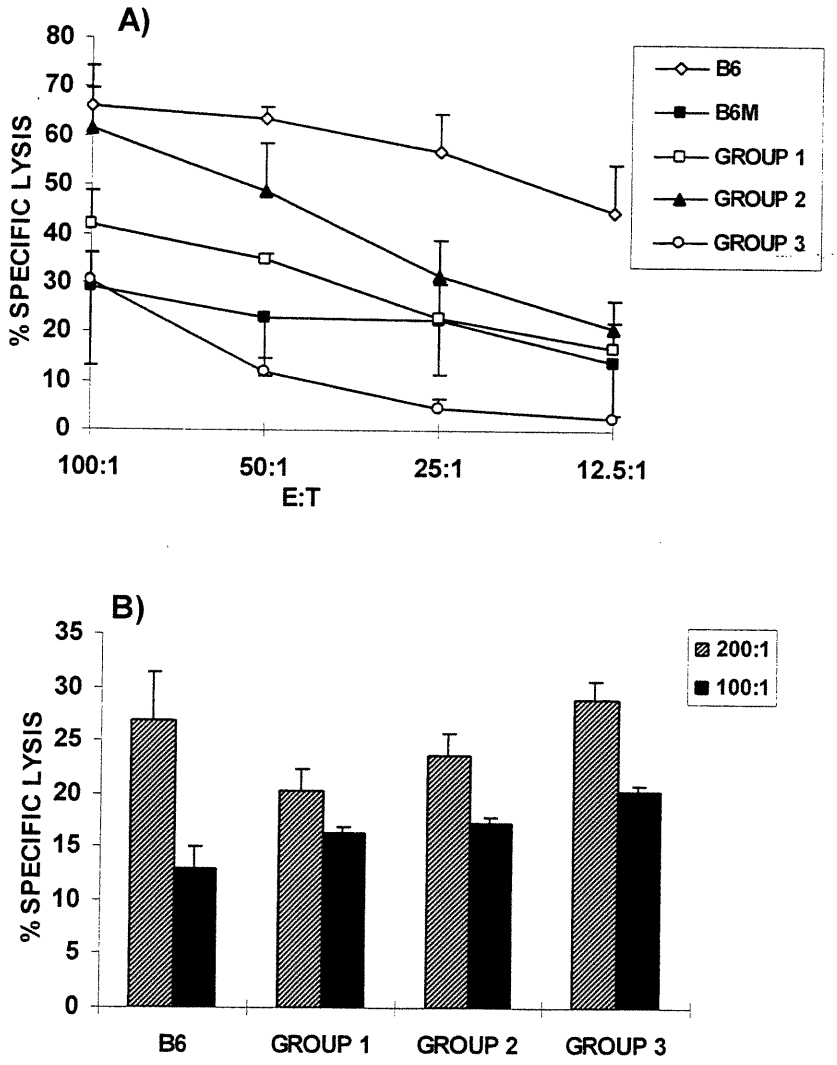

CON A
PHA
POKEWEED
MLR
IL-2
STIMULATION INDEX
GROUP
B6
1
2
3
B6M

**Figure 1-6**


A)
% SPECIFIC LYSIS
80
70
60
50
40
30
20
10
0
100:1
50:1
25:1
12.5:1
E:T
B6
B6M
GROUP 1
GROUP 2
GROUP 3
B)
% SPECIFIC LYSIS
35
30
25
20
15
10
5
0
200:1
100:1
B6
GROUP 1
GROUP 2
GROUP 3

## Article 2

# The effect of graft-versus-host disease on T cell production and homeostasis

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**Summary**

The aim of this work was to decipher how graft-versus-host disease (GVHD) affects T cell production and homeostasis. In $GVHD^+$ mice, thymic output was decreased 4-fold relative to normal mice, but was sufficient to maintain a T cell repertoire with normal diversity in terms of Vβ usage. Lymphoid hypoplasia in $GVHD^+$ mice was caused mainly by a lessened expansion of the peripheral post-thymic T cell compartment. In 5-bromo-2'-deoxyuridine pulse-chase experiments, resident T cells in the spleen of $GVHD^+$ mice showed a normal turnover rate (proliferation and half-life). When transferred into thymectomized $GVHD^-$ secondary hosts, T cells from $GVHD^+$ mice expanded normally. In contrast, normal T cells failed to expand when injected into $GVHD^+$ mice. Thus, the reduced size of the post-thymic compartment in $GVHD^+$ mice was not due to an intrinsic lymphocyte defect but to an extrinsic microenvironment abnormality. We suggest that this extrinsic anomaly is consistent with a reduced number of functional peripheral T cell niches. Therefore, our results show that GVHD-associated T cell hypoplasia is largely caused by a perturbed homeostasis of the peripheral compartment. Furthermore, they suggest that damage to the microenvironment of secondary lymphoid organs may represent an heretofore unrecognized cause of acquired T cell hypoplasia.

[1]*Abbreviation used in this paper:* BrdU, 5-bromo-2'-deoxyuridine.

## Introduction

While GVHD causes damage to many recipient organs, one most important hallmark of this disease is a profound defect in the development of donor-derived T cells which is responsible for a long-lasting immunodeficiency state (1-7). GVHD is initiated by host-reactive T cells that produce copious amounts of cytokines and cause direct cytotoxicity via Fas-, TNF-, and perforin-mediated pathways (8-12). This anti-host allogeneic attack, commonly referred to as acute GVHD, occurs during the first few days/weeks following transplantation and is of limited time duration (13) since donor-derived anti-host T cells undergo massive activation-induced cell death (Brochu S, Rioux-Massé B, Roy DC and Perreault C, submitted). After the acute phase of GVHD has abated, most recipients are left with a chronic immunodeficiency state characterized by frequent opportunistic infections and autoimune manifestations i. e., the so called chronic phase of GVHD (4,13,14). The immune deficiency found during chronic GVHD involves mainly T cells, and is characterized by a severe lymphoid atrophy and inadequate responses to both recall antigens and new epitopes (15-20). It remains unclear how a time-limited anti-host T cell response can induce long lasting (commonly permanent) disturbances in the differentiation and homeostasis of donor-derived T cells.

During the last decade, studies using transgenic mice and transplant chimeras have shed light on the intricacies of T cell differentiation and homeostasis. It was shown that T cell production can proceed along both thymic and extrathymic pathways (21-23). This has been well ascertained by investigations using mice

transplanted with congeneic histocompatible hematopoietic stem cell grafts. In young euthymic recipients, T cell reconstitution is carried out by donor hematopoietic progenitor cells that differentiate into the host's thymus (21,23). In contrast, in athymic recipients, T cell reconstitution entirely depends on extrathymic pathways and is influenced by the presence/absence of post thymic T cells in the graft. When T lymphocytes are present in the graft, their progeny is responsible for the repopulation of host secondary lymphoid organs (21,23). This underscores the fact that mature T cells have a considerable proliferation potential (up to $8x10^{15}$-fold) and can survive long-term in the absence of competing recent thymic emigrants (24-27). However, when the graft does not contain T lymphocytes, reconstitution of peripheral T cell compartments can only proceed through extrathymic maturation of donor hematopoietic progenitors (22,23,28). In various mouse models, extrathymic differentiation of hematopoietic stem cells has been detected in the bone marrow (28,29), the intestinal cryptopatches (30), the liver (22), and the lymph nodes (31). The ability of these organs to replenish and maintain lymph node and spleen T cell compartments is considered to be much inferior to that of the thymus.

The number of peripheral $CD4^+$ and $CD8^+$ T lymphocytes is tightly regulated by poorly defined homeostatic mechanisms which do not only rely on the cellular input into the peripheral compartment (24,32,33). In support to this, thymic output is not influenced by downstream alterations in peripheral T cell pool size or CD4:CD8 ratio (34,35) and, furthermore, the size of peripheral T cell compartments shows relatively modest variations when confronted with a low/absent thymic output or with a major increase in thymic output (e. g., hyperthymic mice and some transgenic

models) (34-40). It thus seems that the number of available T cell niches in secondary lymphoid organs determines the size of peripheral T cell compartments (38,41). The term niche designates an environment that provides local conditions (such as expression of specific chemokines, cytokines, and MHC molecules) required for T cells to seed and survive long-term in the peripheral compartment (41,42). Recently, a number of evidences have been presented suggesting that resident dendritic cells represent fundamental constituents of the peripheral T cell niches (43-46). Because of their abundant expression of MHC class I and class II molecules and their specific chemokine and cytokine expression profile, dendritic cells seem to have a unique ability to control the homing of post-thymic T cells and to provide the continuous TCR ligation required for the survival of naïve and memory T cells in the periphery (33,44,47).

The goal of this work was to decipher the mechanisms responsible for the GVHD-associated hypoplasia of peripheral T cell pools. Therefore, we addressed two questions. Firstly, how does GVHD influence thymic and extrathymic generation of donor-derived T lymphocytes? Knowing that GVHD per se causes thymic dysfunction (17,48,49), we wanted to quantify the impact of thymic lesions on thymic output, and determine whether GVHD also affected extrathymic T cell production. Secondly, how does GVHD influence T cell production, proliferation and survival? In otherwise normal subjects, the size of peripheral T cell compartments can be maintained even in the presence of a severily decreased thymic output. Why, therefore, is chronic GVHD associated with persistent lymphoid hypoplasia? Establishing how GVHD affects the size and the repertoire of peripheral T cell

compartments should be instrumental in understanding why patients with chronic GVHD present more infections, autoimmune diseases and neoplasia (14,50-52). Our results demonstrate that GVHD not only impairs thymic production of new T cells, but also abrogates expansion of mature T cell pools in secondary lymphoid organs. Furthermore, they indicate that the defective expansion of the peripheral post-thymic T cell compartment in $GVHD^+$ mice is not due to an intrinsic T cell proliferative defect, but rather to an extrinsic anomaly consistent with a restriction in the number of functional peripheral T cell niches. These findings allow for the development of a model in which GVHD-associated lymphoid hypoplasia would represent a disease of soil (environment) rather than seed (T cell). Accordingly, damage to the stroma of the thymus and of secondary lymphoid organs inflicted during the acute phase of GVHD would be responsible for a prolonged impairment of the development of donor-derived T cells.

## Materials and methods

*Mice.* The following strains of mice were used: C57BL/6J (H-$2^b$) (Thy-$1.2^+$, Ly $5.2^+$), B6.PL-*Thy-1*$^a$/Cy (B6.PL; Thy-$1.1^+$, Ly $5.2^+$), B6.SJL-*Ptprc*$^a$*Pep3*$^b$/BoyJ (Ly$5^a$) (B6.SJL; Thy $1.2^+$, Ly $5.1^+$), and A.BY-*H2*$^b$ *H2-T18*$^b$/SnJ (ABY; Thy-$1.2^+$, Ly $5.2^+$). Mice, originally purchased from the Jackson Laboratory (Bar Harbor, ME), were bred and housed in specific pathogen-free conditions at the Guy-Bernier Research Center according to the standards of the Canadian Committee for Animal Protection. All mice used as primary cell donors or irradiated recipients were between 8 and 20 weeks of age. For 5-bromo-2'-deoxyuridine (BrdU) incorporation studies, mice were given sterile drinking water containing 0.8 mg/ml BrdU (Sigma Chemical, St-Louis, MO), which was made fresh, changed daily and protected from light.

*Thymectomy.* At 4 to 8 weeks of age, mice were anesthetized by intraperitoneal injection of 75 mg/Kg sodium pentobarbital (Somnotol; MTC Pharmaceuticals, Cambrige, Ontario, Canada). Thymectomy was performed with a suction cannula introduced over the suprasternal notch. Completeness of thymectomy was verified in each animal by visual inspection at the time of sacrifice. Cell transplantation was performed at least 2 weeks after surgery.

*Cell transplantation and T cell depletion.* Bone marrow cells were T cell-depleted and transplanted as previously described (23). Recipient mice received 10 Gy total body irradiation on day 0, the day of transplant. Bone marrow cells were obtained from the tibias and femurs of donor mice and T cell-depleted with a specific anti-Thy-1.2 monoclonal Ab (5a-8; mouse IgG) or with a rabbit anti-mouse T cells

(Thy 1) antiserum, both obtained from Cedarlane (Hornby, Ontario, Canada), and rabbit serum (Low-Tox-M rabbit complement; Cedarlane) as a source of complement. Efficacy of depletion was assessed by flow cytometry. Spleen or axillary, cervical and inguinal LN cells were harvested and washed. The number of T cells injected was determined by flow cytometry using an anti-Thy 1.1 or anti-Thy 1.2 Ab. Bone marrow and spleen or LN cells were given as a single intravenous injection, via the tail vein, in a volume of 0.5 ml.

*Monoclonal Abs.* The following Abs were obtained from PharMingen (Mississauga, Ontario, Canada): biotinylated-anti-CD8α (53-6.7; rat IgG2a) detected with Cy-chrome™-streptavidin, FITC-conjugated anti-TCR-α/β (H57-597; hamster IgG), anti-Vβ3 (KJ25; hamster IgG), anti-Vβ5.1,2 (MR9-4; mouse IgG1), anti-Vβ6 (RR4-7; rat IgG2b), anti-Vβ7 (TR310; rat IgG2b), anti-Vβ8.1,2 (MR5-2; mouse IgG2a), anti-Vβ9 (MR10-2; mouse IgG1), anti-$V\beta10^b$ (B21.5; rat IgG2a), anti-Vβ11 (RR3-15; rat IgG2b), anti-Vβ13 (MR12-3; mouse IgG1), anti-Vβ14 (14-2; rat IgM), anti-$V\beta17^a$ (KJ23; mouse IgG2a), anti-CD45.1 (Ly-5.1; 104; mouse IgG2a), anti-CD45.2 (Ly5.2; A20; mouse IgG2a); PE-conjugated anti-Thy-1.2 (30-H12; rat IgG2b), anti-Thy-1.1 (OX-7, mouse IgG1,k), anti-CD62L (MEL-14; rat IgG2a,k), anti-TCR γ/δ (GL3; hamster IgG), and specific Cy-chrome™-conjugated anti-CD4 (RM4-5; rat IgG2a), anti-CD8α (53-6.7; rat IgG2a) Abs, and their isotypic controls. PE-conjugated anti-CD4 (YTS 191.1, rat IgG2b), anti-CD8α (YTS 169.4, rat IgG2b) Abs and their isotypic controls were purchased from Cedarlane and the FITC-conjugated anti-BrdU Ab from Becton Dickinson (Mountain View, CA).

*Flow Cytometry.* Cell surface staining was performed as previously described

(23). BrdU labeling was performed as described by Tough and Sprent (27). Briefly, after surface staining, cells were resuspended in cold 0.15 M NaCl, fixed by dropwise addition of cold 95% ethanol, incubated for 30 min on ice, and washed with PBS. The cells were then incubated with PBS containing 1% paraformaldehyde and 0.01% Tween 20 for 30 min, pelleted and then incubated for 30 min with 50 K units of DNase I (Sigma Chemical) in 0.15 M NaCl, 4.2 mM $MgCl_2$, pH 5. After washing, cells were incubated with FITC-conjugated anti-BrdU for 30 min and washed. Cells were analyzed on a FACScalibur® (Becton Dickinson) using the CellQuest program (Becton Dickinson) or on a FACScan® (Becton Dickinson) using the Lysis II program (Becton Dickinson).

## Results and Discussion

*The influence of GVHD on T cell development in allogeneic chimeras.* Irradiated euthymic or thymectomized A.BY (Thy1.2, Ly5.2) recipients received a graft containing B6.PL (Thy1.1, Ly5.2) bone marrow cells (as a source of hematopoietic progenitors) with or without mature T cells harvested from the LNs of B6.SJL donors (Thy1.2, Ly5.1). The origin of recipient T cells was determined according to their Thy1/Ly5 phenotype. Recipients were studied on day 100 post-transplant, i. e., after the allogeneic acute phase of GVHD was terminated. We found that two factors affected the level of T cell reconstitution: the presence/absence of a host thymus, and the presence/absence of GVHD (Fig.1). Indeed, based on the number of both $CD4^+$ and $CD8^+$ T cells found in the spleen of day 100 chimeras, the following hierarchy was observed (Fig. 1): $thymus^+$ $GVHD^-$ (group A) > $thymus^-$ $GVHD^-$ (group B) > $thymus^+$ $GVHD^+$ (group C) > $thymus^-$ $GVHD^+$ (group D). These results confirm that, in thymectomized recipients of a T cell-depleted graft, some extrathymic differentiation of donor hematopoietic progenitors may take place but that this cannot compensate for the absence of the classical thymic differentiation pathway (group B vs A) (22,23,28,29,53). More importantly, they show that, in terms of T cell reconstitution of secondary lymphoid organs, the impact of GVHD (groups C and D) is even more deleterious than that caused by the mere absence of thymus (group B).

In $GVHD^+$ euthymic recipients grafted with low or high numbers of donor T cells (group C), the number of spleen T cells was decreased 6 to 12-fold relative to

recipients without GVHD (group A). T cell hypoplasia was slightly more severe when the number of grafted T cells was increased from 0.4 x $10^6$ to 1.6 x $10^6$. Interestingly, most T cells found in euthymic GVHD$^+$ recipients (group C) did not derive from expansion of grafted post-thymic T cells, but rather from the donor hematopoietic stem cells. We could ascertain that the vast majority of these T lymphocytes originated from donor hematopoietic progenitors that had differentiated in the GVHD$^+$ thymus and not in extrathymic sites, because their numbers were decreased 8-fold in athymic GVHD$^+$ recipients (group D vs. C).

In athymic hosts, the occurrence of GVHD (group D) caused an extremely severe T cell hypoplasia with T cell numbers decreased by 5-fold compared to athymic GVHD$^-$ recipients (group B). Among the few T cells that were found in athymic GVHD$^+$ mice, ~55% were derived from grafted mature T cells while ~45% had the phenotype of donor hematopoietic stem cells and where likely derived from the extrathymic differentiation these progenitors. T cells derived from grafted post-thymic T cells were much less abundant (~ 14-fold) in athymic GVHD$^+$ hosts (group D) than what we previously observed in similarly treated syngeneic (GVHD$^-$) recipients (23). Two main conclusions can be drawn from these results. First, GVHD not only impairs reconstitution via the thymic pathway, but also abrogates expansion of grafted post-thymic T cells. Second, although thymus function is impaired in GVHD$^+$ chimeras, intra-thymic maturation still represents the most effective pathway for T cell reconstitution in these mice.

*The TCR Vβ repertoire of GVHD$^+$ mice.* Results from Fig.1 indicate that in GVHD$^+$ mice, the ability of thymic-independent pathways to restore peripheral T cell pools is extremely poor and much inferior to that of the classical thymic pathway. Besides these quantitative considerations, we were interested in determining whether the different origin of T lymphocytes in athymic vs. euthymic GVHD$^+$ chimeras would have any impact on their Vβ profile. This question was addressed because a continuous thymic output can contribute to the maintenance of T cell diversity whereas the repertoire of T cell pools that rely solely on the expansion of post-thymic T cells is more prone to skewing following stochastic encounter with antigens (38,54). Thus, we evaluated by flow cytometry the TCR Vβ profile of T cells (Thy1.1$^+$) that differentiated in the thymus of non-thymectomized GVHD$^+$ recipients, of (Thy1.2$^+$) cells that originated from the expansion of grafted mature T cells in athymic GVHD$^+$ recipients, and of age-matched A.BY controls.

In both controls and euthymic GVHD$^+$ mice, the proportion of CD4$^+$ and CD8$^+$ cells bearing various Vβ elements was remarkably constant and, except for Vβ11$^+$ cells in controls mice, showed very little variation from mice to mice (Fig. 2). In contrast, considerable variability in the usage of Vβ chains was found in athymic GVHD$^+$ mice (Fig. 2). Thus, among CD8$^+$ lymphocytes, the percentage of cells expressing Vβ5 or Vβ6 elements was 14-16% and 7-7.5% respectively in euthymic GVHD$^+$ mice, but 4-20% and 2-14.5% respectively in athymic GVHD$^+$ mice. Analyses based on size heterogeneity or on sequence of the CDR3 region will be required to assess more precisely the diversity and clonality of these T cell

populations (55,56). Nevertheless, our results indicate that the TCR repertoire of T cells derived from the expansion of grafted mature T cells is subject to dramatic skewing in athymic GVHD$^+$ mice. The absence of Vβ skewing in euthymic GVHD$^+$ mice suggest that, even though their thymus is damaged, it has the ability to maintain a Vβ profile that is similar to that of age-matched controls.

*Survival of euthymic vs. thymectomized GVHD$^+$ recipients.* In order to address the biological significance of the residual thymus function detected in non-thymectomized GVHD$^+$ mice, we followed up to day 95 post-transplant the survival of mice from experimental groups presented in Fig. 1 (Fig. 3). No deaths were observed either in thymectomized or non-thymectomized GVHD$^-$ recipients. In contrast, the presence/absence of the thymus significantly influenced the survival of GVHD$^+$ mice. Indeed, during the 100 day observation period, death rates were greater in thymectomized than in non-thymectomized hosts: 25% vs. 0% and 65% vs. 10% for thymectomized vs. non-thymectomized recipients grafted with 0.4 x $10^6$ and 1.6 x $10^6$ donor T cells , respectively. Thus, at least in mice housed under specific pathogen-free conditions, the limited T cell reconstitution afforded by the GVHD$^+$ thymus has a major impact on post-transplant survival.

*Thymus output in GVHD$^+$ mice.* Having found that practically all T cells found in day 100 GVHD$^+$ mice derived from intra-thymic differentiation of donor hematopoietic stem cells, we wanted to quantitatively assess the thymic function of GVHD$^+$ mice. Relative to normal mice, thymus cellularity was decreased by 33% -

50% in $GVHD^+$ mice transplanted with low or high number of T cells, respectively (Fig. 4A). The proportion of immature double-positive vs. single-positive $CD4^+$ and $CD8^+$ thymocytes was similar in $GVHD^+$ vs. normal thymi (Fig. 4B). In order to get a better appraisal of thymus function, we measured the rate of production of recent thymic emigrants following *in vivo* BrdU labeling (27). Euthymic A.BY recipients of B6.PL bone marrow cells with or without $0.4 \times 10^6$ B6.SJL LN T cells were given BrdU-supplemented drinking water for 21 days beginning on day 70 post-transplant, and three-color flow cytometry analyses were performed selectively on $CD62L^+$ T cells. For both the $CD4^+$ and the $CD8^+$ subsets, recent thymic emigrants were defined as $CD62L^+BrdU^{lo}$ cells. The $CD62L^-$ T cell subset contains antigen-experienced activated/memory T cells while the $CD62L^+$ subset contains practically all naïve T cells and some "revertant" antigen-experienced T cells (27,57). The intensity of BrdU labeling provides a convenient way to distinguish recent thymic emigrants ($BrdU^{lo}$) from "older" peripheral T cells ($BrdU^{hi}$) that divide during the BrdU-labeling period. Low BrdU labeling of recent thymic emigrants results from cold target competition in the thymus (27). Indeed, high levels of apoptosis with local breakdown and release of DNA, as found in the thymus but not in secondary lymphoid organs, leads to cold target competition for BrdU incorporation by amino acids released from dying cells.

After administration of BrdU for 21 days, the total number of recent thymic emigrants ($CD4^+/CD62L^+/BrdU^{lo}$ and $CD8^+/CD62L^+/BrdU^{lo}$) per spleen was 4-fold lower in $GVHD^+$ hosts relative to normal mice and $GVHD^-$ recipients (Fig. 4C). Thus, thymus output in $GVHD^+$ mice was significantly decreased but not absent. Interestingly, for both $CD4^+$ and $CD8^+$ T cells, the percentage of $CD62L^+$ elements

that were $BrdU^{lo}$ was similar in mice with or without GVHD (Fig. 4D). This suggest that the reduced size of the $CD62L^+$ compartment, which encompasses naïve and revertant T cells, cannot be wholly ascribed to a decreased input of recent thymic emigrants. If this were the case, as in senescent individuals, one would expect not only the absolute number but also the proportion of $BrdU^{lo}$ elements among $CD62L^+$ cells to be decreased (58).

Results presented in Fig. 1 showed that the mere absence of thymus (group B) does not entail the severe level of lymphoid hypoplasia found in $GVHD^+$ mice (groups C and D). In addition, we found that the thymus of $GVHD^+$ mice was still functional with a thymocyte production equivalent to 25% that of 8 wk old controls or 18 wk old $GVHD^-$ recipients (Fig. 4 C and D). Such a level of thymus export has been shown to be sufficient to sustain the size of T cell compartments in secondary lymphoid organs of otherwise normal senescent mice (39,40). Thus, thymus hypoplasia cannot be held solely responsible for GVHD-associated T cell hypoplasia. These data point to the existence of other factors that perturb peripheral homeostasis of T cell compartments in $GVHD^+$ mice.

*Kinetics of BrdU labeling and disappearance of BrdU-labeled peripheral T cells in $GVHD^+$ mice.* In order to evaluate the rate of division of peripheral T cells, BrdU labeling was performed for 21 days as in the previous experiment. Results for $CD62L^+$ and $CD62L^-$ subsets were analyzed separately because it has been shown that $CD62L^-$ cells divide more rapidly than $CD62L^+$ cells (27), and the proportion of $CD62L^+$ vs. $CD62L^-$ T cells were different in our experimental groups (Fig. 5A).

Strikingly, the kinetics of BrdU labeling was similar in GVHD$^-$ recipients, GVHD$^+$ hosts and normal controls (Fig. 5B). This suggest that the reduced size of the CD62L$^+$ and CD62L$^-$ peripheral compartments is not due to a proliferative defect of resident T cells, otherwise the proportion of dividing (BrdU$^+$) T cells would have been decreased.

Since BrdU is not reused, it was possible to perform pulse-chase experiments. Thus, after being placed on BrdU water for 21 days, mice were transferred to normal water to examine the rate of decay of BrdU-labeled cells up to day 75. This approach has been used notably to show that the peripheral T cell lymphopenia of BB rats is caused by a shortened survival of T cells associated with an increased apoptotic death rate relative to WF rat controls (59). The intensity of BrdU labeling in T cells from GVHD$^+$ and control mice on day 21 and 75 is depicted in Fig. 6. The rate of disappearance of BrdU-labeled T cells was similar in the three experimental groups so that, by day 75, the remaining proportion of BrdU-labeled cells was approximately 20% in normal mice, GVHD$^-$ recipients and GVHD$^+$ hosts (Fig. 7). We deduced from the above BrdU-labeling experiments, that the failure of peripheral homeostatic mechanisms to compensate for the moderate decrease in thymic output (Fig. 4) and to prevent the severe T cell depletion in GVHD$^+$ mice (Fig. 1) could neither be ascribed to an impairment of T cell proliferative activity (Fig. 5), nor to a shortened half-life of BrdU-labeled cells (Fig. 7). These findings suggest that the deficient expansion of the post-thymic T cell compartment in GVHD$^+$ mice was not due to an intrinsic lymphocyte defect but to an extrinsic microenvironment abnormality. This led us to raise the intriguing possibility that the number of peripheral T cell niches might be

decreased in GVHD$^+$ mice. This premise, according to which T cell hypoplasia would represent a problem of soil (environment) rather than seed (lymphocytes), entails two crucial corollaries: i) T cells from GVHD$^+$ mice should expand normally in normal mice, ii) lymphoid hypoplasia of GVHD$^+$ mice should not be corrected by infusion of (host-tolerant) T cells from normal mice.

*Normal expansion of T cells from GVHD$^+$ mice following adoptive transfer into normal hosts*. In order to determine whether T cells from GVHD$^+$ mice would expand in normal mice, we compared the expansion of T cells from GVHD$^+$ mice with that of normal T cells following injection in normal thymectomized/irradiated recipients (with no defect in peripheral niches). Thus, thymectomized/irradiated A.BY or B6.SJL recipients were transplanted with 2 x $10^6$ Thy1.1$^+$ Ly5.2$^+$ T cells harvested from the spleen of non thymectomized GVHD$^+$ mice (cf. Fig. 1C) and $10^7$ T cell-depleted C57BL/6 bone marrow cells. The expansion of T cells from GVHD$^+$ mice was compared to that of normal T cells 30 days following injection (together with T cell-depleted bone marrow cells) into thymectomized/irradiated syngeneic recipients (Fig. 8). In all recipients, the origin of T lymphocytes could be determined according to their Thy 1/Ly 5 phenotype. Strikingly, the level of T cell reconstitution found in syngeneic (B6.SJL) or allogeneic (A.BY) secondary recipients of GVHD$^+$ T cells was similar to that of hosts repopulated with normal T cells (Fig. 8). The numbers of T cells harvested from secondary recipients were increased 2-fold relative to those in day 100 GVHD$^+$ mice. This difference is impressive when one considers that i) the T cell reconstitution of normal secondary hosts was achieved after only 30 days (vs 100 days in GVHD$^+$ mice), and ii) GVHD$^+$ mice had a measurable thymic

output, whereas reconstitution of thymectomized secondary hosts was achieved strictly by expansion of transplanted post-thymic T cells. Hence, T cells from GVHD$^+$ mice expanded normally when transferred to normal hosts. As suggested by the studies presented in Fig. 5, this shows that the failure of T cells to expand in GVHD$^+$ mice is not due to an intrinsic T cell proliferative defect.

Interestingly, the fact that normal reconstitution was achieved not only in syngeneic (B6.SJL) but also in allogeneic (A.BY) secondary hosts indicates that T cells found in the spleen of day 70 GVHD$^+$ mice could not elicit GVHD, and thus were purged of functional host-reactive T cells. As these T cells had differentiated in the thymus of the GVHD$^+$ mice, this observation supports the concept that, at least after the acute phase of GVHD, the thymus of GVHD$^+$ mice efficiently performs negative selection of host-reactive thymocytes (60). Furthermore, the lack of anti-host alloreactive T cells in day 70 GVHD$^+$ mice, argues against the possibility that a persistent GVHD activity per se could be responsible, notably via production of some cytokine(s), for the persistent T cell hypoplasia found in long-term (day 100) chimeras.

*Failure of normal post-thymic T cells to restore the size of the peripheral pool in GVHD$^+$ mice.* We next evaluated whether adoptive transfer of normal post-thymic T cells would correct the T cell lymphopenia of GVHD$^+$ mice. The fate of normal T cells after passive transfer to histocompatible recipients depends on the quantity of available T cell niches. When the size of the peripheral T cell compartment is normal, such that few T cell niches are available, most donor T cells disappear soon after

transfer (61). In contrast, transfer of T cells to "T-less" recipients, in which numerous empty niches are available, is followed by the persistence of a large proportion of donor-derived T cells and a considerable, antigen-driven expansion of these cells, which results in the restoration of the size of the peripheral compartment (26,62).

In order to obtain T cells of B6 origin that were tolerant to A.BY antigens, normal irradiated A.BY mice were transplanted with $10^7$ T cell-depleted C57BL/6 bone marrow cells. Sixty days later, a spleen cell suspension from these chimeras containing 5 x $10^6$ Thy1.2$^+$Ly5.2$^+$ T cells (of C57BL/6 origin) was injected into day 60 GVHD$^+$ secondary hosts, and the fate of Thy1.2$^+$Ly5.2$^+$ T cells was assessed 40 days after transfer. Prior to transfer, no Thy1.2$^+$Ly5.2$^+$ cells were present in GVHD$^+$ secondary hosts which had been constructed by injection of $10^7$ B6.PL bone marrow cells + 0.4 x $10^6$ B6.SJL LN T cells into irradiated non-thymectomized A.BY hosts. As a positive control, the proliferative potential of post-thymic T cells transferred into secondary hosts was evaluated in a group of irradiated/thymectomized GVHD$^-$ recipients. When transferred into GVHD$^-$ secondary hosts, post-thymic T cells showed a major expansion (Fig. 9C), whose magnitude was similar to what has been reported following transfer into irradiated/thymectomized syngeneic recipients (23). In contrast, transfer of host-tolerant C57BL/6 T cells did not increase the size of the peripheral T cell pool of GVHD$^+$ recipients (Fig. 9B). Indeed, 40 days after their transfer into GVHD$^+$ secondary hosts, only trace amounts of Thy1.2$^+$Ly5.2$^+$ T cells were recovered. Thus, supply of post-thymic T cells that had differentiated in a normal GVHD$^-$ thymus and possessed a normal proliferation potential had no influence on the size of the splenic T cell pool in GVHD$^+$ mice. Together with those

presented in Fig. 8, these results demonstrate that the perturbed homeostasis of the peripheral T cell pool in $GVHD^+$ mice is not due to a lymphocyte intrinsic anomaly, but rather to a failure of the peripheral environment to support the seeding and/or expansion of post-thymic T cells.

**Concluding Remarks**

The results presented herein show that both central and peripheral mechanisms contribute to the long lasting T cell hypoplasia found in $GVHD^+$ mice. $GVHD^+$ mice have a decreased thymic output, but this defect cannot be held solely responsible for peripheral T cell hypoplasia. The lack of reconstitution of normal peripheral T cell compartments is largely accounted for by an inability of post-thymic T cells to expand in the secondary lymphoid organs of $GVHD^+$ hosts. Collectively, our results provide convincing evidence that the failure of mature T cells to expand in $GVHD^+$ mice is not due to an intrinsic lymphocyte defect but to an undefined abnormality of the lymphoid microenvironment: i) post-thymic T cells from $GVHD^+$ mice expanded normally in syngeneic or allogeneic secondary hosts, and ii) normal T cells failed to expand in $GVHD^+$ mice.

Until better defined, we think that this microenvironment defect is most consistent with a decrease in the number of functional peripheral T cell niches. As mentioned in the introduction, resident dendritic cells may represent the most crucial elements of these niches. Thus, the possible influence of quantitative and/or qualitative (e. g., expression of MHC molecules, chemokines, cytokines) dendritic cell defects on T cell homeostasis in GVHD deserves further investigation. To our knowledge, these questions have not been addressed in mouse models of GVHD. However, according to the limited information available from human studies, it is noteworthy that chronic GVHD appears to be associated with a major decrease in the numbers of dendritic cells in the skin and secondary lymphoid organs (63-65). Alternatively, we cannot discard the possibility that the microenvironment defect

epitomized herein as a reduction of functional T cell niches could be related to the presence of a toxic and/or absence of supportive soluble factor produced by cells other than dendritic cells. Nevertheless, two elements argue against some involvement of an inhibitory T cell-derived cytokine: no GVHD-inducing T cells were detected in our day 100 $GVHD^+$ mice, and the defect was not transferred to secondary recipients of T cells from $GVHD^+$ mice (Fig. 8).

These considerations emphasize the need for a precise definition of the peripheral T cell niches, whose number likely dictates the size of peripheral T cell pools. Furthermore, it will be important to determine whether a similar microenvironment defect could represent an unrecognized cause of acquired immunodeficiency in other settings. For example, damage to peripheral T cell niches could provide a plausible explanation for the prolonged hypoplasia of secondary lymphoid organs observed after massive T cell responses (66,67). Importantly, a loss of peripheral T cell niches must not be considered irreversible a priori, and may possibly be amenable to therapy. Indeed, recent studies in RIP-LT transgenic mice have demonstrated that engineered local release of lymphotoxin can trigger lymphoid neogenesis characterized by the formation of well organized and functional "tertiary" lymphoid tissue (68,69).

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## Figure Legends

**Figure 1.** The influence of GVHD on T cell development in allogeneic chimeras. Irradiated euthymic (panels A and C) or thymectomized (panels B and D) A.BY (Thy1.2, Ly5.2) recipients received a graft containing $10^7$ T cell-depleted B6.PL (Thy1.1, Ly5.2) bone marrow cells (as a source of hematopoietic progenitors) with or without mature T cells ($0.4 \times 10^6$ or $1.6 \times 10^6$) harvested from the LNs of B6.SJL donors (Thy1.2, Ly5.1). Chimeras' spleen cells were analyzed by three-color staining on day 100 ± 5 post-transplant. The origin of recipient T cells was determined according to their Thy1/Ly5 phenotype. Results are presented as the mean ± SD (3-4 mice per group). Note that the ordinate scale is different in panels A and B vs. panels C and D. nd = not determined.

**Figure 2.** Skewing of Vβ usage by $CD4^+$ and $CD8^+$ T cells in athymic $GVHD^+$ chimeras. Three-color staining was performed using Abs against CD4 or CD8, Thy1.1 or Thy1.2, and specific Vβ elements. Chimeras were studied on day 100 ± 5. Results are presented as the mean ± SD (3-4 mice per group).

**Figure 3.** The influence of GVHD on survival of euthymic and thymectomized recipients. Irradiated thymectomized or sham thymectomized A.BY recipients received a graft (C57BL/6) containing $10^7$ bone marrow cells with either none, $0.4 \times 10^6$, or $1.6 \times 10^6$ LN T cells (10 to 12 mice per group). Survival was assessed daily up to day 95. In $GVHD^+$ mice transplanted with $0.4$ or $1.6 \times 10^6$ donor T cells, the

survival of thymectomized recipients was inferior to that of non-thymectomized recipients ($p < 0.05$, Student's *t* test).

**Figure 4**. The effect of GVHD on thymic cellularity and thymic output. **A)** Thymic cellularity and **B)** proportion of double-positive and single-positive thymocytes in euthymic A.BY recipients of a B6.PL bone marrow graft supplemented with none, 0.4 x $10^6$, or 1.6 x $10^6$ B6.SJL LN T cells. Three mice per group. **C)** Accumulation of recent thymic emigrants ($CD62L^+/BrdU^{lo}$ cells) in the spleen of normal A.BY mice, $GVHD^-$ recipients and $GVHD^+$ hosts. All mice received BrdU-supplemented drinking water for 21 days, beginning on day 70 post-transplant in the case of $GVHD^-$ and $GVHD^+$ mice. $GVHD^-$ and $GVHD^+$ mice were euthymic A.BY recipients transplanted with $10^7$ B6.PL bone marrow cells supplemented with none ($GVHD^-$) or 0.4 x $10^6$ ($GVHD^+$) B6.SJL LN T cells. The data show the values obtained for two mice per time point. **D)** Percentage of $CD62L^+$ elements that were $BrdU^{lo}$ in the three groups presented in panel C.

**Figure 5**. Peripheral T cells from $GVHD^+$ mice have a normal rate of cell division (BrdU incorporation). **A)** Proportion of $CD62L^+$ and $CD62L^-$ cells in the spleen of normal A.BY mice, $GVHD^-$ recipients, and $GVHD^+$ hosts. **B)** Kinetics of BrdU labeling of T cells in the three experimental groups. Mice received BrdU-supplemented drinking water for 21 days as in Fig. 4. Results are presented as the percentage of BrdU-labeled ($BrdU^{lo}$ and $BrdU^{hi}$) elements in four cell subsets: $CD4/CD62L^+$, $CD4/CD62L^-$, $CD8/CD62L^+$, and $CD8/CD62L^-$. * BrdU labeling of $CD8/CD62L^-$ elements could not be studied in the A.BY control group because this

cell subset is practically undetectable in 8 wk old A.BY mice (panel A). Two to three mice per point. The intensity of BrdU-labeling of the various T cell subsets on day 21 is presented in Fig. 6.

**Figure 6**. Intensity of BrdU labeling of T cells from mice placed on BrdU water for 21 days and then on normal water for a further 54 days. The intensity of BrdU labeling (negative, low, high) is presented as a function of CD62L expression (negative or positive) for both the $CD4^+$ and $CD8^+$ T cell subsets. Each result is representative of three experiments.

**Figure 7**. Kinetics of disappearance of BrdU-labeled T cells in $GVHD^-$ and $GVHD^+$ mice. Decline in percent BrdU labeling of T cells after transferring mice to normal water for 54 days. The intensity of BrdU labeling on day 75 is presented in Fig. 6. Two to three mice per point. * The fate of $CD8/CD62L^-$ elements could not be studied in the A.BY control group because this cell subset is practically undetectable in 8 wk old A.BY mice (cf. Fig. 5A).

**Figure 8**. Expansion of T cells from $GVHD^+$ mice following adoptive transfer into normal hosts. Three-color staining was performed on spleen cell suspensions using Abs specific for CD4 or CD8, Ly5.1 or Ly5.2, and Thy1.1 or Thy1.2. **A**) Numbers of $CD4^+$ and $CD8^+$ T cells of B6.PL origin (Thy1.1, Ly5.2) found in the spleen of day 100 non-thymectomized $GVHD^+$ recipients (negative controls), **B**) Numbers of $CD4^+$ and $CD8^+$ T cells of B6.PL origin (Thy1.1, Ly5.2) found in the spleen of irradiated/thymectomized B6.SJL or A.BY secondary hosts, on day 30 after injection

of $10^7$ T-cell-depleted C57BL/6 bone marrow cells + 2 x $10^6$ T cells harvested from the spleen of day 70 GVHD$^+$ mice (test group), **C**) Numbers of Thy1.2$^+$Ly5.2$^+$ T cells found in the spleen of irradiated/thymectomized B6.SJL or A.BY hosts, on day 30 after injection of $10^7$ T-cell-depleted B6.PL bone marrow cells + 2 x $10^6$ T cells harvested from the spleen of normal syngeneic donors (A.BY for A.BY secondary recipients, C57BL/6 for B6.SJL secondary recipients) (positive controls). GVHD$^+$ mice were prepared as in Fig. 1C (irradiation followed by transplantation of $10^7$ B6.PL bone marrow cells + 0.4 x $10^6$ B6.SJL LN T cells). Results are presented as the mean ± SD (3-4 mice per group). * $p < 0.05$, ** $p < 0.005$ relative with numbers of CD4$^+$ or CD8$^+$ T cells in GVHD$^+$ mice (Student's *t* test). No significant difference was found between T cell numbers presented in panels B and C.

**Figure 9**. Adoptive transfer of normal host (A.BY)-tolerant T cells into non-thymectomized GVHD$^+$ mice and thymectomized GVHD$^-$ recipients. **A)** Number of T cells in the spleen of day 100 GVHD$^+$ mice. GVHD$^+$ mice were prepared as in Fig. 1C (irradiation followed by transplantation of $10^7$ B6.PL bone marrow cells + 0.4 x $10^6$ B6.SJL T cells). **B)** Number of T cells in the spleen of day 100 GVHD$^+$ mice previously injected, on day 60, with 5 x $10^6$ host-tolerant T cells of C57BL/6 (Thy1.2,Ly5.2) origin. **C)** Number of T cells in the spleen of thymectomized A.BY mice 40 days after irradiation and injection of $10^7$ T cell-depleted B6.SJL bone marrow cells + 5 x $10^6$ host-tolerant B6.PL T cells. Host (A.BY)-tolerant T cells used in panel B and C experiments were obtained from the spleen of non-thymectomized A.BY hosts, 60 days after irradiation and injection of T cell-depleted C57BL/6 or

B6.PL bone marrow cells. Results are presented as the mean ± SD (3-4 mice per group).

**Figure 2-1**



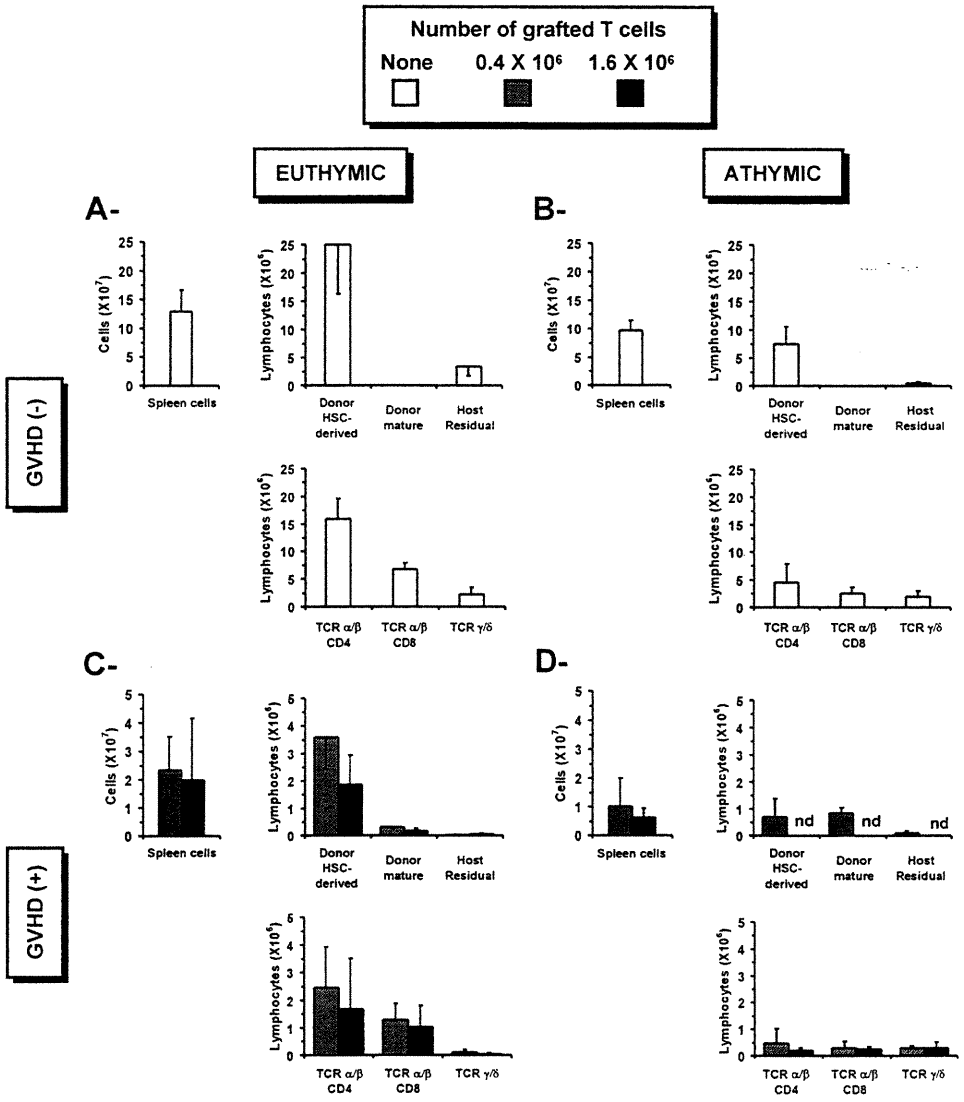

Number of grafted T cells
None
0.4 X 10⁶
1.6 X 10⁶
EUTHYMIC
ATHYMIC
A-
B-
C-
D-
GVHD (-)
GVHD (+)
Cells (X10⁷)
Lymphocytes (X10⁶)
Spleen cells
Donor HSC-derived
Donor mature
Host Residual
TCR α/β CD4
TCR α/β CD8
TCR γ/δ
nd

**Figure 2-2**

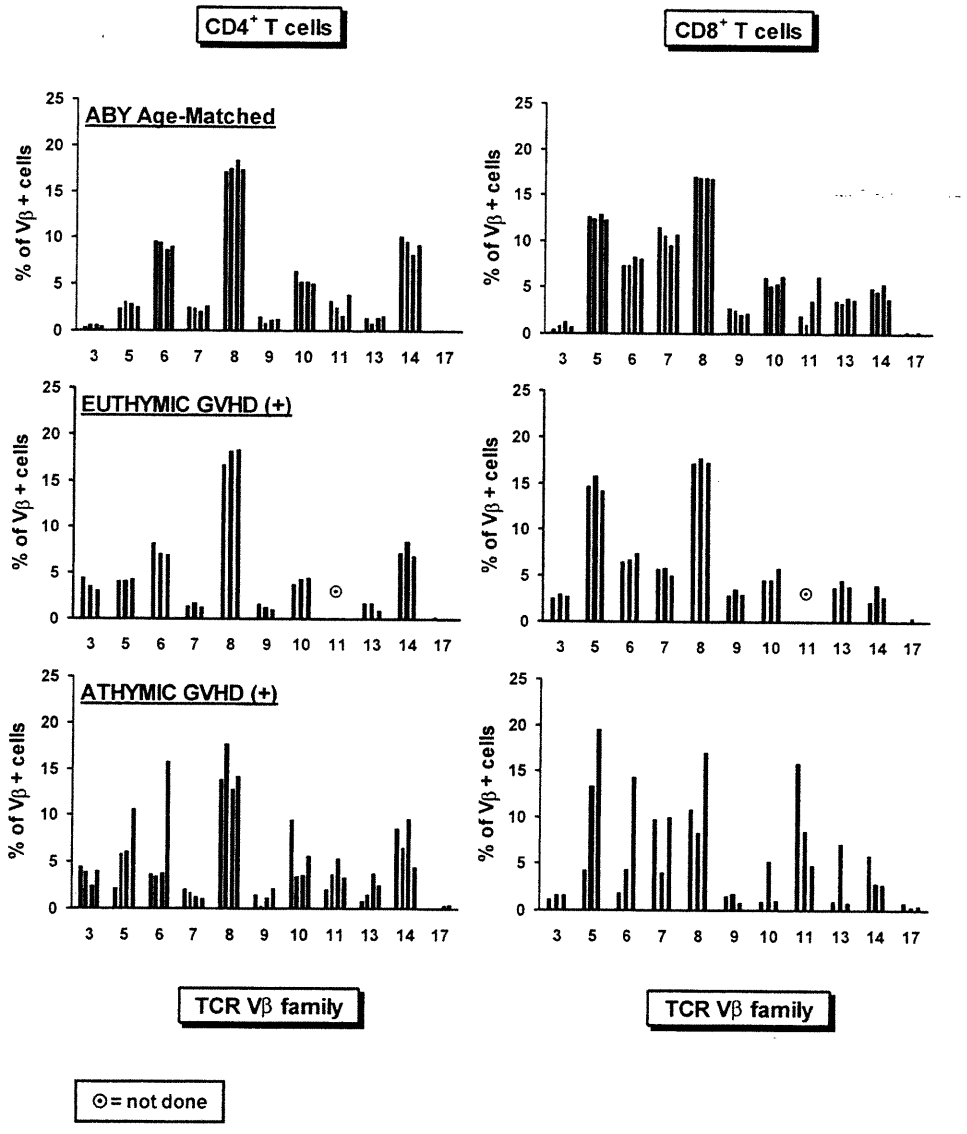

CD4+ T cells
CD8+ T cells
ABY Age-Matched
EUTHYMIC GVHD (+)
ATHYMIC GVHD (+)
% of Vβ + cells
0
5
10
15
20
25
3 5 6 7 8 9 10 11 13 14 17
TCR Vβ family
⊙= not done

**Figure 2-3**

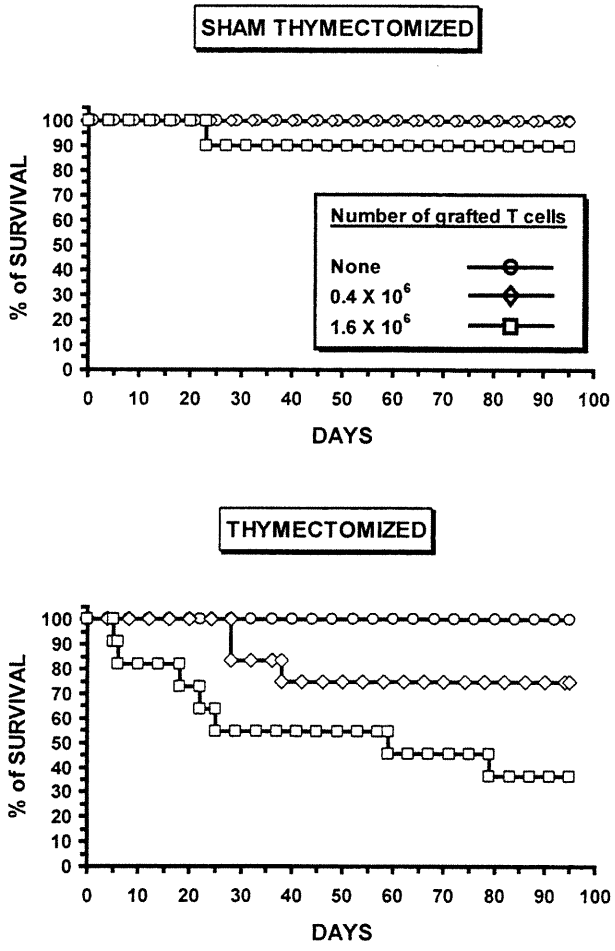

SHAM THYMECTOMIZED
% of SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
DAYS
Number of grafted T cells
None
0.4 X 10^6
1.6 X 10^6
THYMECTOMIZED
% of SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
DAYS

**Figure 2-4**

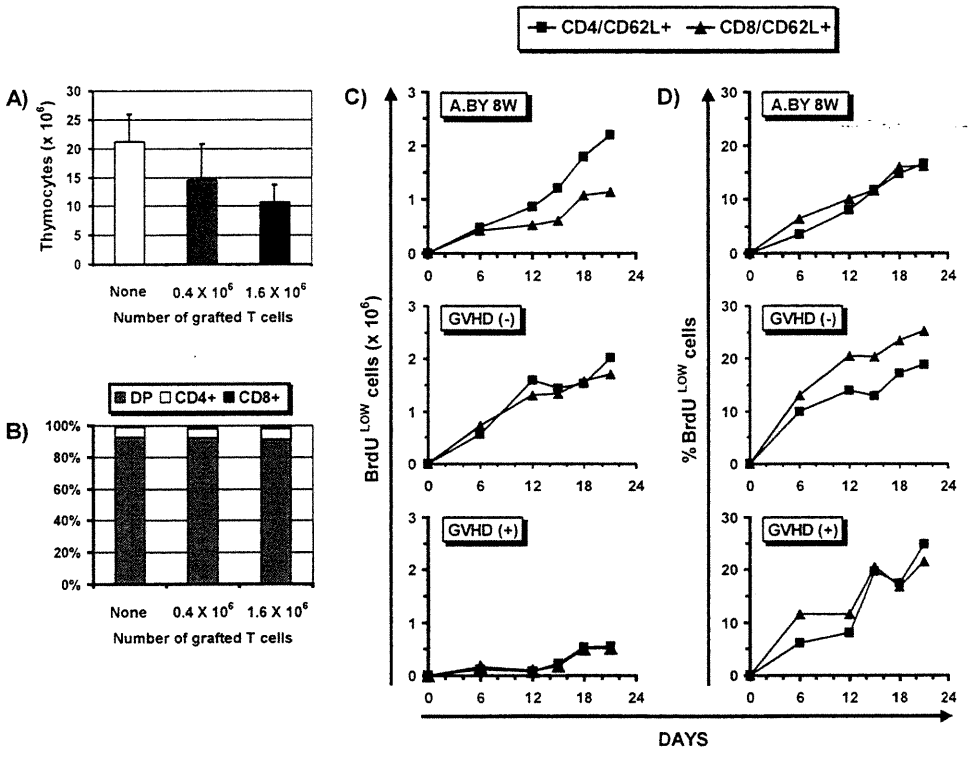

A)
Thymocytes (x 10⁶)
None
0.4 X 10⁶
1.6 X 10⁶
Number of grafted T cells
B)
DP CD4+ CD8+
100%
80%
60%
40%
20%
0%
None
0.4 X 10⁶
1.6 X 10⁶
Number of grafted T cells
CD4/CD62L+
CD8/CD62L+
C)
A.BY 8W
GVHD (-)
GVHD (+)
BrdU LOW cells (x 10⁶)
D)
A.BY 8W
GVHD (-)
GVHD (+)
% BrdU LOW cells
DAYS

**Figure 2-5**

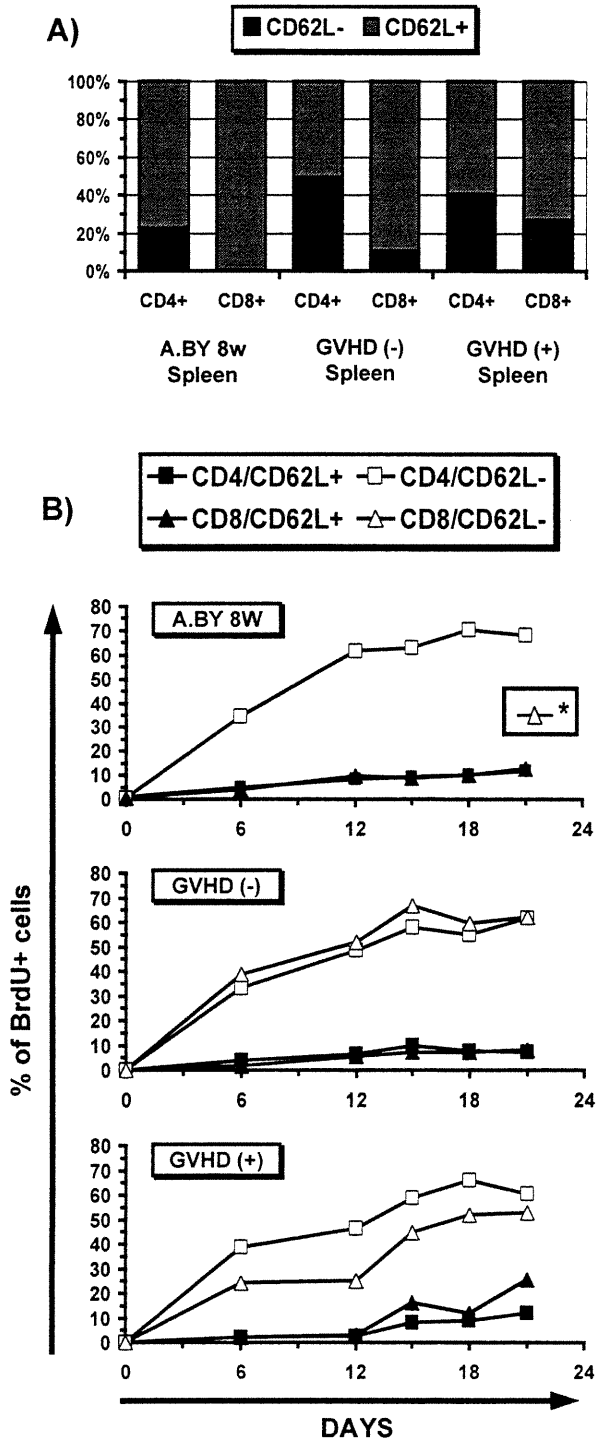

A)
CD62L-
CD62L+
100%
80%
60%
40%
20%
0%
CD4+
CD8+
CD4+
CD8+
CD4+
CD8+
A.BY 8w Spleen
GVHD (-) Spleen
GVHD (+) Spleen
B)
CD4/CD62L+
CD4/CD62L-
CD8/CD62L+
CD8/CD62L-
A.BY 8W
*
GVHD (-)
GVHD (+)
% of BrdU+ cells
0
10
20
30
40
50
60
70
80
0
6
12
18
24
DAYS

**Figure 2-6**

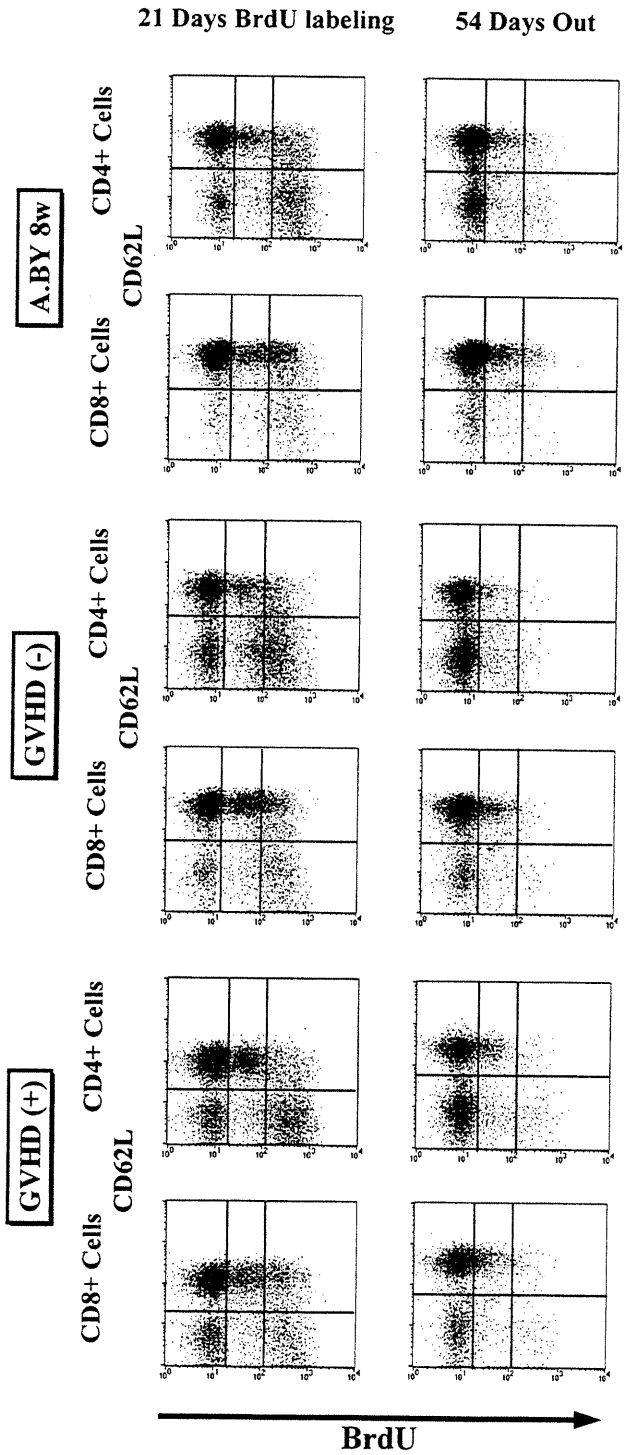

21 Days BrdU labeling
54 Days Out
A.BY 8w
GVHD (-)
GVHD (+)
CD4+ Cells
CD62L
CD8+ Cells
CD62L
BrdU

**Figure 2-7**

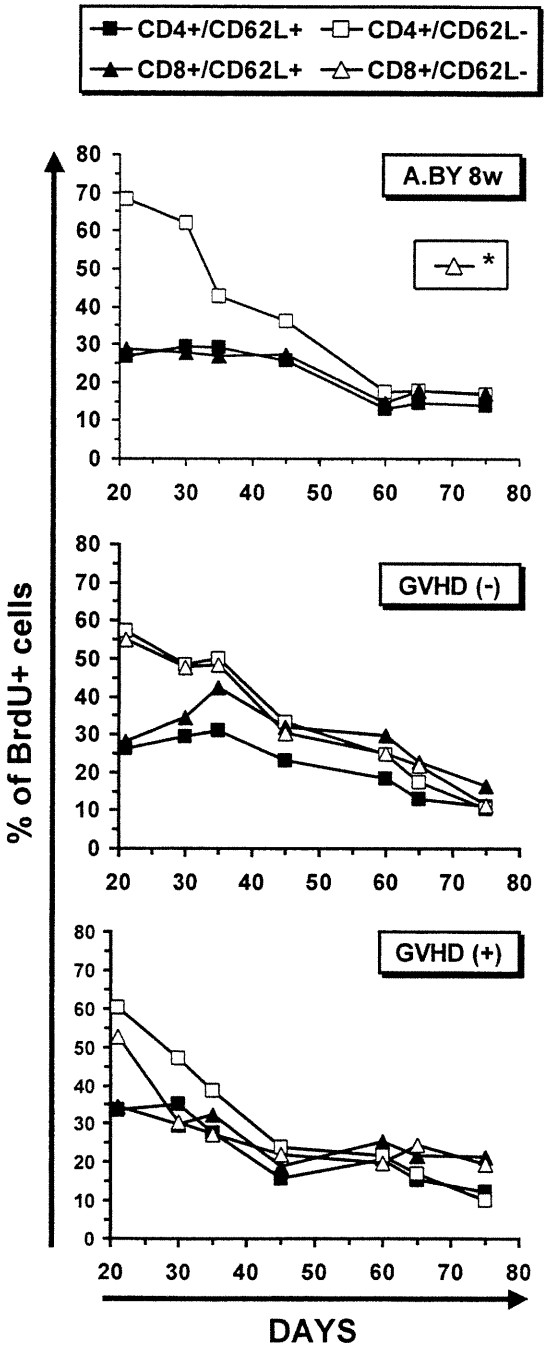

CD4+/CD62L+
CD4+/CD62L-
CD8+/CD62L+
CD8+/CD62L-
A.BY 8w
*
GVHD (-)
GVHD (+)
% of BrdU+ cells
DAYS
0
10
20
30
40
50
60
70
80

Figure 2-8

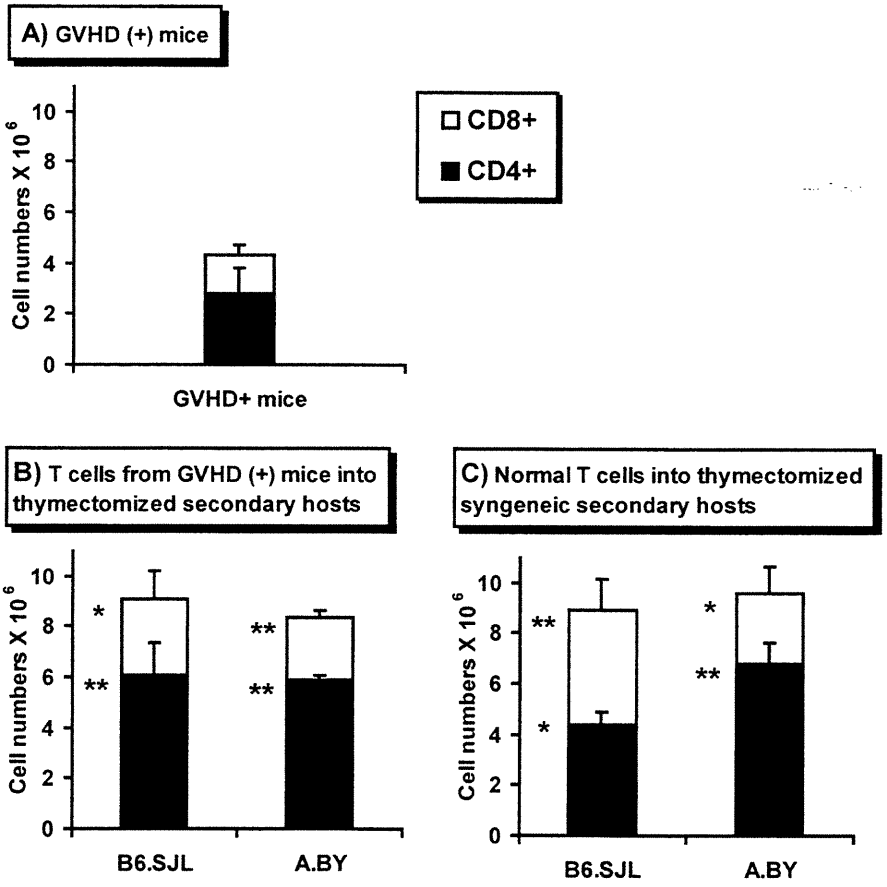
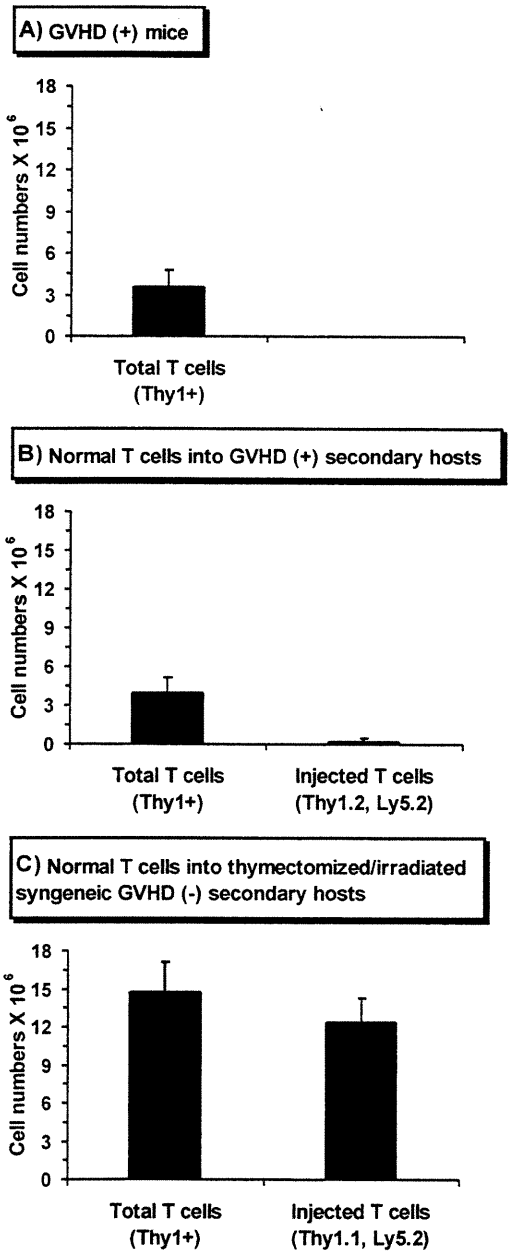

A) GVHD (+) mice
CD8+
CD4+
Cell numbers X 10 6
GVHD+ mice
B) T cells from GVHD (+) mice into thymectomized secondary hosts
C) Normal T cells into thymectomized syngeneic secondary hosts
B6.SJL
A.BY

**Figure 2-9**


A) GVHD (+) mice
Cell numbers X 10^6
18
15
12
9
6
3
0
Total T cells
(Thy1+)
B) Normal T cells into GVHD (+) secondary hosts
Cell numbers X 10^6
18
15
12
9
6
3
0
Total T cells
(Thy1+)
Injected T cells
(Thy1.2, Ly5.2)
C) Normal T cells into thymectomized/irradiated syngeneic GVHD (-) secondary hosts
Cell numbers X 10^6
18
15
12
9
6
3
0
Total T cells
(Thy1+)
Injected T cells
(Thy1.1, Ly5.2)

## Article 3

# Regulation of extrathymic T cell development and turnover by oncostatin M

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## Abstract

Chronic exposure to oncostatin M (OM) has been shown to stimulate extrathymic T cell development. The present work shows that in OM-transgenic mice, i) massive extrathymic T cell development takes place exclusively the lymph nodes (LNs) and not in the bone marrow, liver, intestines, and spleen, and ii) LNs are the sole site where the size of the mature $CD4^+$ and $CD8^+$ T cell pool is increased (6 to 7-fold). Moreover, when injected into OM-transgenic mice, both transgenic and non-transgenic $CD4^+$ and $CD8^+$ T cells preferentially migrated to the LNs rather than the spleen. Studies of athymic recipients of fetal liver grafts showed that lymphopoietic pathway modulated by OM was truly thymus-independent, and that non-transgenic progenitors could generate extrathymic $CD4^+CD8^+$ cells as well as mature T cells under the paracrine influence of OM. The progeny of the thymic-independent differentiation pathway regulated by OM was polyclonal in terms of Vβ usage, exhibited a phenotype associated with previous TCR ligation, and displayed a rapid turnover rate (5-bromo-2'-deoxyuridine pulse-chase assays). This work suggests that chronic exposure to OM i) discloses a unique ability of LNs to sustain extrathymic T cell development, and ii) increases the number and/or function of LN niches able to support seeding of recirculating mature T cells. Regulation of the lymphopoietic pathway discovered in OM-transgenic mice could be of therapeutic interest for individuals with thymic hypoplasia or deficient peripheral T cell niches.

Changes in T lymphocyte function underlie much of the age-related decline in protective immune responses (1). Indeed, senescence-associated thymic atrophy leads to the progressive replacement of virgin T cells by memory cells that display decreased proliferative potential and a restricted repertoire diversity (2-5). Numerous observations suggest that immune competence has a major influence on life span, and that disturbed T cell responses are implicated in the age-related increase in the incidence of infections, cancer, and autoimmune diseases (6-11). The mechanisms responsible for thymic involution are unknown (12). Its occurrence may reflect the fact that, from an evolutionary perspective, thymopoiesis can be considered as an energy-expensive process, and that there is no selective pressure for maintaining the same level of T cell repertoire diversity in aged as in young individuals (12). Importantly, thymic output and the size of peripheral T cell pools are independently regulated. Thus, increase in thymic export (by thymic grafts) does not bring about a commensurate enlargement of peripheral T cell compartments (5,13). The size of peripheral T cell compartments is rather determined by the number of available T cell niches. The term niche designates an environment that provides local conditions (such as expression of specific chemokines, cytokines, and MHC molecules) required for T cells to seed and survive long-term in the peripheral compartment (14,15). Furthermore, thymic output does not increase in the presence of peripheral T cell depletion (16). Hence, the consequences of the progressive age-associated decline in thymic function are magnified in individuals whose peripheral lymphoid compartments have been rendered hypoplastic by various factors such as chemotherapy and human immunodeficiency virus-1 infection (17-21).

In athymic subjects, continuous production of new T cells is afforded by proliferation of post-thymic T cells and by extrathymic T cell development (22-24). In various mouse models, extrathymic differentiation of hematopoietic stem cells has been detected in selected organs such as the bone marrow (25,26), intestinal cryptopatches (27), and the liver (28,29). However, under normal circumstances, the ability of these organs to replenish and maintain LN and spleen T cell compartments is inferior to that of the thymus. Nevertheless, it was recently shown that expression of an oncostatin M $(OM)^3$ transgene, under the control of the proximal Lck promoter or the CD34 gene promoter, causes thymus atrophy and thymus-independent accumulation of immature and mature T cells in LNs. (30-32). OM is a member of the IL-6 family of cytokines that acts as a growth regulator for many types of mammalian cells (33). In normal mouse, this pleiotropic cytokine is produced late in the activation cycle of T cells and macrophages, and its best known activities *in vivo* are antiinflammatory (34,35). Breeding experiments with IL-$6^{-/-}$ and IL-$7r^{-/-}$ deficient mice showed that induction of extrathymic development by the OM transgene occurs in the absence of IL-6, but is strictly dependent on IL-7 receptor signaling (32). Intraperitoneal administration of recombinant human OM produced the same effect in non-transgenic mice (31).

The striking occurrence of extrathymic T cell development in LckOM transgenic mice provides unforeseen evidence for the existence of a lymphopoietic pathway whose regulation could possibly be of therapeutic interest for individuals with senescence- or disease-associated thymic hypoplasia. Thus, the goal of this study was to evaluate the development and turnover of extrathymic T cell produced under the influence of OM. We

found that chronic production of OM endowed LNs with the unique ability to sustain T cell development and to attract mature T cells. These extrathymically produced T cells had a diversified TCR Vβ repertoire, showed a rapid turnover rate, and expressed differentiation markers associated with previous TCR ligation.

**Materials and methods**

*Mice.* C57BL/6J (B6; Thy-1.2$^{+}$) and B6.PL-*Thy-1$^{a}$*/Cy (B6.PL; Thy1.1$^{+}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). LckOM transgenic mice were initially kindly provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA). In LckOM mice, the p56 $^{lck}$ proximal promotor targets expression of the bovine OM gene to thymocytes (30,31). Fertilized oocytes from (C3H x B6) F1 mice were used for pronuclear injections, and transgenic mice were back-crossed with nontransgenic B6 mice. The mice that we obtained from Bristol-Myers Squibb Pharmaceutical Research Institute, and that were used in this work, had been bred in this manner for greater than 13 generations. LckOM mice used in our experiments were heterozygous. As LckOM females develop ovarian failure at about 10 weeks of age, heterozygous transgenic mice were obtained by breeding heterozygous LckOM males with B6 females. The LckOM genotype was confirmed by PCR assay using 200 ng of genomic tail DNA and the following primers: 5'→3' AGTCCCGTACTGCAGGAACA and GCTCACACCATTAAAGTGC. Mice were bred and housed under specific pathogen-free conditions (in sterile ventilated racks in the case of LckOM mice) at the Guy-Bernier Research Center according to the standards of the Canadian Committee for Animal Protection.

*Thymectomy.* At 4-5 weeks of age, mice were anesthetized by intraperitoneal injection of 75mg/Kg sodium pentobarbital (Somnotol; MTC Pharmaceuticals, Cambrige, Ontario, Canada), and the thymus was removed with a suction cannula introduced over the suprastrenal notch. Completeness of thymectomy was verified in

each animal by visual inspection at the time of sacrifice. Cell transplantation was performed at least two weeks after surgery.

*Bone marrow and fetal liver cell transplantation.* Bone marrow collected from the femurs and tibias of LckOM donors was T cell-depleted with a specific anti-Thy-1.2 monoclonal Ab (Cedarlane; Hornby, Ontario, Canada) and rabbit serum (Low-Tox-M rabbit complement; Cedarlane) as a source of complement. Efficacy of depletion was assessed by flow cytometry. Timed pregnancies were established for B6.PL mice and fetal liver cells were collected on day 13 post-coitum. Hematopoietic chimeras were created by injecting $4x10^6$ LckOM bone marrow cells + $4x10^6$ B6.PL fetal liver cells into irradiated (10 Gy) B6 recipients. 5-bromo-2'-deoxyuridine (BrdU) labeling experiments were initiated in hematopoietic chimeras 75-90 days after transplantation.

*Isolation of hepatic and intestinal lymphocytes.* Isolation of hepatic and intestinal intraepithelial lymphocytes was performed using density centrifugation as previously described (29,36).

*mAbs.* The following Abs were obtained from PharMingen (Mississauga, Ontario, Canada): Cy-chrome™ conjugated anti-CD4 (RM4-5; rat $IgG_{2a},\kappa$), and anti-CD8α (53-6.7; rat $IgG_{2a},\kappa$), biotinylated-anti-CD8α (53-6.7; rat $IgG_{2a},\kappa$) detected with Cy-chrome™-streptavidin or APC-streptavidin, biotinylated-anti-Thy1.1 (OX-7; mouse $IgG_1,\kappa$), biotinylated-anti-Vβ3 TCR (KJ25; hamster IgG) detected with FITC-streptavidin, FITC-conjugated anti-Thy1.2 (53-2.1; rat $IgG_{2a},\kappa$) anti-Vβ5.1,2 TCR (MR9-4 ; mouse $IgG_1,\kappa$), anti-Vβ6 TCR (RR4-7; rat $IgG_{2b},\lambda$), anti-Vβ7 TCR (TR310; rat $IgG_{2b},\kappa$), anti-Vβ8.1,2 TCR (MR5-2; mouse $IgG_{2a},\kappa$), anti-Vβ9 TCR (MR10-2; mouse $IgG_1,\kappa$), anti-$V\beta10^b$ TCR (B21.5; rat $IgG_{2a},\lambda$), anti-Vβ11 TCR (RR3-15; rat $IgG_{2b},\kappa$),

anti-Vβ13 TCR (MR12-3; mouse $IgG_1,\kappa$), anti-Vβ14 TCR (14-2; rat IgM,κ), anti-Vβ17$^a$ TCR (KJ23; mouse $IgG_{2a},\kappa$), PE-conjugated-anti-Thy1.1 (OX-7; mouse $IgG_{2a},\kappa$), -Thy1.2 (30-H12; rat $IgG_{2b},\kappa$), -CD19 (ID3; rat $IgG_{2a},\kappa$), -CD44 (IM7; rat $IgG_{2b},\kappa$), -CD45RB (23G2; rat $IgG_{2a},\kappa$), -CD62L (MEL-14; rat $IgG_{2a},\kappa$), -CD122 (IL-2 Receptor β chain) (TM-β1; rat $IgG_{2b},\kappa$) and -NK1.1 (PK136; mouse $IgG_{2a},\kappa$) Abs and their isotypic controls. PE-conjugated-anti-CD8α was purchased from Cedarlane, FITC-conjugated anti-BrdU from Becton Dickinson, (Mountain View,CA), and Cy™5-streptavidin from Jackson Immunoresearch (West Grove, PA).

*Flow cytometry and BrdU labeling.* Cell surface staining and BrdU labeling were performed as previously described (37,38). Analyses were performed with a FACScalibur® flow cytometer using the CellQuest software, or with a FACScan® flow cytometer using the LysisII software (all from Becton Dickinson).

*In vivo cell trafficking*: Spleen cells from 12-20 wk old B6 or LckOM donors were labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) as previously described (39). Splenocytes ($10^8$) were incubated at 37°C for 15 min in PBS (2 ml) supplemented with CFSE (0.5μM), and washed twice in cold PBS. Then, cells were injected into the unirradiated recipients via the lateral tail vein. A spleen cell suspension containing $43 \pm 5 \times 10^6$ CFSE-labeled T lymphocytes was injected, and their spleen and mesenteric LNs were removed 36 h later for flow cytometry analysis.

## Results

*LNs represent the sole site of massive extrathymic T cell development in LckOM mice.* The relative and absolute numbers of lymphocyte subsets found in the thymus, LNs and spleen of LckOM mice and normal B6 controls aged 4-20 wk are depicted in Fig. 1 and 2, respectively. The most dramatic findings were observed in the LNs which, at 12 wk, showed a 30-fold increase in cellularity relative to controls (Table I). This was caused primarily by a massive accumulation of double-positive $CD4^+CD8^+$ lymphocytes that reached a maximum at 12 wk, and to a lesser extent, by a more progressive increase in the numbers of B cells and single-positive $CD4^+$ and $CD8^+$ lymphocytes that rose progressively from 4 to 20 wk. Data depicted in Fig. 1 and 2 concern mesenteric LNs; other LNs (axillar and cervical) showed the same proportions of various lymphocyte subsets but were slightly less hypercellular than mesenteric nodes (data not shown). LckOM spleen were also hypercellular. In the spleen, however, increased cellularity was due essentially to an accumulation of B lymphocytes; there was a minimal accumulation of immature T cells, and no significant increase in the number of $CD4^+$ or $CD8^+$ T cells. Young (4 wk) LckOM mice presented a severe thymic hypoplasia with very low numbers of immature thymocytes. Thymic cellularity increased with age in LckOM mice, but this was due mainly to a major accumulation of B cells and, to a lesser extent, to increasing numbers of single-positive $CD4^+$ and $CD8^+$ T cells. Immature thymocytes were virtually absent from the thymus of old (20 wk) LckOM mice.

Since extrathymic T cell development can take place in the liver (28,29), intestine (27,40), and bone marrow (25,26), we assessed the number of $CD4^+CD8^+$ thymocytes as well as single positive $CD4^+$ and $CD8^+$ T cells in these organs in LckOM mice (Fig. 3). We found no notable increase in the number of $CD4^+CD8^+$, $CD4^+$, or $CD8^+$ T cells in the bone marrow and intestines when compared to B6 mice. A minimal, but statistically significant, accumulation of $CD4^+CD8^+$ cells was evident in the liver. Together, these results indicate that LckOM LNs are remarkable in at least two points. First, assuming that developing thymocytes must go through a $CD4^+CD8^+$ stage, we can conclude that the LNs constitute the sole site where massive extrathymic thymopoiesis occurs in LckOM mice. As judged by the number of $CD4^+CD8^+$ T cells, the level of T cell production in the LNs of LckOM mice is considerable. Thus, in the mesenteric LNs alone, it reaches a level of 214 x $10^6$ at 12 wk of age (Table I). Second, LNs of OM-transgenic mice also present a conspicuous increase in the pool size of mature $CD4^+$ and $CD8^+$ T cells (Fig. 2). Hence, the mean numbers of single-positive T cells in the mesenteric LNs at age 12 and 20 wk were 43 and 92 x $10^6$ in the case of LckOM mice comparatively with 7 and 12 x $10^6$ for B6 mice (Table I and data not shown).

*$CD4^+$ and $CD8^+$ T lymphocytes are $CD44^{hi}$ in LckOM mice.* Analysis of expression of CD44, CD45RA or RB, CD62L and IL-2Rβ gives important information regarding previous antigen encounter by T cell populations. As depicted in figure 4, the phenotype of LN $CD4^+$ and $CD8^+$ cells was strikingly different in LckOM mice relative to B6 controls. In LckOM mice, most $CD4^+$ T cells were $CD44^{hi}$, $CD45RB^{lo}$, $CD62L^{lo}$ and IL-$2R\beta^{lo}$, a phenotype found following TCR engagement either by non-self antigens

or self-ligands (41-43). In addition, the vast majority of $CD8^+$ T cells were $CD44^{hi}$, $CD45RB^{hi}$, $CD62L^{hi}$ and $IL\text{-}2R\beta^{hi}$. The $CD44^{hi}CD62L^{hi}$ phenotype is found in two types of $CD8^+$ cells: revertants and class I-restricted T cells triggered by self-ligands (42-44). Thus, the phenotype of both $CD4^+$ and $CD8^+$ T cells of LckOM mice does not correspond to that of resting cells, but rather suggests that these cells have sustained significant levels of TCR signaling by heretofore undetermined ligands. Parenthetically, an "activated phenotype" can also be found in NK T cells which harbor a $CD4^+CD8^-$ or $CD4^-CD8^-$ phenotype (45-47). However, their $NK1.1^-$ phenotype shows that LckOM T cells do not correspond to NK T cells (Fig. 4). Interestingly, while aforementioned phenotypic analyses have been done on LckOM LN cells, similar results were observed in LckOM spleen cells, and in LNs and spleen of irradiated B6 mice transplanted with LckOM hematopoietic progenitors (data not shown).

*Extrathymic T cells have a polyclonal Vβ repertoire and a rapid turnover rate.* In LckOM mice aged 12-20 wk, the total numbers of single-positive $CD4^+$ and $CD8^+$ T cells is significantly increased relative to normal mice (Fig. 2). Therefore, we asked whether these mature T cells had a polyclonal origin and how could their expansion be explained in kinetic terms. Functional *in vitro* studies on cytokines of the IL-6 family suggest that OM could possibly have pleiotropic effects on T cell development *in vivo*. Thus, OM has been shown to support the differentiation of $CD34^+$ cells into $CD3^+$ T cells (48). In addition, IL-6, which shares the gp130 receptor subunit with OM (49), can prolong T cell survival (15), and can provide costimulation for naïve T cells (50) (51) by preventing apoptosis (52). Therefore, to address these questions, we created hematopoietic chimeras

by injecting a 1:1 mixture of B6.PL fetal liver cells and T cell-depleted LckOM bone marrow cells into lethally irradiated thymectomized B6 mice, and performed studies specifically on Thy1.1$^+$ cells (of B6.PL origin). Under these experimental conditions, Thy1.1$^+$ cells were 100% of extrathymic origin as they were derived from the differentiation of fetal liver cells in athymic hosts. Furthermore, Thy1.1$^+$ cells were not transgenic themselves, but rather developed under the paracrine influence of OM (Fig. 5).

Among spleen Thy1.1$^+$ cells, both CD4$^+$ and CD8$^+$ T cells expressed a TCR Vβ repertoire that was as diverse as that of age-matched B6 and LckOM controls when assessed by flow cytometry analysis (Fig. 5). Although analyses based on size heterogeneity or on sequence of the CDR3 region will be required to assess more precisely the diversity of extrathymic T cells (53,54,54), our results indicate that CD4$^+$ and CD8$^+$ extrathymic T cells have a polyclonal origin.

BrdU pulse-chase experiments were performed to evaluate the turnover of extrathymic T cells in chimeras. Specifically, we sought to determine whether OM-dependent expansion of extrathymic T cell compartments was due to prolonged survival of resting cells or to an increased proliferation rate. During the pulse period, chimeras and control mice were given BrdU-supplemented water for 20 days (38,55). Again, analyses in chimeras were performed specifically on Thy1.1$^+$ cells. Results for CD62L$^+$ and CD62L$^-$ subsets were analyzed separately since CD62L$^-$ cells divide more rapidly than CD62L$^+$ cells (38,55), and because similar to LckOM mice (Fig. 4), the proportion of CD4$^+$CD62L$^-$ cells was much increased in chimeras relative to B6 controls. The key

finding was that BrdU-labeled $CD4^+$ and $CD8^+$ cells accumulated more rapidly among extrathymic T cells than in controls. Thus, when $CD62L^+$ and $CD62L^-$ subsets in chimeras were compared to their normal counterparts in euthymic controls, the rate of appearance of BrdU-labeled cells was more rapid for extrathymic T cells than classic T cells (Fig. 6). In contrast, the kinetics of BrdU incorporation by $Thy1.1^+CD4^+CD8^+$ thymocytes in chimeras' mesenteric LNs was similar to that of $CD4^+CD8^+$ cells in the thymus of B6 mice (data not shown). After being placed on BrdU water for 20 days, mice were transferred to normal water to examine the rate of decay of BrdU-labeled cells up to day 70. The disappearance of BrdU-labeled T cells was swifter for extrathymic T cells than for classic T cells (Fig. 6). This was conspicuous in the first 10 days after BrdU withdrawal, when the proportion of $BrdU^+$ elements was relatively stable in B6 controls but sharply decreased in extrathymic T cells. Collectively, these results indicate that extrathymic T cells proliferate actively and have a high turnover rate.

*LNs of LckOM attract $CD4^+$ and $CD8^+$ T cells.* In LckOM mice, LNs differ from the spleen as well as other organs, not only in that they are the sole site of extrathymic T cell development, but also because the numbers of LN $CD4^+$ and $CD8^+$ T cells are increased approximately 6 to 7-fold relative to age matched B6 mice (Fig. 2). The selective expansion of the LN single positive T cell compartment is likely due, at least to a minimal extent, to the accumulation of T cells produced *in situ*. However, another explanation would be the preferential homing of recirculating extrathymic T cells to the LNs. To evaluate the latter possibility, we assessed the *in vivo* distribution of CFSE-labeled splenocytes from B6 and LckOM donors 36 h after injection into B6 and LckOM

hosts. Figure 7A depicts the results from these studies in the form of mesenteric LN/spleen ratios calculated from the absolute numbers of injected $CD4^+$ and $CD8^+$ that were recovered from these two sites. The notable finding was that, whatever their source (B6 or LckOM) or their type ($CD4^+$ or $CD8^+$), the proportion of T cells that home to the LNs was greatly increased in LckOM recipients. Increased mesenteric LN/spleen ratios in OM-transgenic recipients were due to both an increased accumulation of T cells in the LN and decreased homing to the spleen (Fig. 7B). It was also observed that the propensity to home to the LN rather than the spleen was greater for B6 than for LckOM T cells. The latter characteristic was T cell autonomous since, when B6 and LckOM splenocytes were co-injected, their respective recovery from the mesenteric LNs and spleen was exactly alike that shown in Fig. 7 (data not shown). The preferential LN homing of T cells injected into LckOM hosts was quite remarkable considering that the size of the T cell pool in LckOM LNs was already increased and that, in a variety of experimental models, the recovery of injected T cells was found to be inversely related to the number of host T cells already present in lymphoid organs (22,43,56,57).

**Discussion**

Extrathymic T cell development in LckOM mice points to the existence of a novel pathway of T cell maturation whose unique characteristics raise fundamental issues concerning the regulation of T cell production and homeostasis. From a topographical point of view, the LNs of these mice are most peculiar. They are the sole site of a massive extrathymic T cell production, and they display an unusual propensity to attract recirculating $CD4^+$ and $CD8^+$ T cells. The single-positive progeny of this extrathymic pathway is polyclonal, shows a phenotype associated with earlier antigen encounter, and displays a rapid turnover rate.

Why T cell development normally takes place in the thymus is not known yet. No adhesion molecule –ligand pair has been identified on T cell precursors or thymic stroma that explains convincingly a selective entry or preferential survival of T cell precursors in the thymic microenvironment (58-61). Accordingly, the reason why extrathymic T cell production induced by OM is limited to the LNs, and particularly the mesenteric LNs, is not inherently obvious. The fact that we found no evidence of extrathymic T cell development in other sites reported to have some ability to support T cell production (namely the liver, bone marrow and intestines), suggests that chronic exposure to OM induces changes that uniquely affect LN stromal (non-lymphoid) cells. An alternative possibility would be that the LN stroma normally expresses a unique structure/molecule that is essential for the homing and development of OM-conditioned pre-thymic cells. The absence of immature thymocytes in the spleen of LckOM mice discloses

unanticipated heterogeneity in the ability of secondary lymphoid organs to sustain T cell development. The latter observation is consistent with recent evidences that the rules governing the development of organized structure in the spleen and LNs are different. Thus, mice deficient either in osteoprotegerin ligand (a TNF-family molecule) or in transcription factor Id2 lack LNs but have a normal spleen, while the reverse is observed in Hox11-deficient mice (62-64). Likewise, B cell/T cell segregation is differentially affected in the spleen vs LNs of $LT\alpha^{-/-}$ and $TNFR\text{-}I^{-/-}$ mice (65). Moreover, some $CD4^-$ $CD8^-$ intrathymic thymocytes (but not pre-thymic progenitors present in fetal liver) can, when injected into thymectomized non-transgenic mice, develop into both $CD4^+CD8^+$ and single-positive T cells into the LNs but not into the spleen (66). Clearly, further investigations must be pursued to decipher the molecular interactions responsible for the striking ability of LNs to support extrathymic T cell development under the influence of OM.

When transplanted into thymectomized hosts together with OM-transgenic bone marrow, non-transgenic fetal liver cells yielded a major accumulation of $CD4^+CD8^+$ T cells in the LNs and generated mature T cells with a polyclonal Vβ repertoire. This suggests that significant levels of thymic-independent positive selection takes place extrathymically (presumably in the LNs) under the paracrine influence of OM, otherwise $CD4^+CD8^+$ would die by neglect (67,68). This observation is consistent with evidence that thymic epithelial cells are not the only cells that can support positive selection, and that *in vivo* positive selection can be mediated by hematopoietic cells (69,70). Nevertheless, it remains to be determined whether the extrathymic pathway modulated

by OM follows the same rules regarding positive and negative repertoire selection as the classical thymic pathway. Other important questions that must be addressed concern the immunocompetence of extrathymic T cells and whether or not they are self tolerant. Since reconstitution of nu/nu mice with LckOM bone marrow restored immune responsiveness to allogeneic mouse melanoma cells, the progeny of the OM-dependent pathway shows at least some level of immunocompetence (31). However, it remains to be determined whether T cells that have differentiated in the LNs can generate protective immune responses against microbial pathogens as efficiently as conventional T cells do.

When injected into 12-20 wk old LckOM mice, T cells harvested from the spleen of normal or LckOM donors preferentially homed to the LNs rather than the spleen. This was somewhat unexpected since i) in LckOM recipients the size of the T cell pool was normal in the spleen but increased 6 to 7-fold in the LNs, and ii) injected T cells usually home preferentially to lymphoid organs that contain less T cells (22,43,56,57). This bias is attributed to the higher number of available (or "empty") T cell niches in T-depleted as opposed to T-replete lymphoid organs. Thus, one logical extension of our findings is that the number of T cell niches increases under the influence of sustained OM production. Recently, a number of indications have been presented suggesting that resident dendritic cells represent fundamental constituents of the peripheral T cell niches (71-74). Because of their abundant expression of MHC class I and class II molecules and their specific chemokine and cytokine expression profile, dendritic cells seem to have a unique ability to control the homing of post-thymic T cells and to provide the continuous TCR ligation required for the survival of naïve and memory T cells in the periphery (72,75-77).

Interestingly, OM and Flt3 ligand act synergistically to enhance the *in vitro* proliferation of hematopoietic stem cells committed to macrophage/dendritic cell formation (78). Therefore, it will be of great interest to evaluate the influence of OM on the number, phenotype and function of dendritic cells *in vivo*. The postulated ability of OM to increase the number of functional T cell niches would be, to our knowledge, unprecedented, and could be of medical interest in circumstances where the number of such niches is deficient (38).

T cells that have developed extrathymically under the influence of OM display two striking features that are perhaps related: these T cells have a rapid turnover rate and the phenotype of antigen-experienced cells ($CD44^{hi}CD45RB^{lo}CD62L^{lo}$ for $CD4^+$ cells, and $CD44^{hi}CD45RB^{hi}IL2R\text{-}\beta^{hi}$ for $CD8^+$ cells). As stated above, a $CD44^{hi}$ activated phenotype is indicative of previous TCR interaction either with conventional non-self antigen or with peripheral self-ligands (42-44). Two findings argue against the possibility that $CD4^+$ and $CD8^+$ extrathymic T cells have been primed en masse by environmental antigens. First, we observed the same "non-naïve" phenotype (depicted in Fig. 4), without conspicuous skewing of the Vβ repertoire, in LckOM mice aged 4 to 18 wk (data not shown). The second argument is based on the CD62L phenotype of $CD8^+$ elements. Indeed, although some $CD8^+$ cells that respond to non-self antigens can revert to a $CD62L^{hi}$ phenotype, a $CD8^+$ compartment composed primarily of $CD44^{hi}CD62L^{hi}$ elements has been found, to our knowledge, in only one situation: following expansion driven by self-ligands in lymphopenic hosts (44). In the latter situation, it has been proposed that, consecutive to lymphopenia, the increased level of available (empty) T

cell niches may allow greater accessibility to niches APCs presenting self-ligands or growth factors that promote T cell division (43,44). LckOM are certainly not lymphopenic. Thus, we surmise that the activated phenotype of LckOM T cells supports the concept that LckOM mice show a major increase in the number and/or function of T cell niches. This strengthens the need to study the effect of OM on the numbers, phenotype and function of dendritic cells. In this regard, it is notewhorty that IL-6, which belongs to the same family as OM, has been reported to modify the processing of self-ligands by dendritic cells, and to increase the presentation of otherwise cryptic epitopes (79). Such mechanism could be instrumental in expanding the size of the peripheral T cell compartment by increasing the reactivity of T cells toward self-ligands.

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**Footnotes**

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[3]Abbreviations used in this paper: B6, C57BL/6J; B6.PL, B6.PL-*Thy-1*$^a$/Cy; BrdU, 5-bromo-2'-deoxyuridine; CFSE, carboxy-fluorescein diacetate succinimidyl ester; OM, oncostatin M

**Figure Legends**

**Figure 1**. Proportion of lymphocyte subsets in the thymus, mesenteric LNs and spleen of LckOM mice and B6 controls. Based on three-color staining, cells were defined as double negative T cells ($Thy1^+CD4^-CD8^-$), double positive T cells ($Thy1^+CD4^+CD8^+$), single positive T cells ($Thy1^+CD4^+CD8^-$ or $Thy^+CD4^-CD8^+$), or B lymphocytes ($Thy1^-CD19^+$). Results represent the mean of 3-4 mice per group.

**Figure 2**. Absolute numbers of lymphocyte subsets in the thymus, mesenteric LNs and spleen of LckOM mice and B6 controls. Populations were defined as in Fig. 1. Results represent the mean of 3-4 mice per group.

**Figure 3**. T lymphocyte subsets in the bone marrow (tibiae + femurs), liver, and intestine of LckOM and B6 mice. Populations were defined as in Fig. 1. DP = $Thy1^+CD4^+CD8^+$ cells. The number (mean ± SD) of DP cells in the various organs is shown above the bars. Three to four mice per group. * $p < 0.05$, Student's *t* test.

**Figure 4**. Expression of differentiation markers (CD44, CD45RB, CD62L, IL-2Rβ and NK1.1) by $CD4^+$ and $CD8^+$ mesenteric LN T lymphocytes from 6 wk old LckOM (bold line) and B6 mice (dotted line). Three-color staining was performed with anti-CD4, anti-CD8, and either anti-CD44, anti-CD45RB, anti-CD62L, anti-IL-2Rβ or anti-NK1.1 antibodies. The percentage of LckOM cells on right side of the marker is indicated. These results are representative of three such experiments.

**Figure 5.** TCR repertoire of extrathymic T cells. Hematopoietic chimeras were created by injecting a 1:1 mixture of B6.PL fetal liver cells and T cell-depleted LckOM bone marrow cells into lethally irradiated/thymectomized B6 mice. **A)** Presence of $CD4^+CD8^+$ cells in the mesenteric LNs of hematopoietic chimeras, 75 days after transplantation. **B)** A large proportion of $CD4^+CD8^+$ cells originate from non-transgenic fetal liver cells (i.e., are $Thy1.1^+$). Dot plot histogram gated on $CD4^+CD8^+$ cells. **C)** Vβ expression patterns in $CD4^+$ and $CD8^+$ splenocytes from euthymic B6 mice (thymic T cells), LckOM mice, and $Thy1.1^+$ cells (derived from B6.PL fetal liver cells) of hematopoietic chimeras (extrathymic T cells). These results represent the mean of 5 - 7 individuals per group. Error bars represent SD.

**Figure 6**. Incorporation (pulse) and decay (chase) of BrdU label in extrathymic vs conventional $CD4^+$ and $CD8^+$ spleen T cells. Normal B6 mice and hematopoietic chimeras were given BrdU water for 20 days, then BrdU was chased for 50 days by transferring mice to normal drinking water. At various time points, splenocytes were harvested and analysed by four-color staining: CD4 or CD8, Thy1.1, CD62L and BrdU. In hematopoietic chimeras, created as in Fig. 5, analyses were done on $Thy1.1^+$ T cells i.e., extrathymic T cells derived from B6.PL fetal liver cells. Each point represents the mean of 2-3 individuals.

**Figure 7.** Migration of CFSE-labeled LckOM and B6 T cells. Spleen cell suspensions containing $43 \pm 5 \times 10^6$ CFSE-labeled T lymphocytes derived from LckOM or B6 mice

were injected through the tail vein of LckOM or B6 recipients. Recipients were sacrificed after 36 h to assess the numbers of CFSE-labeled T cells in the spleen and mesenteric LNs. **A)** The mesenteric LN/spleen ratio was calculated from the absolute number of $CFSE^+$ T cells recovered from these two sites. Each dot represents one individual. The bar indicates the mean of the group. MLN/spleen ratio differences in LckOM vs B6 recipients were significant ($p < 0.05$, Student's *t* test) for $CD4^+$ and $CD8^+$ T cells from B6 as well as LckOM donors. **B)** Absolute number (mean x $10^6 \pm$ SD) of $CD4^+$ and $CD8^+$ B6-derived T cells harvested from the spleen and mesenteric LN of B6 and LckOM recipients. Five to seven mice per group.

Figure 3-1

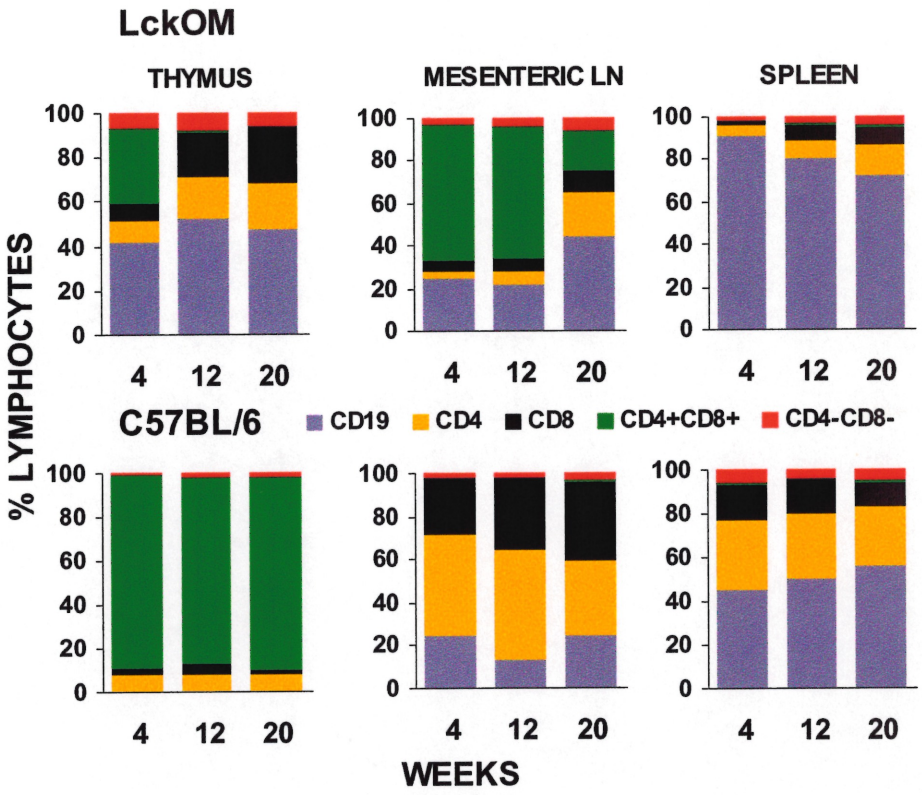

LckOM
THYMUS
MESENTERIC LN
SPLEEN
C57BL/6
CD19
CD4
CD8
CD4+CD8+
CD4-CD8-
% LYMPHOCYTES
WEEKS
0
20
40
60
80
100
4
12
20

Figure 3-2

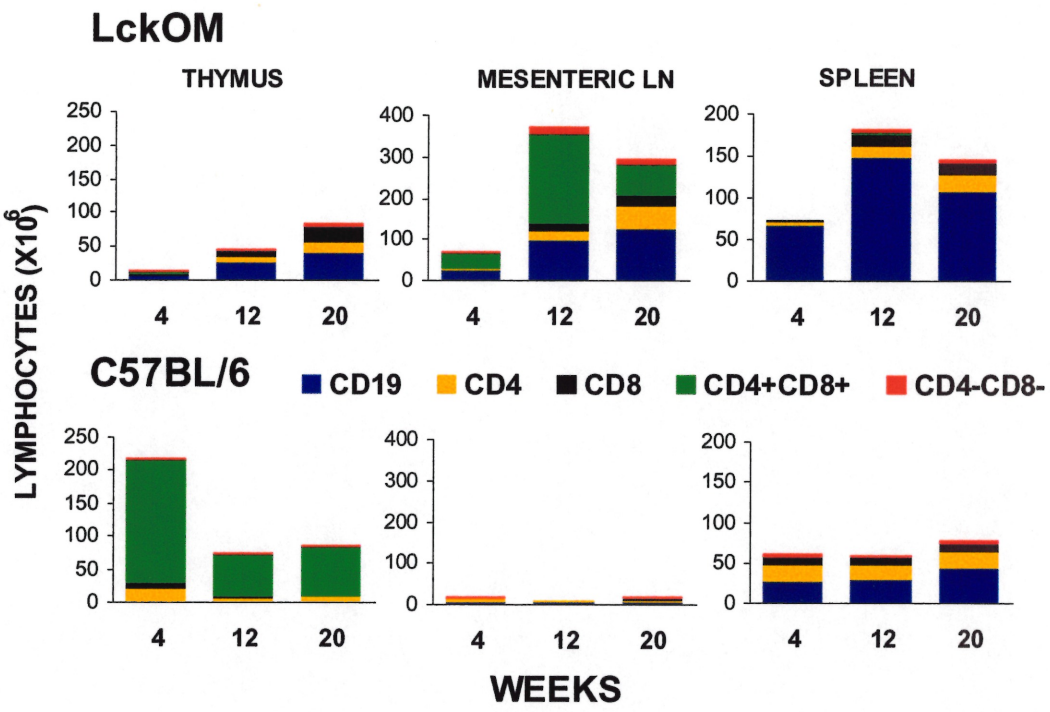

LckOM
THYMUS
MESENTERIC LN
SPLEEN
LYMPHOCYTES (X10⁶)
C57BL/6
CD19
CD4
CD8
CD4+CD8+
CD4-CD8-
WEEKS

**Tableau 3-1**

Table I. *Absolute number of lymphocytes in the thymus, mesenteric lymph nodes and spleen of 12- week old LckOM and C57BL/6 mice.*

| Organ | Phenotype | **LckOM** | **C57BL/6** |
|---|---|---|---|
| | | Lymphocytes $x10^6 \pm SD^c$ | |
| **Thymus** | **$CD19^+$** | 24.32±12.24 | 0.39±0.29 |
| | **$CD4^+$** | 8.53±0.96 | 5.59±0.19 |
| | **$CD8^+$** | 8.83±3.31 | 3.52±1.83 |
| | **DP [a]** | 0.50±0.32 | 63.57±7.7 |
| | **DN [b]** | 3.49±0.12 | 1.63±0.2 |
| **Mes LN** | **$CD19^+$** | 95.62±83.28 | 3.87±1.91 |
| | **$CD4^+$** | 24.34±20.48 | 4.23±0.65 |
| | **$CD8^+$** | 18.18±6.63 | 2.82±0.7 |
| | **DP [a]** | 214.8±121.9 | 0.11±0.12 |
| | **DN [b]** | 17.41±13.41 | 0.32±0.17 |
| **Spleen** | **$CD19^+$** | 147.3±143.5 | 29.07±13.66 |
| | **$CD4^+$** | 14.61±12.79 | 17.85±9.72 |
| | **$CD8^+$** | 12.57±12.36 | 9.46±4.18 |
| | **DP [a]** | 2.25±3.23 | 0.21±0.02 |
| | **DN [b]** | 4.84±3.03 | 2.44±1.0 |

[a] DP, double positive T lymphocyte Thy1.2$^+$CD4$^+$CD8$^+$

[b] DN, double negative T lymphocyte Thy1.2$^+$CD4$^-$CD8$^-$

[c] SD, Standard Deviation (n>3)

**Figure 3-3**

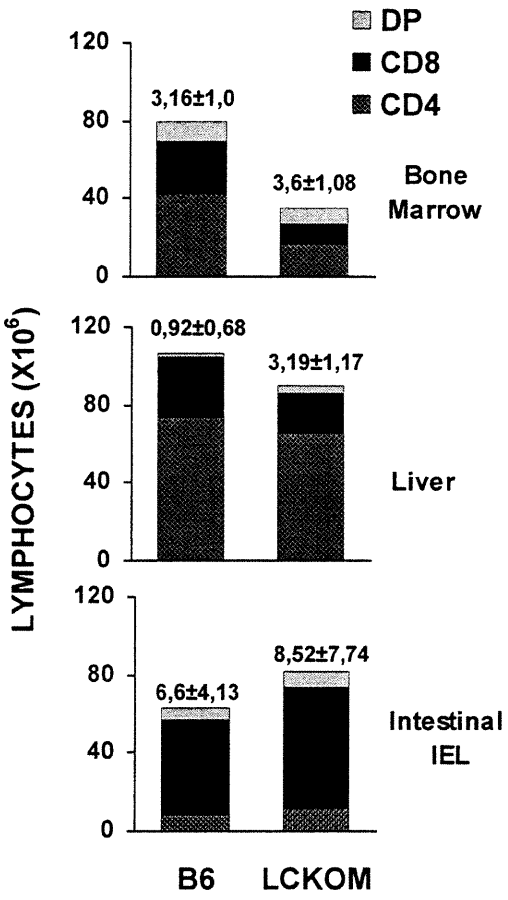

DP
CD8
CD4
LYMPHOCYTES (X10⁶)
120
80
40
0
3,16±1,0
3,6±1,08
Bone Marrow
0,92±0,68
3,19±1,17
Liver
6,6±4,13
8,52±7,74
Intestinal IEL
B6
LCKOM

**Figure 3-4**

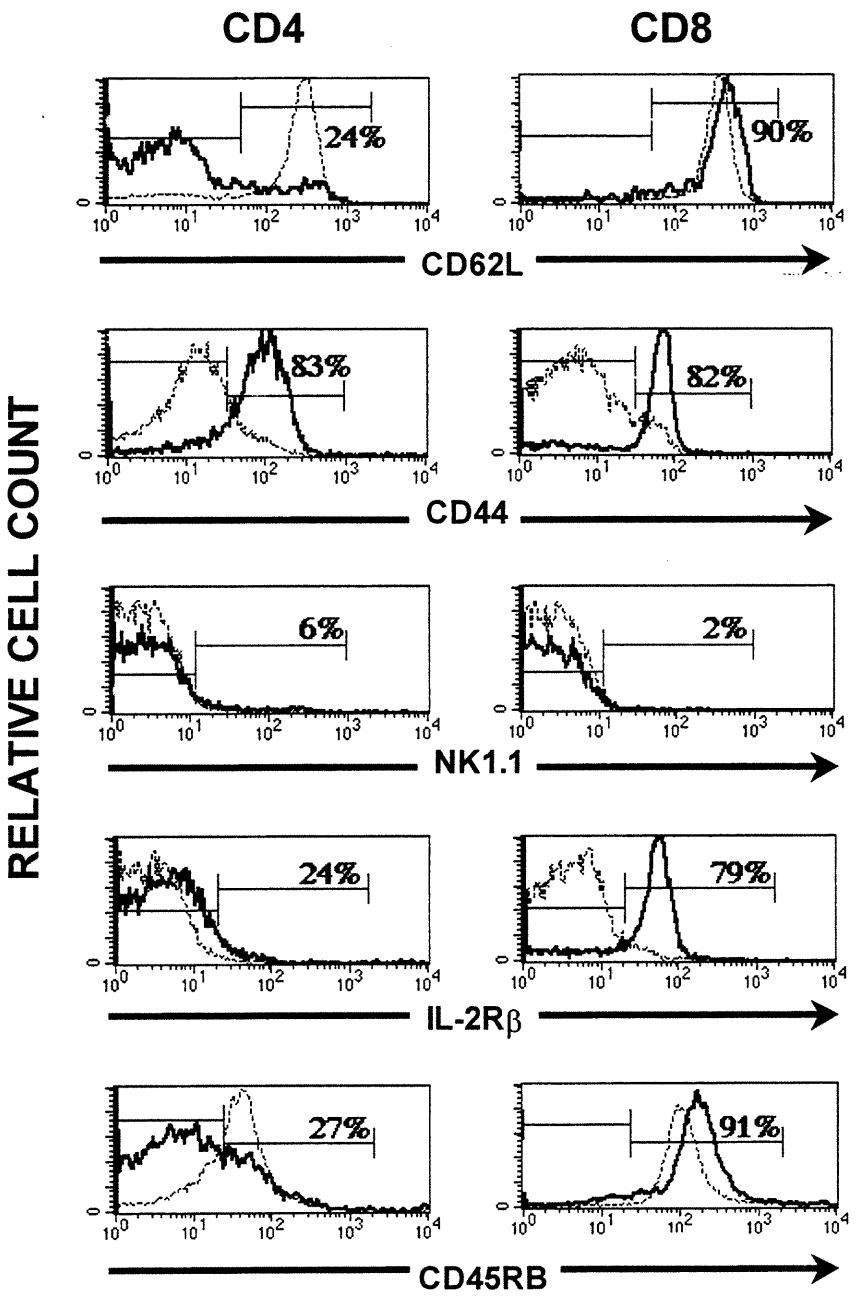

CD4
CD8
RELATIVE CELL COUNT
24%
90%
CD62L
83%
82%
CD44
6%
2%
NK1.1
24%
79%
IL-2Rβ
27%
91%
CD45RB

**Figure 3-5**

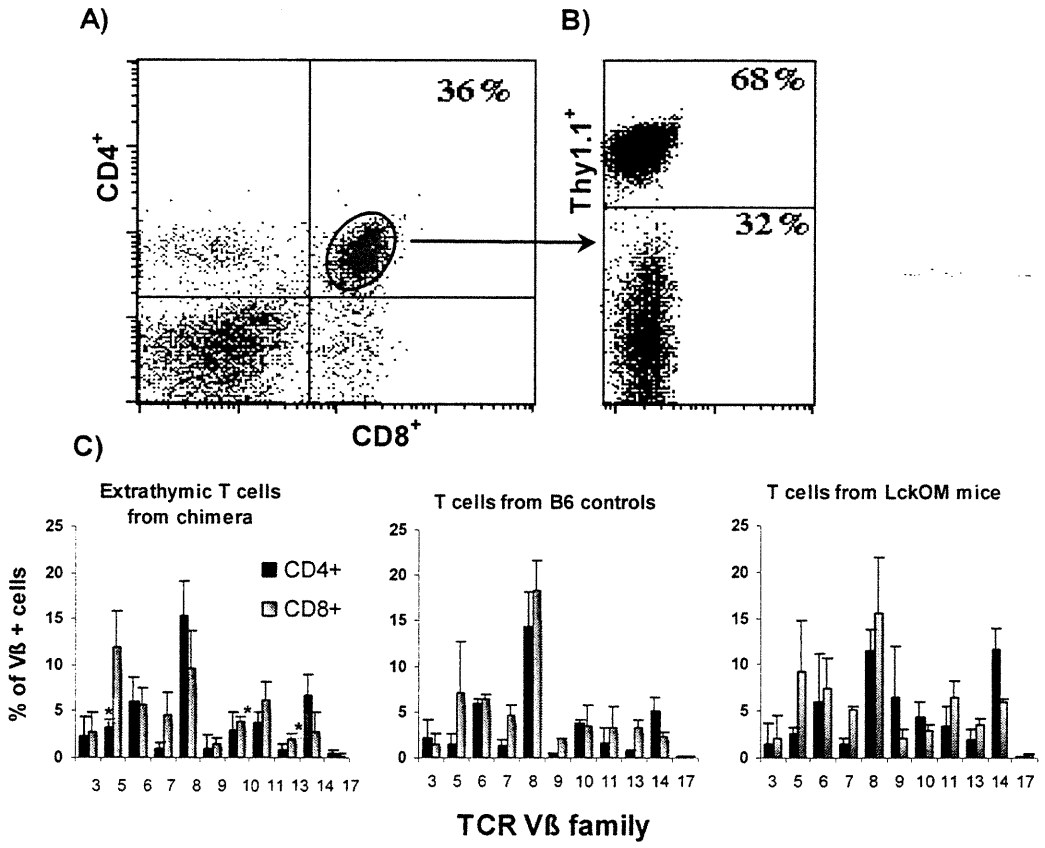

A)
B)
36%
68%
32%
CD4+
CD8+
Thy1.1+
C)
Extrathymic T cells from chimera
T cells from B6 controls
T cells from LckOM mice
CD4+
CD8+
% of Vß + cells
TCR Vß family

**Figure 3-6**

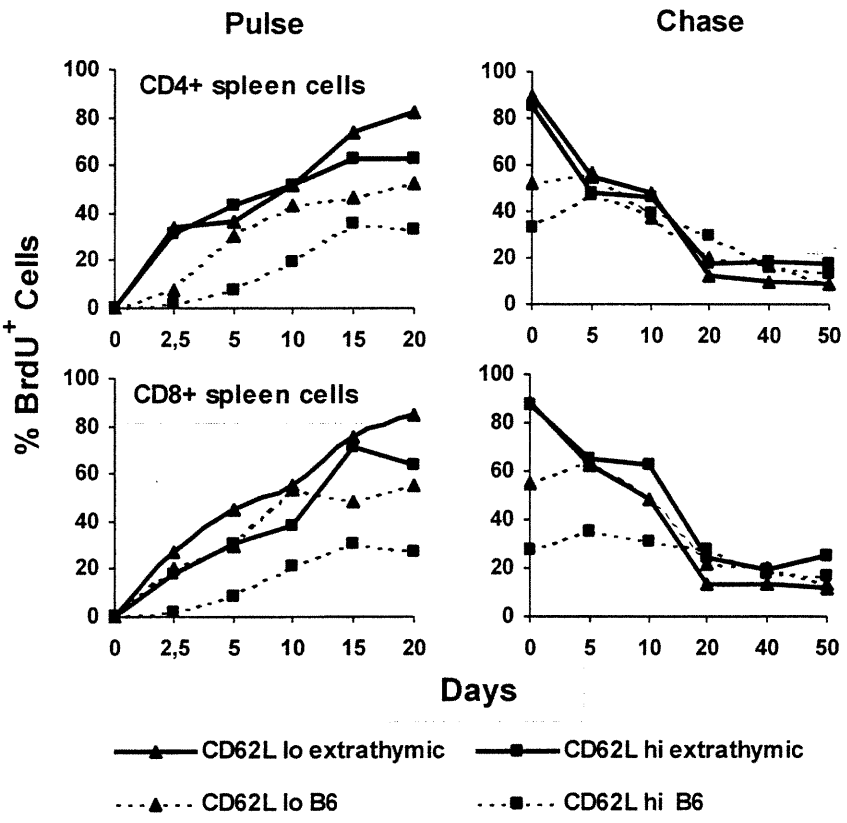
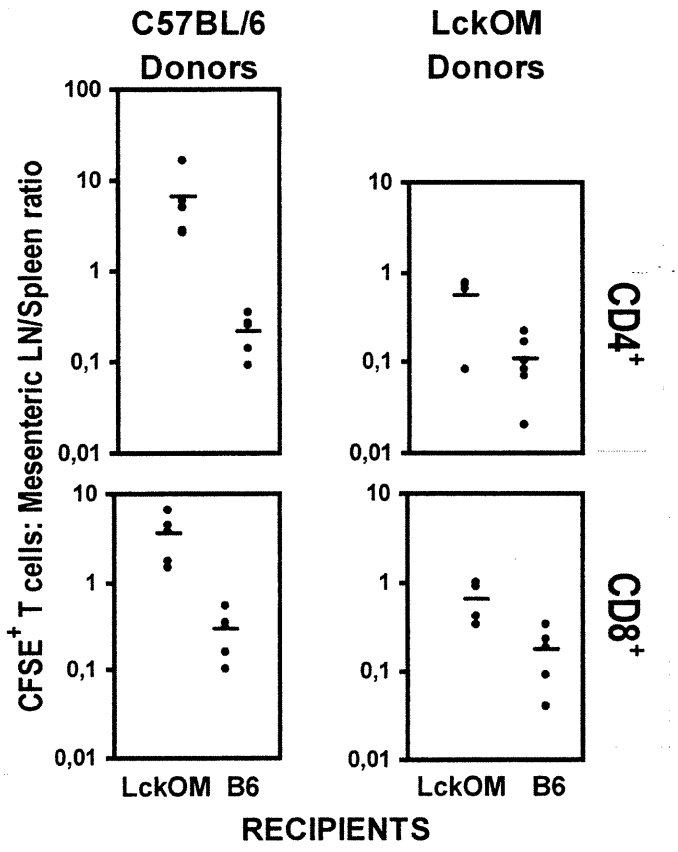

Pulse
Chase
CD4+ spleen cells
CD8+ spleen cells
% BrdU+ Cells
Days
0
2,5
5
10
15
20
40
50
CD62L lo extrathymic
CD62L hi extrathymic
CD62L lo B6
CD62L hi B6

**Figure 3-7**

A)




B)

| | Number of CFSE$^+$ T Lymphocytes (mean x10$^6$±SD) | | | |
|---|---|---|---|---|
| **Phenotype** | **B6 LN** | **LckOM LN** | **B6 Spleen** | **LckOM Spleen** |
| **CD4$^+$** | 0,53±0,33 | 1,43±1,12 | 2,40±0,80 | 0,27±0,19 |
| **CD8$^+$** | 0,44±0,24 | 1,01±0,89 | 1,34±0,29 | 0,14±0,14 |

## Discussion

### 1. Différenciation/expansion thymique et extrathymique des lymphocytes T chez des receveurs de moelle osseuse irradiés.

Chez l'humain, le thymus commence à s'atrophier dès l'âge de un an et sa fonction ne cesse de diminuer jusqu'à l'âge adulte (23;25;130-132). Chez les jeunes patients, la reconstitution immunitaire post-greffe médullaire est généralement plus rapide et le nombre de cellules T naïves beaucoup plus élevé. Il est probable que le thymus participe à la production de cellules T naïves chez ces individus, ce qui permet une augmentation du nombre de cellules T naïves et l'apparition d'un répertoire T beaucoup plus diversifié (39;66;124;133-137). Ces individus profitent donc d'une reconstitution immunitaire plus efficace ce qui diminue le risque d'infection et améliore leur taux de survie. De plus, la présence d'une production thymique semble permettre le contrôle des cellules T auto-réactives périphériques (22) ce qui, dans le contexte d'une greffe de moelle osseuse, pourrait permettre de diminuer la sévérité de la GVH. C'est pourquoi certains auteurs affirment que la présence d'une fonction thymique adéquate semble être l'élément déterminant du pronostic favorable observé chez les jeunes patients. Le développement d'approches thérapeutiques pouvant permettre une régénération du thymus devrait donc s'avérer essentiel à l'amélioration des résultats cliniques lors de déplétion des cellules T (138).

Afin d'évaluer l'impact de l'hypoplasie thymique sur l'ontogénie des lymphocytes T post-greffe en absence de réaction de GVH, nous avons étudié

le phénotype et la fonction des cellules T chez des souris euthymiques et athymiques ayant reçu des progéniteurs médullaires avec ou sans cellules T matures (139). Chez les receveuses thymectomisées, l'origine des cellules T dépend de la composition du greffon. Quand ce dernier contient des cellules T matures, celles-ci prolifèrent et repeuplent le pool T périphérique de la receveuse. Chez les receveuses thymectomisées dont le greffon ne contient pas de cellules T, les lymphocytes T proviennent exclusivement de la maturation extrathymique des progéniteurs hématopoïétiques greffés. Ces souris présentent une lymphopénie importante et leurs cellules T possèdent un phénotype CD44/CD45 de type mémoire/activé.

Cette approche nous a également permis d'observer que chez les receveuses euthymiques, les cellules T proviennent de la différenciation des progéniteurs hématopoïétiques dans le thymus du receveur. Ces souris possèdent un système immunitaire pratiquement normal, autant qualitativement que quantitativement. Chez ces souris, les cellules nouvellement produites par le thymus possèdent un avantage certain sur les autres lymphocytes T car elles inhibent l'expansion/accumulation des cellules T matures du greffon et le développement extrathymique des progéniteurs hématopoïétiques. Comment expliquer que ces cellules puissent inhiber l'accumulation des cellules T d'origine extrathymique? Différentes études ont démontré que des cellules naïves injectées à des receveurs lymphopéniques se divisent, colonisent l'espace périphérique et acquièrent un phénotype de type mémoire/activé (19-21). Notre hypothèse est que, suite à la greffe, les cellules nouvellement produites par le thymus prolifèrent dès leur sortie du thymus et acquièrent un

phénotype mémoire/activé. Dès lors, ces cellules nouvellement produites entrent en compétition directe avec les cellules provenant de l'expansion des cellules T matures du greffon et les remplacent graduellement. L'expansion des lymphocytes T matures du greffon est le résultat de deux phénomènes. Premièrement, la prolifération homéostatique des lymphocytes T non-alloréactifs et, deuxièmement, l'expansion des cellules T alloréactives suite à leur stimulation. Donc, les cellules T nouvellement produites par le thymus pourraient graduellement remplacer les lymphocytes T alloréactifs lors d'une GVH et ainsi réduire l'amplitude de la réaction alloréactive et améliorer le taux de survie des patients.

Par la suite, nous pensons que le pool T périphérique augmente lentement et que les cellules nouvellement produites par le thymus conservent leur phénotype naïf. De plus, des résultats de Goldrath *et al.* (19) suggèrent que l'activation homéostatique des cellules T naïves est réversible. Ils ont démontré, dans un modèle murin, que lorsque la cellularité du compartiment lymphoïde périphérique redevient normale, les cellules T arrêtent de se diviser et retrouvent les caractéristiques phénotypiques et fonctionnelles de cellules naïves. Les cellules naïves retrouvées chez les receveuses euthymiques proviennent donc soit directement du thymus soit de le conversion à un phénotype naïf des cellules activées lors de l'expansion homéostatique. Une étude détaillée et précoce, en cytométrie en flux, de la cinétique de reconstitution et des niveaux de prolifération et d'apoptose des différentes populations de lymphocytes T périphériques, devrait permettre de démontrer 1) le mode de remplacement des cellules T d'origines extra-thymique par les

cellules T nouvellement produites par le thymus et 2) la conversion à un phénotype naïf des cellules activées lors de l'expansion homéostatique.

Finalement, toutes les cellules d'origine extrathymique présentent un phénotype de type mémoire/activé. Il est possible que le même type de compétition se déroule chez ces populations cellulaires, ce qui permettrait aux cellules provenant d'une expansion périphérique d'inhiber le développement extrathymique des cellules souches hématopoïétiques.

Nos résultats montrent également que quantitativement, la reconstitution immune chez les souris thymectomisées ne dépasse jamais le nombre de cellules mémoire/activées retrouvées chez un sujet normal. Cette observation suggère 1) la présence d'une régulation homéostatique et donc la présence d'une compétition intercellulaire et 2) que ces cellules requièrent les mêmes conditions afin de survivre et proliférer que les cellules matures retrouvées chez un sujet normal. Donc, il est possible que les cellules nouvellement produites par le thymus, suite à leur activation homéostatique, compétitionnent avec les lymphocytes T déjà présents en périphérie. Tout ajout de nouvelles cellules doit être compensé par une perte cellulaire si le nombre de cellules demeure constant. De plus, les cellules provenant d'une maturation ou d'une expansion extrathymique présentent toutes une prolifération très diminuée en présence de mitogènes ou d'alloantigènes (139). Cette caractéristique permet possiblement aux cellules nouvellement produites par le thymus de prendre tout l'espace périphérique.

Notre seconde hypothèse est que la présence du thymus et d'une production thymique inhibe la prolifération homéostatique des cellules matures

du greffon et permet leur dilution graduelle par les cellules nouvellement produites par le thymus. Cette hypothèse provient d'observations démontrant que le thymus influence le développement extrathymique des cellules T de l'intestin (140;141). Une étude de la prolifération cellulaire post-greffe à l'aide du BrdU démontrerait 1) si les cellules provenant d'une maturation ou d'une expansion extrathymique présentent effectivement un niveau de prolifération diminué et 2) si les cellules nouvellement produites par le thymus inhibent effectivement leur prolifération. De plus, l'étude de la reconstitution immune dès les premiers jours suivant la greffe aurait permis de décrire clairement le mode de remplacement des cellules matures du greffon.

La plupart des patients qui subissent une transplantation médullaire présentent une atrophie thymique secondaire à l'âge et la maladie. De plus, la chimiothérapie, l'irradiation et la GVH affectent également la production thymique de lymphocytes T chez ces patients. Jusqu'à tout récemment, la fonction thymique chez des individus adultes était considérée comme étant inexistante ou très faible. Des études récentes ont par contre démontré la présence d'une production thymique substantielle chez des individus dont l'âge variait entre un an et 60 ans (22;25;28;34;35;130). Bien que ces résultats montrent une variabilité interindividuelle importante et que l'interprétation des résultats soit complexe (142), il semble maintenant difficile d'attribuer les différences observées chez les jeunes patients et patients adultes à une seule différence au niveau de la fonction thymique. Il est par contre possible que le thymus adulte soit plus sensible aux traitements et à la GVH que le thymus de jeunes patients. De plus, la production thymique de ces individus est

possiblement insuffisante et n'arrive pas à permettre le maintien de cellules T naïves périphériques. Finalement, l'espace périphérique nécessaire au maintien des cellules naïves est possiblement réduit chez l'adulte. Ceci expliquerait 1) le déficit en cellules naïves observé chez l'adulte malgré la présence d'une production thymique substantielle et 2) le déficit en cellules naïves post-greffe observé chez ces mêmes individus.

Nos résultats montrent que la voie de différenciation ontogénique utilisée post-greffe médullaire dépend de deux facteurs : 1) la présence/absence du thymus et 2) la composition cellulaire du greffon. De plus, ils permettent de mettre en évidence la présence d'une compétition cellulaire et d'une régulation homéostatique des populations lymphocytaires T. Puisque ces facteurs affectent de façon quantitative et qualitative la maturation des cellules T, ils doivent avoir une influence déterminante sur l'apparition d'une réaction du greffon contre l'hôte et le niveau d'immunocompétence des patients.

## 2. Effets de la réaction du greffon contre l'hôte sur la production et la régulation homéostatique des différentes populations de cellules T

De nombreuses études ont démontré la présence d'atteintes à la structure et à la fonction thymique suite à une greffe de moelle osseuse allogénique (125;143-149). Diverses hypothèses ont été soulevées afin d'expliquer les effets délétères de la GVH sur la fonction thymique. Une destruction du squelette épithélial, une réduction du nombre de cellules d'origine hématopoïétiques stromales, dont les cellules dendritiques, et finalement, une inhibition du cycle cellulaire des pro et pré-thymocytes suite à la

présence d'une réponse inflammatoire sont les principaux facteurs invoqués (125;143-146;149). L'hypoplasie lymphoïde observée lors d'une GVH est beaucoup plus sévère que celle observé suite à une greffe de type syngénique. Cette différence est généralement associée à une atteinte thymique provenant de la GVH (124;125;150). Par contre, des résultats de Brochu *et al.* (129) montrent que l'expansion des cellules T matures alloréactives et non-alloréactives du greffon est réduite par la GVH car ces cellules entrent rapidement en apoptose et disparaissent. Une reconstitution immune via l'expansion des cellules T matures du greffon est donc compromise par la GVH et démontre l'importance de la fonction thymique sur la reconstitution immune post-greffe médullaire.

Cette étude visait donc à déterminer l'effet de la GVH sur les différentes voies de différenciation ontogénique utilisées post-greffe médullaire et ainsi définir clairement les causes de l'hypoplasie lymphoïde associée à la GVH. Nos résultats démontrent que la production thymique post-greffe médullaire chez des individus présentant une GVH est équivalente à 25% de la production thymique observée chez des sujets sans GVH. Il a été démontré qu'un tel niveau d'exportation thymique est suffisant à maintenir le compartiment lymphocytaire T au niveau des organes lymphoïdes secondaires de souris sénescentes (39). Nous avons également montré que le niveau de prolifération des cellules T périphériques était normal chez ces souris. Donc, pourquoi le nombre de cellules T périphérique demeure-t-il si bas chez les sujets atteints de GVH? Les expériences subséquentes ont démontré que les cellules T provenant d'un donneur atteint de GVH proliféraient de façon normale lorsque

transférées chez un receveur thymectomisé et irradié. Par contre, des lymphocytes T provenant d'un donneur sain greffés à un receveur présentant une GVH ne parviennent pas à reconstituer le compartiment lymphocytaire T périphérique. Nous suggérons donc que l'hypoplasie lymphoïde associée à la GVH provient en partie 1) d'une disparition des cellules T matures non-alloréactives du greffon et 2) d'une diminution de la production thymique mais également 3) d'une perturbation de l'environnement nécessaire au maintien des populations cellulaires périphériques.

De nombreuses études suggèrent que l'environnement, ou niche, spécialisé procurant les conditions requises à la survie et au développement des populations de cellules T périphériques se compose principalement de trois éléments (130). Premièrement, ces cellules requièrent une liaison fréquente entre leur récepteur TCR et les molécules du CMH (151). Deuxièmement, la présence de peptides du soi présentés par les molécules du CMH est nécessaire tout comme lors de la sélection positive au niveau du thymus (152). De plus, certains résultats suggèrent que ces molécules doivent être présentées à la surface de cellules spécialisées comme les cellules dendritiques (18). Finalement, certains résultats suggèrent la présence d'une compétition pour des ressources limitées au niveau des cellules présentatrices d'antigènes: facteurs solubles, molécules du CMH ou facteurs de co-stimulation (78;79). Cette observation est importante car elle suggère que les ressources de cet environnement sont limitées et qu'il est plausible que la GVH puisse diminuer quantitativement les signaux disponibles. L'implication de facteurs solubles dont les chémokines et les cytokines, Il-7, Il-15 et Il-2 par exemple, est

probable mais n'a pas été clairement démontrée (78;151). Certains résultats de Napolitano *et al.* (153) démontrent que la production d'Il-7 par des cellules dendritiques des ganglions lymphatiques est augmentée lors d'une déplétion des cellules T. De plus, certains résultats très récents (154;155) démontrent l'importance de l'Il-7 sur la reconstitution immune post-greffe médullaire. L'Il-7 stimulerait la production thymique et l'accumulation périphérique des cellules T et améliorerait la compétence immunitaire des sujets lymphopéniques. L'Il-7 semble donc être un élément important de la régulation homéostatique des cellules T.

Goldrath et Bevan (156) proposent trois hypothèses pouvant expliquer la présence d'une régulation homéostatique périphérique des cellules T. Premièrement, les lymphocytes T pourraient réguler leur nombre via des signaux inhibiteurs présentés l'un à l'autre ou, deuxièmement, inhiber les fonctions stimulantes des cellules délivrant les signaux associés à la survie des cellules T. Il est improbable que ces deux hypothèses puissent expliquer l'hypoplasie lymphoïde observée lors d'une GVH. Ces mécanismes supposent la présence, chez des sujets atteints de GVH, de cellules T pouvant réduire anormalement le nombre ou la fonction des niches périphériques. Ce type de cellule aurait donc été transféré lors de la greffe de cellules T provenant de receveurs atteints de GVH à des receveurs sains. Par contre, la troisième hypothèse soulevée par ces auteurs suggère la présence d'une compétition pour des signaux délivrés par certaines cellules au niveau des ganglions lymphatiques. Cette compétition permettrait le contrôle du nombre de cellules T périphérique. Nous pensons que l'hypoplasie lymphoïde associée à la GVH

provient d'une diminution importante des cellules stimulantes ou des signaux requis à la sélection positive périphérique des cellules T.

Notre hypothèse est donc que lors d'une GVH, les dommages causés aux ganglions lymphatiques engendrent une diminution durable des signaux responsables du maintien des cellules T périphériques. Nous suggérons que ces dommages provoquent une diminution du nombre de cellules dendritiques et donc du nombre de molécules du CMH associées à des peptides du soi au niveau des ganglions lymphatiques. Ces dommages seraient également responsables d'une diminution de la quantité d'Il-7 produite et présente au niveau des ganglions lymphatiques. L'hypoplasie lymphoïde observée post-greffe médullaire est généralement durable. Une étude histologique (immunohistochimie et hybridation *in situ*) et en cytométrie en flux des organes lymphoïdes secondaires permettrait de quantifier le nombre de cellules dendritiques, le niveau d'expression des molécules du CMH et de l'Il-7 et de déterminer les types cellulaires exprimant ces molécules. De plus, l'utilisation de la technique de RT-PCR permettrait également d'évaluer, suite à un tri cellulaire, le niveau d'expression de l'Il-7 selon le type cellulaire. Ces expériences permettront de vérifier si les dommages causés par la GVH 1) diminuent réellement le nombre de cellules dendritiques, 2) si ces dommages sont durables et 3) si ces dommages diminuent la quantité d'Il-7 produite et présente au niveau des ganglions lymphatiques. Tester directement notre hypothèse étant difficile, nous avons plutôt opté pour l'étude d'un modèle murin transgénique qui présentait la particularité d'induire une différenciation de type «thymique» au niveau des ganglions lymphatiques.

### 3. Développement extrathymique des cellules T chez la souris LckOM

Nous avons démontré que les ganglions de la souris LckOM sont particuliers à bien des égards. Le développement extrathymique des cellules T (caractérisé par la présence de cellules immatures doubles positives $CD4^+CD8^+$) ne se retrouve qu'au niveau des ganglions lymphatiques et non au niveau de la moelle osseuse, du foie, de l'intestin ou de la rate. De plus, les ganglions lymphatiques sont le seul endroit où le nombre de cellules T matures $CD4^+$ et $CD8^+$ est augmenté (6 à 7 fois). Lorsqu'injectés chez des souris LckOM, les lymphocytes T matures $CD4^+$ et $CD8^+$ provenant de la rate de souris donneuses normales ou LckOM migrent préférentiellement vers les ganglions lymphatiques et non vers la rate. Le comportement migratoire de ces cellules T est très surprenant car des lymphocytes T greffés migrent préférentiellement vers les organes lymphoïdes qui contiennent le moins de cellules T (157-160). Notre hypothèse actuelle explique ces résultats en assumant que les cellules T injectées ne migrent pas vers les organes lymphoïdes contenant le moins de cellules T mais vers ceux présentant la plus grande quantité de niches périphériques libres. Donc, nos résultats suggèrent que le nombre de niches périphériques libres au niveau des ganglions lymphatiques de la souris LckOM est augmenté sous l'influence d'une production soutenue d'OM.

Afin d'évaluer le niveau de prolifération et la diversité du répertoire T des cellules d'origine extrathymique chez la souris LckOM, nous avons créé des chimères hématopoïétiques auxquelles nous avons injecté des cellules

de foie fœtal de souris B6.PL (Thy 1.1) mélangées à des cellules de moelle osseuse T-déplétées provenant de souris LckOM (Thy 1.2). Chez ce modèle, où les receveurs sont irradiés et thymectomisés, les cellules Thy 1.1 sont toutes d'origine extrathymique car elles proviennent de la différenciation de cellules de foie fœtal chez un receveur athymique. De plus, ces cellules ne sont pas transgéniques mais se développent sous l'influence de l'OM. L'accumulation de cellules immatures et matures Thy 1.1 au niveau des ganglions lymphatiques de ces chimères démontrent clairement que la production de cellules T sous l'influence de l'OM est indépendante du thymus et que la présence du transgène n'est pas requise pour le développement de ces cellules. Ces cellules présentent un répertoire T polyclonal ainsi qu'un niveau de prolifération rapide.

Nos travaux ont révélé que l'exposition chronique à l'OM permet aux ganglions lymphatiques de soutenir le développement extrathymique des cellules T. De plus, cette exposition chronique semble induire une augmentation du nombre ou de la fonction des niches périphériques ce qui permet le maintien d'un plus grand nombre de cellules T matures en périphérie. La découverte d'une méthode permettant de stimuler la production de cellules T naïves afin de compenser l'atrophie graduelle du thymus permettrait de maintenir la diversité du répertoire des cellules T chez des individus dont la fonction thymique est déficiente mais également une reconstitution immune efficace chez des individus immunodéficients. De plus, une augmentation du nombre ou de la fonction des niches périphériques permettrait d'améliorer la reconstitution immune chez des individus dont les

niches périphériques sont réduites comme dans le cas des patients souffrant de GVH (150).

## 4. Comment l'OM induit-elle le développement extrathymique des cellules T?

Les études futures devraient permettre de déterminer comment une exposition chronique à l'OM permet d'induire le développement extrathymique des cellules T au niveau des ganglions lymphatiques et, de plus, définir ce que sont les niches périphériques et comment l'OM augmente leur fonction. Notre hypothèse est que ces deux phénomènes proviennent d'une modification du patron d'expression de certaines chémokines ou récepteurs de chémokines au niveau des ganglions lymphatiques des souris LckOM.

Depuis quelque années, de nombreuses études ont démontré que les chémokines et leurs récepteurs constituent des éléments essentiels au niveau de la régulation de la maturation, du déplacement et de la localisation tissulaire des leucocytes. De plus, ces molécules participent activement au développement des organes lymphoïdes primaires et secondaires (82;87;97). Par exemple, l'interaction du récepteur CCR7 avec les chémokines ELC et SLC est nécessaire à la localisation des cellules T naïves et des cellules dendritiques au niveau des ganglions lymphatiques (91;94;115;118;119;161;162). Les cellules T naïves et les cellules dendritiques doivent respectivement transiter via les HEV et les vaisseaux lymphatiques afférents. De plus, des études récentes indiquent que l'expression ectopique des chémokines SLC ou BLC permet le

développement spontané de tissus lymphoïdes par le recrutement de lymphocytes et qu'une réponse différentielle à la chémokine SLC permet la ségrégation des zones T et B à l'intérieur de ce tissu lymphoïde (163;164). La migration des lymphocytes T vers la peau et l'intestin requiert par contre les chémokines TARC et TECK et leurs récepteurs respectifs CCR4 et CCR9 (116;165). Finalement, *in vivo*, l'entrée et la migration des thymocytes à travers la jonction corticomédullaire, ainsi que la sortie des thymocytes du thymus requièrent des signaux provenant de un ou plusieurs récepteurs couplés aux protéines G, probablement des récepteurs de chémokines (42;108;109). L'identité des chémokines responsables de l'entrée des progéniteurs T au niveau du thymus n'est pas encore connue mais des études récentes de chémotaxie *in vitro* ont démontré que les chémokines SDF-1$\alpha$ (récepteur: CXCR4) et TECK (récepteur: CCR9) sont des candidates intéressantes du fait qu'elles agissent préférentiellement sur les thymocytes immatures (42;43;110;166).

Les cytokines n'ont habituellement pas d'activité chémotactique mais peuvent modifier le patron d'expression de chémokines et de leurs récepteurs (82). Le TNF et la lymphotoxine $\alpha/\beta$ régulent par exemple l'expression des chémokines BLC, ELC et SLC (120), le TGF-$\beta$, l'expression du récepteur CCR7 (167) et l'OM l'expression de JE (MCP-1) (168). De plus, une étude récente a démontré que l'IL-4 et l'IL-10 stimulent ou inhibent respectivement, sur les cellules T $CD4^+$, l'expression du récepteur de SDF-1$\alpha$, CXCR4 (169).

Finalement, l'expression de certaines chémokines peut être régulée par la sécrétion de certaines cytokines produites par les lymphocytes T (170).

L'étude de la biologie des chémokines est encore récente mais évolue très rapidement avec plus de 50 chémokines identifiées jusqu'ici. De plus, 16 récepteurs ont déjà été caractérisés. Parmi ces molécules, les candidates potentielles sont celles qui ont démontré un certain rôle au niveau de la localisation et du trafic des cellules T, de leurs progéniteurs ou des cellules dendritiques, et qui sont exprimées par les cellules dendritiques ou les HEV. Chez les souris LckOM, la production extrathymique de cellules T ainsi que l'augmentation du nombre de lymphocytes T n'est observée qu'au niveau des ganglions lymphatiques et il n'y a aucun autre organe qui ne présente ces caractéristiques. Cette observation soulève la question suivante; quelles structures sont uniques aux ganglions lymphatiques? Nous pensons qu'il y a deux éléments propres aux ganglions lymphatiques. Premièrement, les vaisseaux lymphatiques afférents qui permettent aux cellules dendritiques activées de migrer vers les ganglions lymphatiques (171;172) et deuxièmement, les HEV (173;174). Certaines études récentes ont démontré les propriétés angiogéniques de l'OM ainsi que ses effets positifs sur la prolifération des progéniteurs hématopoïétiques destinés à une différenciation en cellules dendritiques (175;176). Nos observations (non publiées) montrent, au niveau des ganglions lymphatiques de la souris LckOM, une augmentation marquée du nombre de vaisseaux sanguins observée par immunohistochimie ainsi qu'une augmentation importante des cellules dendritiques myéloïdes observée par FACS. Les vaisseaux sanguins

des ganglions lymphatiques étant responsables de l'entrée sélective de certains types cellulaires via l'expression de chémokines, nous pensons qu'une modification du patron d'expression de ces molécules pourrait être responsable du phénotype observé chez les ganglions de la souris LckOM. De plus, les cellules dendritiques sécrètent de nombreuses chémokines et expriment de nombreux récepteurs de chémokines. Il est possible que l'entrée sélective de ces cellules au niveau des ganglions permette l'accumulation d'une chémokine pouvant permettre l'entrée des progéniteurs T, leur survie ou leur maturation. Il est également possible qu'un tel dépôt permette la localisation de progéniteurs T aux environs d'un type cellulaire auquel ils n'ont pas accès habituellement, ce qui permettrait leur développement.

Les molécules dont nous voulons étudier l'expression sont les chémokines ELC, JE, MDC, MIP-1β, MIP-3α, SDF-1, SLC, TARC, TECK, BLC et leurs récepteurs CCR1, CCR2, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CXCR4 et CXCR5. Le niveau d'expression sera évalué par RT-PCR semi-quantitatif au niveau des ganglions lymphatiques, de la rate et du thymus de souris LckOM et de souris contrôles (C57BL/6). La comparaison de l'expression au niveau de ces trois organes devrait permettre d'identifier une où plusieurs molécules dont l'expression au niveau des ganglions lymphatiques de la souris LckOM est particulière. La comparaison du niveau d'expression entre le ganglion lymphatique de la souris LckOM et les thymus et ganglions contrôles devrait permettre de déceler un profil d'expression

semblable à celui observé dans le thymus contrôle mais qui diffère de celui observé dans les ganglions lymphatiques normaux. De plus, l'absence de développement extrathymique des cellules T au niveau de la rate LCKOM nous permettra de comparer le profil d'expression des chémokines entre deux organes lymphoïdes exposés à l'OM et dont la composition cellulaire est semblable. Ces comparaisons devraient nous permettre d'identifier toutes modifications propres aux ganglions lymphatiques LckOM et susceptibles de participer au phénotype des souris LCKOM.

La présence d'une expression différentielle de certaines molécules au niveau des ganglions lymphatiques des souris LckOM sera par la suite caractérisée par immunohistochimie et hybridation *in situ*. Ces techniques devraient nous permettre de définir la nature ainsi que la localisation des cellules produisant ces molécules. Les types cellulaires dont la distribution sera déterminée par immunohistochimie sont les cellules T simples et doubles positives, les cellules B, les cellules dendritiques myéloïdes et lymphoïdes, les fibroblastes et les cellules HEV. De plus, il a été démontré que les chémokines formaient des gradients sous forme de dépôts au niveau de la matrice extracellulaire (107). L'immunohistochimie devrait donc nous permettre dévaluer la distribution tissulaire de ces dépôts et permettre une évaluation quantitative sommaire. Une quantification précise sera entreprise par "western blot" afin de déterminer la quantité de protéine présente à l'intérieur de l'organe. Cette étape, complémentaire à la PCR, est nécessaire car il a été démontré que certaines chémokines et récepteurs peuvent être

stockés à l'intérieur des cellules (99;177) ou se retrouver sous forme de dépôts au niveau de la matrice extracellulaire (107).

La suite des travaux est dépendante des résultats obtenus lors des étapes précédentes. Suite à l'identification d'une expression différentielle d'une ou plusieurs chémokines ou récepteurs au niveau des ganglions lymphatiques de la souris LckOM, l'évaluation de l'importance d'une telle modification sera entreprise. L'activité chémotactique des chémokines ou récepteurs identifiés sera déterminée *in vitro* sur les différentes populations de cellules T immatures et matures. De plus, la construction de transgènes codant pour les molécules d'intérêt permettra la transfection des types cellulaires produisant ces molécules. Les lignées cellulaires ainsi tranfectées pourront être injectées à l'intérieur de ganglions de souris contrôles et l'activité biologique déterminée. De plus, la production de souris trangéniques exprimant ces mêmes molécules sous le contrôle de promoteurs spécifiques pourrait s'avérer nécessaire. Ces souris permettraient de démontrer clairement l'implication de la ou les molécules étudiées.

Finalement, si aucune chémokine ou récepteur à chémokine ne présente un niveau d'expression particulier, l'utilisation de la technique d'hybridation soustractive sera utilisée afin d'identifier le ou les gènes impliqués dans le développement du phénotype observé au niveau des ganglions lymphatiques de la souris LckOM. Cette technique permet de limiter le nombre de gènes (ADN complémentaires) suspects à ceux dont l'expression est restreinte aux ganglions de la souris LckOM.

## Conclusion

La reconstitution immunitaire post-greffe médullaire est quantitativement et qualitativement dépendante de la présence d'une fonction thymique efficace et de la présence d'un espace périphérique adéquat. De plus, la GVH affecte de façon importante ces deux éléments ce qui limite la reconstitution immunitaire des patients souffrant de GVH. Peu d'information est disponible concernant les mécanismes par lesquels la GVH affecte la fonction thymique et le nombre de niches périphériques. L'étude de la souris LckOM devrait permettre de définir clairement 1) les raisons pour lesquelles le thymus est le seul organe pouvant soutenir la maturation des lymphocytes T, 2) comment une telle maturation peut être stimulée au niveau des ganglions lymphatiques et finalement 3) permettre de définir la structure des niches périphériques. Ces données devraient permettre de déterminer comment la GVH affecte ces systèmes et donc comment la GVH affecte la reconstitution immunitaire des patients greffés médullaires.

L'OM est une cytokine dont les effets pleïotropiques provoquent des effets secondaires importants, ce qui empêche son utilisation directe à des fins thérapeutiques. Par contre, nous prévoyons que l'étude de la production extrathymique des cellules T au niveau des ganglions lymphatiques de la souris LckOM permettra d'élargir nos connaissances sur le développement des lymphocytes T. Ce modèle permettra éventuellement de définir les conditions requises au développement extrathymique des lymphocytes T, ce qui pourrait s'avérer déterminant à une approche thérapeutique visant à faciliter la reconstitution immunitaire chez des patients lymphopéniques. De

plus, la compréhension des mécanismes par lesquels une exposition chronique à l'OM permet l'augmentation du nombre de niches périphériques pourrait également s'avérer cliniquement utile. L'étude de ce modèle permettra de pallier aux deux principaux obstacles reliés aux déficiences immunologiques observées chez les patients greffés médullaires : l'atrophie thymique et la diminution du nombre de niches périphériques disponibles. Finalement, il est possible d'émettre l'hypothèse selon laquelle une augmentation du nombre de niches périphériques chez des individus âgés serait suffisante à maintenir un répertoire de cellules T diversifié et donc à améliorer la condition immune des ces personnes. En conclusion, le maintien d'une production constante de cellules T et d'un espace périphérique suffisant chez des individus âgés et greffés médullaires permettra de réduire l'incidence de certaines maladies auto-immunitaires, infections récurrentes ou cancers. En plus d'une amélioration notable des conditions de vie de ces personnes, il est possible d'envisager un allongement de leur espérance de vie ainsi qu'une réduction des coûts reliés au vieillissement de la population.

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