# The role of lymphatic endothelial cells in T cell tolerance

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# ABSTRACT

The adaptive immune response can recognize a tremendous variety of antigens. Therefore, robust self-tolerance mechanisms are critical to prevent autoimmunity. Tolerance begins during T cell development in the thymus and continues in lymph nodes. Recently, we demonstrated that lymphatic endothelial cells (LEC) transcribe tyrosinase mRNA and present tyrosinase on MHC I molecules to CD8 T cells, inducing deletional tolerance of tyrosinase-reactive T cells. LEC express intermediate levels of MHC II molecules, suggesting they may play a role in CD4 T cell tolerance as well. Here, we demonstrate that LEC do not directly present epitopes from β-galactosidase or hemagglutinin on MHC II to CD4 T cells, although they do present epitopes from these antigens on MHC I to CD8 T cells. Instead, LEC transfer these antigens to dendritic cells, which induce CD4 T cell anergy. LEC express multiple components of the MHC II processing pathway, including invariant chain and cathepsin L, but do not express H2-M, suggesting that they are unable to load antigenic peptides onto MHC II molecules. MHC II is a ligand for the LAG-3 inhibitory pathway, and we demonstrate that deletional tolerance of β-galactosidase-specific CD8 T cells requires both the PD-1/PD-L1 and LAG-3/MHC II pathways. This suggests that a major role of MHC II molecules on LEC is to maintain CD8 T cell tolerance. We also examined whether LEC-induced tolerance is abrogated under inflammatory conditions. LEC express toll-like receptors 3 and 4, and in vivo ligation of these receptors leads to upregulation of PD-L1 but not CD70, CD80, CD86 or 4-1BBL. TLR3 ligation had no effect on tolerance induction by LEC. Surprisingly, however, treatment with an agonistic CD40 antibody led to the accumulation, rather than deletion, of tyrosinase specific T cells, and bone marrow chimeras demonstrated that this was due to the effects of CD40 on hematopoietic cells.

We also investigated which APC immunogenically presents tyrosinase leading to autoimmune vitiligo, and found that $CD8\alpha^+$ or $CD103^+$ cross-presenting dendritic cells are responsible for vitiligo induction in adults, but not in neonatal animals. Finally, we showed that inhibiting LEC-induced tolerance to tyrosinase by blocking the PD-1/PD-L1 pathway enhances melanoma immunotherapy.

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| Aire | Autoimmune regulatory element |
| AP-2 | Adapter protein 2 |
| APC | Antigen presenting cells |
| β-gal | β-galactosidase |
| B6 | C57Bl/6 |
| Batf3 | Basic leucine zipper transcriptional factor ATF-like 3 |
| BEC | Blood endothelial cells |
| Bg1 | β-galactosidase-specific CD8 T cells |
| Bg2 | β-galactosidase-specific CD4 T cells |
| BMDC | Bone marrow-derived dendritic cell |
| BrdU | Bromodeoxyuridine |
| BSA | Bovine serum albumin |
| C-LEC | Colon resident LEC |
| CFSE | 5-carboxyfluorescein diacetate, succinimidyl ester |
| CIITA | Class II transactivator |
| CLIP | Class II associated Ii peptide |
| Clone 4 | Hemagglutinin-specific CD8 T Cells |
| CPD | Cell proliferation dye |
| CTV | Cell trace violet |
| D | Day |
| D-LEC | Diaphragm resident LEC |
| DAMP | Damage associated molecular patterns |

| | |
|---|---|
| DAPI | 4',6-Diamidino-2-Phenylindole |
| DC | Dendritic cells |
| DMSO | Dimethyl sulfoxide |
| ER | Endoplasmic reticulum |
| eTAC | Extra-thymic Aire-expressing cells |
| FACS | Fluorescence activated cell sorter |
| FCS | Fetal calf serum |
| FH | Tyrosinase-specific CD8 T cells |
| FMO | Fluorescence minus one |
| FRC | Fibroblastic reticular cells |
| GM-CSF | Granulocyte macrophage colony stimulating factor |
| gMFI | Geometric mean fluorescence intensity |
| Gy | Gray |
| HA | Hemagglutinin |
| HUVEC | Human umbilical vein endothelial cells |
| ICAM | Intercellular adhesion molecule; |
| iFABP | Intestinal fatty acid binding protein |
| IFN | Interferon |
| Ii | Invariant chain |
| Ins2 | Insulin 2 |
| IP | Intraperitoneal |
| IV | Intravenous |
| kg | Kilogram |
| LAG-3 | Lymphocyte activation gene-3 |

| | |
|---|---|
| LEC | Lymphatic endothelial cells |
| LN | Lymph node |
| LN-LEC | Lymph node resident LEC |
| LNSC | Lymph node stromal cells |
| LPS | Lipopolysaccharide |
| LTβR | Lymphotoxin β receptor |
| Lyve-1 | Lymphatic vessel endothelial hyaluronan receptor 1 |
| MACS | Magnetic activated cell sorting |
| MAdCAM-1 | Mucosal addressin cell adhesion molecule 1 |
| MDP | Melanocyte differentiation proteins |
| mg | Milligram |
| MHC I | Major histocompatibility complex, class I |
| MHC II | Major histocompatibility complex, class II |
| MIIC | MHC II compartment |
| mTEC | Medullary thymic epithelial cells |
| ng | Nanogram |
| NO | Nitric oxide |
| OT-I | Ova-specific CD8 T cells |
| OVA | Ovalbumin |
| PAMP | Pathogen associated molecular patterns |
| PBS | Phosphate buffered saline |
| PD-1 | Programmed death 1 |
| PD-L1 | Programmed death ligand 1 |
| Poly(I:C) | Polyinosinic:polycytidylic acid |

| | |
|---|---|
| Ppy | Pancreatic polypeptide |
| Prox1 | Prospero homeobox 1 |
| PTA | Peripheral tissue antigens |
| RAG | Recombinase activating gene |
| S1P | Sphingosine-1-phosphate |
| S1PR1 | Sphingosine-1-phosphate receptor-1 |
| TAP | Transporter associated with antigen presentation |
| TCR | T cell receptors |
| TGF-β | Transforming growth factor-β |
| TLR | Toll-like receptor |
| TNFα | Tumor necrosis factor α |
| $T_{reg}$ | Regulatory T cell |
| TS1 | Hemagglutinin-specific CD4 T cells |
| μg | Microgram |
| VEGF | Vascular endothelial growth factor |

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# INTRODUCTION

## Antigen Presenting Cells and T cell tolerance induction in the thymus and lymph nodes

T cell receptors (TCR) are stochastically generated in the thymus, which enables the immune system to recognize a tremendous diversity of foreign antigens. However, it also creates T cells with the potential to recognize and attack host tissues expressing normal self-antigens. To prevent autoimmunity, T cells that recognize self-antigens are tolerized through intrinsic mechanisms such as deletion, anergy, and induction of a regulatory T cell ($T_{reg}$) phenotype, or through extrinsic suppression by already differentiated $T_{reg}$[1]. Tolerance induction occurs in the thymus at the time of T cell development, but also occurs in lymph nodes (LN) draining peripheral tissues. Deletion and $T_{reg}$ induction are thought to be the dominant mechanisms of tolerance in the thymus, while anergy, deletion and $T_{reg}$ induction all occur in LN[2]. Tolerance is induced by antigen presenting cells (APC) that present ubiquitously expressed or tissue-restricted antigens on MHC molecules. While it was initially thought that dendritic cells (DC) were the primary tolerance-inducing APC, it has become clear recently that several other cell types can also serve this role. Among them are the lymphatic endothelial cells (LEC) that reside in LN. This work investigates the role of LEC in CD4 and CD8 T cell tolerance, the functionality of the MHC II antigen presentation pathway in LEC, whether inflammation alters the tolerogenic properties of LEC, and the effects of bypassing or overwhelming LEC-induced tolerance.

## Antigen presentation on MHC I and MHC II molecules

T cells recognize peptides presented in the context of an MHC molecule. CD8 T cells recognize antigens presented by MHC I molecules, while CD4 T cells recognize

antigens presented by MHC II molecules. MHC I molecules are composed of a polymorphic heavy chain stabilized by $\beta_2$-microglobulin. MHC I molecules are formed in the endoplasmic reticulum (ER), where they are stabilized by chaperone proteins until an 8-10 amino acid peptide binds[3]. Intracellular or membrane-bound proteins are degraded by cytosolic and nuclear proteasomes, and the resulting peptides are transported into the ER by the transporter associated with antigen presentation (TAP)[4]. Tapasin links the nascent MHC I molecules with TAP, and facilitates peptide loading[5–7]. Traditionally, antigens presented on MHC I molecules were thought to originate from the cell presenting them. It is now appreciated that some specialized APC can present endocytosed antigens on MHC I through a process known as cross-presentation[8]. $CD8\alpha^+$ and $CD103^+$ DC are particularly efficient at this process[9–13]. Endocytosed antigens can either be degraded in the cytosol by proteasomes or in endosomes by cathepsin L and other proteases, and can be loaded onto MHC I in either the ER or endosomes[8,14–18]. Once the MHC I molecule has bound a peptide, it is transported through the Golgi to the plasma membrane[19]. MHC I antigen presentation occurs constitutively in all nucleated cells, including LEC, and is an important aspect of their ability to induce antigen-specific tolerance in CD8 T cells[19,20].

Constitutive expression of MHC II molecules is restricted to professional APC including DC, B cells, and macrophages, as well as a few other specialized cell types, including LEC[19,21]. The α and β chains of the MHC II molecule are synthesized in the ER, where they bind invariant chain (Ii), which blocks the peptide-binding groove and stabilizes the complex. The immature Ii-MHC II complex can either go directly to the late endosomal MHC II loading compartment (MIIC) from the trans-Golgi network, or it can be initially transported to the plasma membrane[22–25]. The cytoplasmic tail of Ii contains

two di-leucine sorting motifs, which are bound by the adapter protein 2 (AP-2) at the plasma membrane or by adapter protein 1 in the trans-Golgi[26–28]. The Ii:MHC II complexes at the plasma membrane are internalized through clathrin mediated endocytosis[26,27], and targeted to the MIIC. In the MIIC, Ii is degraded by cathepsin L or S, until only the class II-associated Ii peptide (CLIP) remains in the MHC II peptide binding groove[29–32]. H2-M (also known as HLA-DM in humans) facilitates the exchange of CLIP for antigenic peptides, and the activity of H2-M is modulated by a homologous molecule known as H2-O in mice or HLA-DO in humans[33–35]. Peptides presented by MHC II molecules were originally thought to derive from endocytosed or membrane-bound proteins; however, it is now appreciated that cytoplasmic proteins can be targeted into the MIIC through autophagy[36–38]. Once loaded, MHC II complexes are transported to the plasma membrane. Ii-free mature MHC II molecules are internalized from the cell membrane through an AP-2, clathrin, and dynamin-independent pathway into $Arf6^{+}Rab35^{+}$ recycling tubular endosomes[39]. The less acidic and less proteolytic environment in the early endosomes favors the generation of a different set of peptides from that generated in late endosomes[40,41]. These peptides can be loaded onto empty recycling MHC II molecules independently of H2-M, and the lack of H2-M in the early endosomes may allow lower affinity peptides to remain bound to MHC II and be presented[41–46]. The functionality of the MHC II antigen-processing pathway has been extensively studied in cell lines and professional APC[3,19,47–49]. LEC express surface MHC II molecules[21], but the expression and functionality of other components of the MHC II presentation pathway in LEC has not yet been investigated. This was a major issue addressed in this thesis.

**Antigen acquisition, cross-presentation and tolerance induction by DC**

The MHC I antigen presentation pathway was traditionally thought to predominantly present cytosolic and nuclear proteins, while the MHC II antigen presentation pathway was specialized for the presentation of endocytosed and membrane bound proteins[50]. More recently, it has become appreciated that peptides from cytosolic proteins can be loaded into the MHC II presentation pathway through autophagy, and peptides from endocytosed antigens can be loaded into the MHC I presentation pathway through cross-presentation [8,36–38]. Although many types of cells, including LEC, can weakly cross-present antigens[51,52], DC are particularly efficient at this process and are likely the major cross-presenting cell type in vivo[53]. In particular, $CD8\alpha^+$ resident DC and $CD103^+$ migratory DCs are most efficient at antigen cross-presentation[9–13]. Basic leucine zipper transcriptional factor ATF-like 3 (Batf3)-deficient mice lack both $CD8\alpha^+$ and $CD103^+$ DCs, and DC from these mice are deficient in cross-presentation and have an impaired ability to generate anti-viral and anti-tumor CD8 T cell responses[10,54].

Early work on peripheral tolerance induction focused on DC which engulf apoptotic cells in the peripheral tissues and cross-present the acquired peripheral tissue antigens (PTA) in the draining LN[55]. Initial work by Kurts et al demonstrated that in the steady-state, pancreatic antigens are cross-presented in the draining LN, leading to antigen-specific CD8 T cell deletion[56–58]. However, a combination of toll-like receptor (TLR) ligation and CD4 T cell help prevented CD8 T cell deletion and induced autoimmune diabetes[59,60]. Additionally, in other models, the provision of CD4 T cell help or TLR ligation also transiently prevents CD8 T cell tolerance[61–63]. This led to the idea that when DC encounter TLR ligands or receive help from CD4 T cells the DC undergo a

maturation process which upregulates costimulatory molecules and cytokine production, and subsequently leads to immunogenic instead of tolerogenic CD8 T cell activation[64–67]. This suggests that other tolerogenic cells such as LEC could potentially be matured into immunogenic APC, and this question will be examined in this thesis.

In addition to inducing tolerance in the periphery, DC also induce tolerance in the thymus. DC can acquire antigen in the periphery and migrate into the thymus[68], or thymic resident DC can capture circulating antigen[69]. DC can also acquire PTA transcribed by medullary thymic epithelial cells (mTEC)[70–75]. The exact mechanism of antigen transfer between mTEC and DC is unclear, but is enhanced by the presence of the autoimmune regulatory element (Aire)[73]. DC present these antigens to developing T cells, leading to tolerance. Cross-tolerance is a mechanism by which DC are specialized for acquiring antigen from other cells and presenting it to induce T cell tolerance in both the thymus and periphery.

**Direct Expression of Peripheral Tissue Antigens by LEC and other APC**

All APC have the ability to present endogenous antigens derived from ubiquitously expressed proteins, but vary in their ability to present PTA, which are defined as antigens from proteins normally expressed in fewer than 5 tissues[76]. Additionally, we have defined PTA as antigens that have no known function in the APC being studied, to exclude lineage-related antigens. A particular challenge has been to understand how tolerance to PTA would develop, since it is expected that these antigens will be largely absent from the thymus. Early models suggested that tolerance to PTA primarily occurred in the LN, where DC could acquire and present antigens from the draining tissue[55]. However, it was subsequently shown that mTEC transcriptionally

express PTA in the thymus[77,78]. More recently, several groups including our own have shown that multiple cell subpopulations in LN also transcriptionally express PTA. PTA are expressed by extrathymic Aire expressing cells (eTAC) and several LN stromal cell (LNSC) subsets including LEC, blood endothelial cells (BEC), and fibroblastic reticular cells (FRC)[79–81]. Interestingly, each of these subpopulations presents distinct PTA, although the overall size and overlap of their PTA repertoires has not been determined. Nonetheless, this mechanism broadens the presentation of PTA to all LN, not just those draining a particular tissue, and enables efficient system-wide peripheral tolerance induction.

The transcriptional regulation of PTA expression is best understood in mTEC in the thymus, where it is controlled by Aire[78]. Mutations in Aire cause the multi-organ autoimmune disease known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED)[82,83]. Using single-cell PCR assays, it was demonstrated that Aire stochastically induces expression of a PTA in 1-3% of total mTEC[84,85]. Limiting the number of PTA expressed in each cell is likely advantageous to ensure that each antigen is adequately represented on the limited number of MHC molecules on the cell surface, and to avoid possible deleterious consequences for mTEC functionality from expressing a large number of functionally specialized proteins. Some PTA transcripts have different start sites in mTEC compared to peripheral tissues, and Aire-regulated genes within a cell are clustered based on chromosomal position rather than by cell of origin[76,84,86]. These results suggest that Aire operates by opening up a region of the chromosome to additional transcriptional regulators, rather than inducing mTEC to differentiate towards and express PTA from an alternative cell lineage. Interestingly, while Aire also regulates PTA expression in eTAC, the PTA repertoires of mTEC and

eTAC are distinct[79], suggesting that these additional transcriptional regulators may differ between the two cell types.

The transcriptional regulation of PTA in LNSC is not well understood. Aire is not expressed in LEC, FRC, or BEC[80,81], and expression of PTA in these subsets does not change in Aire$^{-/-}$ mice[80]. Aire is a member of the SAND family of transcription factors[87], and Yip et al[88] demonstrated that Deaf1, another SAND family member, controls the expression of 600 genes in LNSC of pancreatic LN. Most of these genes were distinct from genes regulated by Aire in eTAC. Deaf-1 regulated genes included pancreatic polypeptide (Ppy) and insulin 2 (Ins2), which are PTA normally expressed in pancreatic islets. Ppy and Ins2 are primarily expressed in LEC and FRC, respectively, and at much lower levels in CD31$^{neg}$podoplanin$^{neg}$ cells[80,89]. However, Deaf1 is expressed in all LNSC subsets[81], suggesting that additional transcriptional regulators that differ among these subpopulations control the specificity of PTA expression. Deaf1 also controls PTA translation by regulating the transcription of the eukaryotic translation initiation factor *Eif4g3*[89]. Further work is needed to determine the extent to which Deaf1 controls expression of PTA in non-pancreatic LN, and whether other members of the SAND family also play a role in controlling PTA expression in LEC and other LNSC. The overall PTA repertoire of LEC and other LNSC remains to be determined, as does the pattern of PTA expression in individual LEC. While PTA expression in mTEC provides a logical model for how PTA expression in LEC may operate, the master transcriptional regulator is different and future studies will illuminate what other similarities and differences exist.

**Forms of T cell tolerance**

T cell tolerance can take many forms, including deletion, anergy, or Treg induction. The form of tolerance induced has been shown to depend on several factors, including TCR avidity, availability of costimulatory or inhibitory pathways, and the cytokine environment[71,90–94]. mTEC, DC, and eTAC have all been shown to induce multiple forms of tolerance[2,79,95,96], while to date LEC have only been shown to induce deletion in vivo[21]. A question of interest is whether LEC can also induce other forms of tolerance, based on the molecules they express or the particular microenvironmental niche in the LN that they occupy.

**LEC induce tolerance through the inhibitory PD-1/PD-L1 pathway combined with a lack of costimulation**

The demonstration that LEC express PTA and function as tolerance-inducing APC in LN for CD8 T cells was the culmination of our laboratory's work over several years to understand tolerance to PTA expressed in both melanocytes and melanomas, termed melanocyte differentiation proteins (MDP). Tyrosinase is a MDP that has been identified as a target in both autoimmune vitiligo and melanoma immunotherapy[97–99]. Our lab initially became interested in mechanisms of tolerance to tyrosinase-specific T cells while investigating methods to enhance anti-melanoma immune responses. Tyrosinase-specific T cells have been isolated from the peripheral blood of melanoma patients[100,101], and tyrosinase is one of several MDP being targeted for active vaccination approaches[102–104]. However, since tyrosinase is also a normal melanocyte self-protein, tolerance mechanisms will hinder the development of an anti-melanoma immune response. Therefore, our lab began investigating how tyrosinase-specific T cells (known

as FH T cells) are tolerized, with the goal of eventually inhibiting the tolerogenic pathways and thereby enhancing the efficacy of anti-tumor vaccination approaches.

Our laboratory demonstrated that tolerance to tyrosinase is not induced in the thymus, and is not induced in the periphery by conventional DC or Langerhans cells[20]. Instead, radioresistant LEC transcribe tyrosinase mRNA and present $Tyr_{369}$ to CD8 T cells, inducing initial T cell proliferation followed by complete deletion[20,80]. While this process of abortive proliferation has been shown in many models of CD8 deletional tolerance, the mechanisms involved in driving this outcome have been somewhat unclear. Some previous work had established that peripheral tolerance could be induced by antigen engagement in the absence of costimulation[105–109]. Other studies pointed to the engagement of inhibitory pathways, including the PD-1/PD-L1, LAG-3/MHC II, or BTLA/HVEM pathways[110–114]. While investigating the mechanisms involved in LEC-induced deletion of CD8 T cells, we found that both a lack of costimulation and engagement of inhibitory pathways were involved and interdependent[21]. In collaboration with Dr. Eric Tewalt, I demonstrated that LEC do not express any of the costimulatory molecules that normally drive immunogenic accumulation of activated T cells, such as CD80, CD86, OX-40L, 4-1BBL, or CD70. However, they express multiple ligands that can activate inhibitory pathways, and express a particularly high level of PD-L1. Indeed, deletion of FH T cells is strictly dependent on engagement of the PD-1/PD-L1 pathway. However, it is antigen activation in the absence of co-stimulation that leads to rapid, high-level upregulation of PD-1 on FH T cells, which is required for deletion to occur. PD-1 ligation in turn prevents the upregulation of the IL-2R, leading to CD8 T cell death. High level PD-1 upregulation and T cell deletion can be prevented if costimulatory signals are provided using an agonistic anti-4-1BB antibody, demonstrating that active

inhibition and the lack of costimulation synergize to induce deletion. Importantly, FH T cells that are rescued from LEC-mediated deletion gain effector function and induce autoimmune vitiligo. Although I collaborated with Dr. Tewalt to determine the mechanism of LEC-induced deletion, this data has been published[21] and will not be included in this thesis. Our work unites previous work that showed that both inhibitory pathways and deficient costimulation could lead to tolerance. Given that LEC express multiple PTA, these results suggest that impairment of LEC-induced tolerance could have a role in the induction of numerous autoimmune disorders. PD-1$^{-/-}$ mice develop late-onset autoimmunity[115,116], suggesting that although other inhibitory pathways or tolerogenic cells can partially compensate for the loss of the PD-1 pathway initially, self-reactive T cells escape tolerogenic mechanisms and are poised to induce autoimmunity. While there is considerable interest in PD-1/PD-L1 as a mechanism to suppress pathology in peripheral tissue and in the genesis of clonal exhaustion in tumors[117–119], these results establish a central role of this pathway in peripheral tolerance induction.

**LEC localized in peripheral tissue and LN subregions differ in their ability to induce tolerance**

LEC form lymphatic vessels in peripheral tissues in addition to lymphatic sinuses in the LN. I assisted Dr. Jarish Cohen in determining whether tissue lymphatics share the tolerogenic properties of LEC in the LN (LN-LEC), although this work has been published[120] and will not be incorporated into this thesis. We found that the LEC which form vessels in the diaphragm (D-LEC) and colon (C-LEC) express substantially less tyrosinase mRNA than LN-LEC, and do not induce proliferation of FH T cells in vitro[120]. Additionally, 6 out of 7 other PTA tested were more highly expressed in LN-LEC compared to D-LEC or C-LEC. Furthermore, D-LEC and C-LEC express substantially

less PD-L1 than LN-LEC. Collectively, these results suggest that tolerance induction is a specialized property of LN-LEC not shared by LEC in tissue lymphatics.

Within the LN, LEC are found in the subcapsular sinus, the cortical sinus, and the medullary sinus. The afferent lymphatics drain into the subcapsular sinus, which forms a thin structure at the outer edge of the LN[121]. DC enter the LN parenchyma through the floor of the subcapsular sinus, while T cells in the afferent lymph pass through the subcapsular sinus to the medullary sinus, where they enter the LN[122]. To exit the LN, lymphocytes first enter blunt-ended cortical sinuses, which are interspersed throughout the T and B cell zone[123]. Lymphatic fluid in the cortical sinuses flows towards the medullary sinus, and lymphocytes ultimately leave the LN through the medullary sinus in the efferent lymph[124–126,123,127]. We showed that LEC in these different sinuses can be distinguished by differential expression of PD-L1, ICAM-1, MAdCAM-1, and LTβR: subcapsular sinus LEC are PD-L1$^{hi}$ICAM-1$^{hi}$MAdCAM-1$^{+}$LTβR$^{lo}$, medullary sinus LEC are PD-L1$^{hi}$ICAM-1$^{hi}$MAdCAM-1$^{neg}$LTβR$^{+}$, and cortical sinus LEC are PD-L1$^{int}$ICAM-1$^{int}$MAdCAM-1$^{neg}$LTβR$^{+}$ [120]. In addition to expressing high levels of PD-L1, medullary LEC are the only subset that expressed a sufficient level of tyrosinase to activate FH T cells. Since the medullary sinus is an exit from the LN, this suggests a model in which LEC function as gatekeepers, engaging and inducing deletion of activated self-reactive CD8 T cells as they attempt to leave.

The specific microenvironmental influences that control the phenotypic distinctions between LEC in the periphery and in different LN sinuses remain to be fully understood. Within the LN, we have shown that high level expression of PD-L1 on medullary LEC and MAdCAM-1 on subcapsular sinus LEC is dependent on LTβR

signaling and B-cells, but not DC, and that these two signals are independent of one another[120]. Interestingly, the presence of T cells showed the opposite effect. Tyrosinase expression was not affected by any of these manipulations. We also found that, while a medullary region is present by postnatal day 7, LEC from these neonatal mice do not present tyrosinase and the expression of PD-L1 on LEC is substantially lower than that in adult mice. Combined, these results indicate that the tolerogenic phenotype of LN-LEC develops after the neonatal period in a way that is influenced by, but not entirely dependent on, the effects of lymphocytes and LTβR signaling. Previous work has shown that thymic tolerance is most critical during the perinatal period[128] and that naïve neonatal T cells directly access peripheral tissues and are tolerized there, instead of in draining LN[129]. This suggests that the relative importance of each tolerogenic site and the associated tolerogenic APC shifts from neonatal to adult animals[130]: during the initial waves of neonatal T cell development the majority of tolerance occurs in the thymus, and T cells specific for PTA not expressed in the thymus can be tolerized directly in the peripheral tissues. Later in life, as thymic output decreases and the peripheral tissues become inaccessible to naïve T cells, peripheral tolerance by LNSC, eTAC, and DC in LN becomes relatively more important to ensure continual tolerance of circulating T cells.

**Functions of LEC within LN**

In addition to tolerizing self-reactive T cells, LEC also influence several other aspects of the immune response. LN and tissue LEC are important sources of IL-7[131–134], which is a homeostatic survival cytokine for naïve and memory T cells. IL-7 secretion by LEC is enhanced during lymphopenia, LN remodeling, and revascularization after transplant, and this assists in re-establishing normal LN

architecture and cellularity after an infection or other perturbation[132,133]. IL-7 promotes survival and differentiation of memory CD8 T cells[135,136], suggesting that increased IL-7 production during LN remodeling in the resolution phase of an infection could potentially play a role in enhancing T cell memory. Additionally, both LN-based and tissue-based LEC express IL-7R and respond to IL-7 in an autocrine fashion, which is required for normal lymphangiogenesis and efficient lymphatic drainage[134]. IL-7R$^{-/-}$ animals have thin and highly branched lymph vessels, suggesting IL-7 might stabilize larger lymphatic vessels. IL-7 also induces VEGF-D secretion in cancer cells, suggesting IL-7 may enhance canonical lymphangiogenesis through the VEGF pathway[137,138]. Through these mechanisms, LEC help control the size of the T cell compartment.

LEC also control T cell egress from the LN. Lymphocytes express a receptor for sphingosine-1-phosphate (S1P), a lipid that is present in relatively high concentration in plasma, but is generally at a low concentration in LN. However, LEC synthesize S1P, providing a high local concentration in their vicinity[139]. Expression of the S1P receptor, S1PR1, on lymphocytes is endocytically downregulated by prolonged exposure to S1P in plasma and by the activation marker CD69[140,141], but is re-expressed upon entry into LN. Signaling through S1PR1 as lymphocytes encounter LEC allows them to overcome CCR7-mediated retention signals and leave the LN[124,126]. S1P is upregulated when tenascin C, a marker of inflammation induced by a variety of pathogen or damage associated molecular patterns, binds to α9-integrin on LN cortical and medullary sinus LEC, and blockade of α9-integrin inhibits lymphocyte egress[142]. Reciprocal regulation of CD69 and S1P1 prevents activated T cells from leaving the LN during an immune response, and may also serve a similar function during self-tolerance. FH T cells tolerized by LEC undergo several rounds of proliferation before expressing the high-level

of PD-1 that mediates deletion. Therefore, retention in the LN may encourage the T cells to remain in proximity with PD-L1$^{+}$ LEC. Alternatively, self-reactive T cells may leave their initial activating LN, and encounter PD-L1 on LEC in a downstream LN to complete the tolerogenic process. Regardless, production of IL-7 and S1P are two additional ways that LEC regulate the immune response, by controlling the homeostasis of naïve and memory lymphocytes as well as their ability to exit the LN.

**LEC restrain T cell proliferation in response to inflammation**

Inflammation has a myriad of effects on lymphatics and the immune response. TLR ligation induces macrophages to secrete VEGF-C/D, which binds to VEGFR-3, triggering proliferation of tissue LEC and lymphangiogenesis[143,144]. IFNγ, TNFα, and TLR ligands increase expression of chemokines and adhesion molecules on LEC, thereby enhancing cell recruitment and migration towards the LN[145–149]. Inflammation has the potential to adversely affect tolerance, as inflammatory cytokines such as IFNγ and TNFα can mature DC, leading to the upregulation of costimulatory molecules and the potential for immunogenic presentation of self-antigens acquired in the periphery. LEC help dampen cytokine-induced DC maturation, as TNFα stimulated LEC decrease the expression of the costimulatory molecule CD86 on immature or TNFα stimulated DC and decrease the ability of the DC to stimulate T cell proliferation[150]. Although the exact mechanism is uncertain, it requires adhesion of the DC to the LEC through ICAM-1/Mac-1 interactions. TNFα stimulated LEC do not affect DC matured with lipopolysaccharide (LPS), suggesting that this mechanism only occurs in the absence of pathogen associated molecular patterns (PAMP), thus potentially contributing to the resolution of inflammation after clearance of infection. LN LEC and FRC also respond to the pro-inflammatory cytokines IFNγ and TNFα by secreting nitric oxide (NO), which limits the

proliferation but not effector activity of already activated T cells[151,152]. Additionally, IFNγ-stimulated cultured human LN-LEC produce indoleamine 2,3 dioxygenase and suppress CD4 T cell proliferation[153]. Both of these may curtail excessive T cell expansion to prevent disruption of LN architecture. Interestingly, NO production by FRC also reduced proliferation of self-reactive OT-I CD8 T cells in vivo[152]. Although the effects on deletion were not investigated, this mechanism may also ensure that the proliferating self-reactive T cells do not expand too rapidly and potentially overwhelm the tolerogenic capacity of the LNSC. However, self-reactive CD8 T cells generally do not produce IFNγ or TNFα after tolerogenic activation[154] (unpublished data), so the relevance of this mechanism may also vary depending on the characteristics and activation state of the T cells.

TLR ligation also alters the ability of LEC and FRC to induce tolerance. Primary murine LEC and FRC express TLR3, and treatment with the TLR3 ligand polyinosinic:polycytidylic acid (Poly(I:C)) upregulates PD-L1 on LEC and FRC but does not change expression of CD80 or CD86[81]. Poly(I:C) downregulates the PTA OVA in FRC of iFABP-OVA mice, leading to reduced OVA-specific CD8 T cell proliferation in vitro; however, the functional consequences in vivo were not evaluated. Interestingly, other PTA were either down or up-regulated by TLR3 signaling in both LEC and FRC. PTA downregulation may be an attempt to maintain the ignorance of self-reactive T cells until after inflammatory conditions have passed, while the upregulation of PD-L1 may help enforce tolerance of any self-reactive T cells that get activated. The significance of PTA upregulation remains unclear. Additionally, although Poly(I:C) does not upregulate costimulatory molecules on LEC, further work is needed to determine if other inflammatory circumstances can lead to immunogenic activation of T cells recognizing

PTA or endocytosed antigens presented by LEC. Combined, these studies suggest that LN-LEC respond to inflammation by dampening T cell proliferation, which likely helps ensure continued T cell tolerance and protects LNSC from damage during an overly vigorous immune response. In this thesis, I will test whether TLR3, TLR4 or CD40 ligation can induce LEC maturation and alter the induction of tolerance in vivo.

**LEC-induced tolerance: a new target for cancer immunotherapy?**

Studies performed to date suggest that LEC can enhance tumor growth by increasing tumor metastasis to the LN through the formation of tumor draining lymphatics[155]. Our work demonstrating that LEC induce tolerance of tyrosinase-specific T cells also suggests that PTA presented by LEC may tolerize tumor-reactive T cells and limit the anti-tumor immune response. Additionally, LEC have been shown to cross-present tumor antigens and induce dysfunctional CD8 T cell responses[52]. This implies that inhibiting LEC-induced tolerance may provide a method of boosting anti-tumor immunotherapy. Indeed, while tyrosinase is a target of melanoma immunotherapy[102–104], LEC-mediated self-tolerance to tyrosinase limits active immunotherapy[156–158]. PD-1 inhibitory antibodies represent one approach to mitigating these effects, and incidentally already show great promise as a monotherapy independently of cancer vaccines in clinical trials[119,159]. These antibodies are currently being tested for their ability to revitalize exhausted effector T cells and prevent tumor immune evasion[159]. However, our work has established that PD-1 blockade also inhibits LEC-induced tolerance, and this suggests that anti-PD-1 blockade may particularly complement efforts to specifically target tyrosinase using cancer vaccines or T cell adoptive therapy. This combination therapy may provide a synergistic benefit by inhibiting tolerance and simultaneously preventing T cell exhaustion. Increasing our understanding of the role of LEC in T cell

tolerance may provide new opportunities to enhance cancer immunotherapies. One goal of this thesis is to test the hypothesis that inhibiting LEC-induced tolerance by blocking the PD-L1 pathway will enhance melanoma immunotherapy and lead to enhanced tumor control.

# THESIS RATIONALE AND PROPOSAL

Our lab has demonstrated that LEC induce tolerance to tyrosinase-specific CD8 T cells, but their role in CD4 T cell tolerance has not been examined. MHC II expression is traditionally thought to be restricted to professional APC as well as a few other types of cells, including thymic epithelial cells and some endothelial cells. LEC express MHC II, but the functionality of the MHC II pathway in LEC and the ability of LEC to induce CD4 T cell tolerance is completely unknown. Therefore, the major goal of this project was to investigate whether LEC can present epitopes from PTA on MHC II, and to determine if LEC play a role in CD4 T cell tolerance. To address these questions, we created a number of mouse model systems where β-galactosidase (β-gal) or hemagglutinin (HA) were expressed as PTA in LEC. Transgenic CD4 and CD8 T cells specific for β-gal or HA allowed us to investigate the ability of LEC to mediate CD4 and CD8 tolerance. We adoptively transferred β-gal or HA specific CD4 T cells into mice expressing β-gal or HA in LEC, and determined whether the cells were tolerized, remained ignorant, or were immunogenically activated in vivo. We also investigated the form of tolerance induced. We determined whether CD4 tolerance was due to direct MHC II antigen presentation by LEC, or whether other cells acquired antigens expressed in LEC. To determine if LEC were presenting MHC II peptides, we used MHC $II^{-/-}$ bone marrow chimeras to eliminate the potential for antigen presentation by hematopoietically-derived APC. Additionally, we used flow cytometry-based cell sorting to isolate LNSC and DC subpopulations from mice expressing β-gal or HA in LEC. These APC were co-cultured with antigen-specific T cells to determine which APCs present β-gal or HA on MHC I and MHC II molecules. We used qPCR, flow cytometry and immunofluorescence microscopy to investigate whether LEC have a deficiency in any component of the MHC

II antigen presentation pathway. These studies elucidated how CD4 T cells specific for PTA expressed in LEC are tolerized. In other models, help from non-tolerant CD4 T cells can break CD8 T cell tolerance, highlighting the importance of tolerizing both CD4 and CD8 T cells[63]. This work substantially enhanced our understanding of the role of LEC in T cell tolerance.

Additionally, we investigated how LEC-induced CD8 T cell tolerance is altered when steady-state conditions are perturbed. Early work with DC-induced tolerance demonstrated that steady-state DC acquire tissue antigens and migrate to the LN, where they present these antigens in a tolerogenic fashion. During an infection, recognition of PAMP and damage associated molecular patterns (DAMP) induces the upregulation of costimulatory molecules, which switches DC from tolerogenic to immunogenic APC. LEC and FRC express a variety of TLR that recognize DAMP and PAMP[81]. Additionally, LEC and FRC have been reported to express CD40[81], which a costimulatory molecule that activates professional APC. Therefore, we investigated whether inflammatory conditions mimicked by TLR or CD40 ligation could induce LEC or FRC maturation and induce immunogenic rather than tolerogenic CD8 T cell activation.

The tyrosinase-specific FH CD8 T cells developed by our lab have also been used as a model for autoimmune vitiligo. When FH mice are crossed with tyrosinase$^{+}$ mice, vitiligo develops beginning in the neonatal period[97]. Vitiligo induction is dependent on CD8 T cells, with CD4 T cells playing a negative regulatory role. However, it is not understood how these T cells become activated in an immunogenic context. Although DC do not present tyrosinase in the steady-state, it is likely that they cross-present tyrosinase in the context of autoimmune melanocyte destruction. We tested whether DC

cross-presentation of tyrosinase was important for vitiligo development by comparing disease progression in tyrosinase$^{+}$ FH mice with BatF3$^{-/-}$ tyrosinase$^{+}$ FH mice, which lack the cross-presenting CD8α$^{+}$ and CD103$^{+}$ DC. We investigated which cells present antigen, as well as the activation status and infiltration of FH T cells in the skin to test whether DC cross-presentation of tyrosinase induced greater effector function in FH T cells.

Tyrosinase has been identified as a target for melanoma immunotherapy[102–104]. Additionally, PD-1 blocking antibodies are currently enjoying great success in melanoma clinical trials as a method of revitalizing exhausted tumor-specific T cells[119,159]. Our work demonstrating that LEC induce tolerance of FH T cells through PD-1 suggests that blocking PD-1 in the context of melanoma may enhance cancer immunotherapy by preventing FH T cell deletion. To test this hypothesis, we compared tumor outgrowth in tyrosinase$^{neg}$ (albino), tyrosinase$^{+}$ and PD-L1$^{-/-}$ tyrosinase$^{+}$ mice to determine whether LEC-induced tolerance hinders tumor control.

Combined, these studies illuminated the role of LEC in peripheral T cell tolerance, and the ways in which LEC behave similarly or differently from other tolerogenic APC. This knowledge can eventually be used to target LEC-induced tolerance to either enhance cancer immunotherapy or strengthen tolerogenic mechanisms to prevent autoimmunity.

# MATERIALS AND METHODS

### Mice

C57Bl/6 (B6) and CD45.1 (B6-LY5.2/Cr) were from NCI. MHC II$^{-/-}$ (B6.129-*H2-Ab1*$^{tm1Gru}$ N12) and MHC I$^{-/-}$ (B6.129P2-*H2-Kb*$^{tm1}$ *H2-Db*$^{tm1}$ N12)[160] mice were from Taconic (NIAID Exchange Program). Lyve-1-cre (*B6;129P2-Lyve1*$^{tm1.1(EGFP/cre)Cys}$*/J*)[139], β-gal (B6.129S4-*Gt(ROSA)26Sor*$^{tm1Sor}$/J)[161], EYFP (B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J), and Clone 4 (CBy.Cg-Thy1a Tg(TcraCl4,TcrbCl4)1Shrm/ShrmJ, Jax)[162] mice were from Jax. Bg1[163] and Bg2[164] mice from Christopher Norbury (Pennsylvania State University) were crossed to Thy1.1 mice (Jax). Prox1-creERT2[165] mice were from Taija Makinen (Cancer Research UK), Rosa26$^{tm(HA)1Libl}$ mice[166] were from Roland Liblau (Institut National de la Santé et de la Recherche Médicale), and TS1[167] mice were from Andrew Caton (University of Pennsylvania). Thy1.1 tyrosinase$^{+}$ mice carrying the chimeric MHC I AAD molecule (referred to here as tyrosinase$^{+}$), C$^{38R145L}$ albino (tyrosinase$^{neg}$) and Thy1.2 mice with CD8 T cells recognizing tyrosinase presented by AAD (referred to here as FH) on an albino and tyrosinase$^{+}$ background have been previously described[156]. Batf3$^{-/-}$ mice[10] were crossed to TFH mice to yield Batf3$^{-/-}$ x TFH mice. PD-L1$^{-/-}$ mice[114] were provided by L. Chen, and bred to tyrosinase$^{+}$ mice to create PD-L1$^{het}$ tyrosinase$^{+}$ or tyrosinase$^{+}$PD-L1$^{-/-}$ mice. PD-1$^{-/-}$ mice[114] were provided by T. Honjo, and crossed with mice expressing tyrosinase-specific CD8 T cells. Thy1.2 iFABP-OVA[168] mice were provided by V. Vezys. OT-I RAG-1$^{-/-}$ (Taconic) mice were crossed to Thy1.1 mice (Jackson Laboratory). CD40$^{-/-}$ (Jackson Laboratory) and C57Bl/6 mice (NCI-Frederick Animal Production Program) are commercially available. All mice other than Clone 4, Clone 6.5, and Rosa26$^{tm(HA)1Libl}$ are on a B6 background. The BALB/c Clone 4 and Clone 6.5 mice were crossed to B6 Thy1.1 mice, and the resulting BALB/c x B6 background

mice were used for adoptive transfers into Prox1-creER$^{T2}$ x Rosa26$^{tm(HA)1Libl}$ (BALB/c x B6) recipients. Prox1-creER$^{T2}$ was induced with tamoxifen chow (TAM400, Harlan) for 2 weeks prior to experiments, and mice were maintained on TAM400 throughout the course of the experiment. Mice used are listed in Table 1.

**LNSC, DC, and skin isolation**

LN (pooled inguinal, axillary, brachial, cervical and mesenteric) or skin were digested for 45-60 minutes at 37°C using 0.42 U/mL Liberase TM (Roche) and 60 U/mL DNase I (Sigma) in DMEM (Cellgro) with 2% FCS, essential and non-essential amino acids, sodium pyruvate and HEPES. Red blood cells were lysed (Sigma). For LN, CD45$^+$ cells were labeled with CD45 magnetic beads in AwesomeMACS (PBS with 0.5% BSA, 2mM EDTA, L-glutamate, sodium pyruvate, essential and non-essential amino acids, and 4.5 g/L dextrose) and separated using the Deplete AutoMACS protocol (Miltenyi Biotec). For in vitro co-cultures, DC and macrophages were enriched using CD11c$^+$ and CD11b$^+$ beads (Miltenyi Biotec) prior to the CD45 depletion. Cells were stained for either analytical flow cytometry (see below), or electronically sorted using Influx (BD), FACSVantage (BD) or iCyt Reflection (Sony) cell sorters for in vitro co-cultures and qPCR.

**In vitro co-culture**

Antigen-specific T cells were enriched using CD4 or CD8 magnetic beads (Miltenyi Biotec) and labeled with 1 μM CFSE or cell proliferation dye eF670 (CPD, eBioscience). T cells were co-cultured with electronically sorted LNSC, CD45$^+$, B cells, DC or macrophages at a 1:2 (T cell:APC) ratio for 4 days in RPMI-1640 with 10% FCS, 2 mM L-glutamine, 30 mM HEPES, 10 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, 1x

essential and non-essential amino acids, and 0.05 mg/mL gentamycin containing IL-2 (10 U/mL) and IL-7 (1 pg/mL for CD8 T cells and 1 ng/mL for CD4 T cells).

**Adoptive transfer**

Thy1.1$^{+}$ Bg1, Bg2, Clone 4, TS1, or OT-1 or Thy1.2$^{+}$ FH antigen-specific T cells were positively selected with CD4 or CD8 beads or CD25$^{neg}$ CD4$^{+}$ cells were negatively selected using a regulatory T cell isolation kit (Miltenyi Biotec). Cells were labeled with Cell Trace Violet (CTV, Invitrogen), and 1x10$^{6}$ Bg1, Bg2, Clone 4, TS1, or Thy1.2$^{+}$ FH or 5x10$^{5}$ OT-I T cells were injected intravenously. 3 x 10$^{6}$ FH cells were used for tumor control experiments where indicated. Thy1.2$^{+}$ or Thy1.1$^{+}$ CTV-labeled congenic cells were used as an injection control in some experiments. Skin-draining LN (inguinal, axillary, brachial) were harvested from recipient mice 1, 3, 6, 7, 14, or 33 days after adoptive transfer. For tumor control experiments, all mice received 1,500 CU of recombinant human IL-2 every other day for 12 days, beginning at the time the first group of mice in the experiment received an adoptive transfer.

**In vivo antibody and IFNγ treatment**

Mice were injected with 100 μg anti-CD28 (clone 37.51, BioXCell) IP at days 0 and 2. 100 μg anti-LAG-3 (C9B7W, BioXCell) and/or anti-PD-L1 (10B5, University of Virginia Lymphocyte Culture Center or BioXCell) were injected IP at days -1, 1, 3 and 5. IFNγ (5 x 10$^{4}$ U, Peprotech) was injected IV at days -1 and 1. 25 μg anti-4-1BB (3H3) and 50 μg anti-OX40 (OX86)[109] were injected IP at day 1, and 25 μg anti-CD40 (FGK4.5, BioXCell) was injected IP at days 0 and 3. Mice received 25 μg of the agonistic CD40 antibody FGK4.5 (BioXCell) intraperitoneally (IP) every other day beginning the day prior to adoptive transfer or 2 days prior to LNSC isolation. FTY720 (gift from V. Brinkmann,

Novartis Pharma AG, Basel, Switzerland) was administered at 1 mg/kg IP daily. Mice were treated with 100 µg Poly(I:C) intravenously (IV) the day before adoptive transfer or with 250 µg Poly(I:C) IV 2 days before LNSC isolation. Mice treated with immune complexes received either 1.5 µg IL-7 with 7.5 µg anti-IL-7, 1.5 µ IL-15 with 7 ug msIL-15Rα–Fc, 1 µg rhIL-2 with 5 ug anti-IL-2 every other day, or 1 µg of IL-12.

**Bone marrow chimeras**

Mice were irradiated (6.5 Gy x 2) and reconstituted with a minimum of 2 x $10^6$ bone marrow cells depleted of $CD4^+$ and $CD8^+$ T cells (Miltenyi Biotec) to prevent graft-versus-host disease. Chimeras were maintained on sulfa water for 3 weeks, and allowed to reconstitute for at least 8 weeks prior to use.

**Flow cytometry:**

Antibodies used include: Ii(In-1), H2-M (2E5A), Rat IgG1 (R3-34) (all from BD Biosciences); (podoplanin (8.1.1, Biolegend); UEA-1 (Vector labs); CLIP (15G4, Santa Cruz); H2-$O^{169}$ (Mags.Ob3, Lisa Denzin); 10.1.1 (UVA lymphocyte culture center); CD45 (30-F11), CD31 (390), MHC I (AF6-88.5.5.3), MHC II (M5/114.15.2), CD8 (53-6.7), CD4 (GK1.5), Thy1.1 (HIS51), Thy1.2 (53-2.1), CD45.1 (A20), CD11c (N418), CD11b (M1/70), CD80 (16-10A1), CD69 (H1.2F3), CD62L (MEL-14), CD44 (IM7), CD25 (PC61.5), Y-AE (eBioY-Ae), PD-1 (RMP1-30), LAG-3 (eBioC9B7W), BTLA (8F4), Rat IgG2b (eB149/10H5), CD40 (HM40-3), CD70 (FR70), CD80 (16-10A1), CD86 (GL1), 41BBL (TKS-1) (all from eBioscience). Intracellular staining for PD-1, LAG-3, BTLA, Ii, H2-M and H2-O was done using the Cytofix/Cytoperm kit (BD Bioscience), and Ki67 (SolA15) and FoxP3 (FJK-16s) were stained using Treg permeabilization buffers (eBioscience). Annexin V was stained using the eBioscience kit. DAPI (Sigma) or

live/dead aqua (Invitrogen) were used to distinguish live cells. Tyrosinase$_{369}$ HLA-A2 tetramer [5] was used to identify tyrosinase-specific cells. Cells were acquired on a FACSCanto II (BD Biosciences) and data analyzed using FlowJo (Tree Star).

**Cathepsin L assay**

LN-LEC or D-LEC were preincubated with the cathepsin L inhibitor 1-naphthalenesulfonyl-IW-CHO (Calbiochem) or DMSO vehicle control for 20 minutes at 37°C, followed by a 20 minute incubation with the cathepsin L substrate (CBZ-Phe-Arg)$_2$-rhodamine 110 (Invitrogen) at 37°C prior to being acquired on a FACSCanto II.

**Immunofluorescence microscopy**

LNs were enzymatically digested, depleted of CD45$^+$ cells, stained with 10.1.1 extracellularly and cathepsin L (EPR8011, Abcam), biotin-anti-rabbit (Vector labs) and streptavidin Dylite 550 (Thermo Scientific) intracellularly prior to being cytospun onto slides. Images were taken using an Axio Imager 2 with Apotome (Carl Zeiss).

**qPCR**

Cells were electronically sorted into RNA Protect (Qiagen). mRNA was purified using either the RNeasy Mini or Plus Micro kits (Qiagen), and cDNA was synthesized using the iScript cDNA synthesis kit (Biorad). Amplification of I-A$^b$ (H2-Ab1), H2-Mα, H2-DMβ2, and HPRT was performed using iQ SYBR Green Supermix (BioRad) and amplification of I-Eα and HPRT was performed using TaqMan Master Mix (Life Technologies) on a MyiQ qPCR Detection System (BioRad). SYBR Green reactions were run at 95°C for 10 min followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. TaqMan reactions were run at 50°C for 2 min, 95°C for 10min, and 40 cycles of 95°C for 15 sec and 60°C for 1min. Primers used were the following: *Hprt* forward, 5'- AGGTTGCAAGCTTGCTGGT-3', and

reverse, 5'-TGAAGTACTCATTATAGTCAAGGGCA-3'; *H2-Mα* forward, 5'-CTCGAAGCATCTACACCAGTG-3', and reverse, 5'-TCCGAGAGCCCTATGTTGGG-3'; *H2-DMβ2* forward, 5'-GTTGGCTTCTTCAGATGGCG-3', and reverse, 5'-TGCCGTCCTTCTGGGTAGG-3', H2-Ab1 forward, 5' – GGTGTGCAGACACAACTACG, and reverse, 5' – CGACATTGGGCTGTTCAAGC; *Tyrosinase* forward, 5'-CCAGGCTCCCATCTTCAGC; reverse, 5'-CCTGTGAGTGGACTGGCAAAT; *GAPDH* forward, 5'-AGGTCGGTGTGAACGGATTTG and reverse, 5'-TGTAGACCATGTAGTTGAGGTCA. Taqman probes (Life Technologies) used were Mm00446968_m1 for HPRT and Mm00772352_m1 for I-Eα. Ct values for were normalized to HPRT (H2-Mα, H2-DMβ, I-Eα, I-$A^b$) or GAPDH (tyrosinase) and relative expression compared to B cells, dendritic cells, or untreated LEC was reported as $2^{-\Delta\Delta Ct}$.

**BMDC**

Bone marrow cells were isolated from the tibias and femurs of B6 mice, cultured in vitro in RPMI-1640 with 10% FCS, 2 mM L-glutamine, 30 mM HEPES, 0.05 mM β-mercaptoethanol, and 0.05 mg/mL gentamycin, supplemented with 1000 U/mL GM-CSF (BD Biosciences) and 100 U/mL IL-4 (eBioscience). Medium was changed on day 2 and the top half of the medium was changed on day 5 of culture. On day 7, BMDC were harvested, enriched for $CD11c^+$ cells (Miltenyi Biotec), and activated overnight with NIH 3T3 cells expressing $CD40L^{170}$ (provided by R. Lapoint, University of Montreal). Activated BMDC were pulsed with 50 μg/mL of $\beta\text{-gal}_{721\text{-}739}$ (AENLSVTLPAASHAIPHLT, GenScript) for 3 hours, washed, and 1 x $10^5$ BMDC were injected IV.

**Vitiligo scoring**

Juvenile and adult mice were scored twice a week by 2 independent observers, using the criteria for juvenile vitiligo reported by Gregg et al[97] and a modified version of the adult scoring system.

**Tumor implantation**

B16-F1 melanoma cells expressing AAD (B16-AAD) have been previously described[99]. 1 x $10^5$ B16-AAD cells were implanted subcutaneously into the shaved flank of tyrosinase$^{neg}$, tyrosinase$^{+}$FH$^{neg}$ , or tyrosinase$^{+}$PD-L1$^{-/-}$ mice. Tumors were palpated or measured three times a week. Mice were considered tumor free until a tumor could be palpated, and mice were euthanized when exceeded 16 mm in any dimension or ulcerated, in accordance with the guidelines set by the University of Virginia institutional animal care and use committee.

**Statistical Analysis**

Statistical analysis of data was performed using GraphPad Prism 5. P values $< 0.05$ were considered statistically significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Two-tailed unpaired T tests were used for comparisons between 2 groups, a one-way ANOVA with Tukey post-test was used for 3 or more groups, and a two-way ANOVA with Bonferroni post-test was used for experiments with 2 variables.

## Table 1: Mouse strains used

| Antigen expressing mice: | | | | | | |
|---|---|---|---|---|---|---|
| **Full name** | **Referred to as:** | **Antigen expressed:** | **Tolerance induced by:** | **Other sites of antigen expression** | **Thy alelle** | **Notes** |
| Lyve-1-cre x β-gal$^{stop\text{-}flox}$ | Lyve-1 x β-gal | β-gal | LEC | BEC, DC, macrophages, T cells | Thy1.2 | Constitutively active Lyve-1 cre |
| Prox1-creER$^{T2}$ x β-gal$^{stop\text{-}flox}$ | Prox-1 x β-gal | | LEC | Liver, heart | Thy1.2 | CreER$^{T2}$ induced by tamoxifen |
| | Tyrosinase$^{+}$ or Tyrosinase$^{+}$FH$^{neg}$ | Tyrosinase | LEC | Melanocytes | Thy1.1 | Endogenous PTA. AAD$^{+}$ |
| C$^{38R145L}$ | Tyrosinase$^{neg}$ | none | -- | -- | Thy1.1 | Albino (tyrosinase$^{neg}$) mice. AAD$^{+}$ |
| Prox1-creER$^{T2}$ x HA$^{stop\text{-}flox}$ | Prox-1 x HA | HA | LEC | Liver, heart | Thy1.2 | CreER$^{T2}$ induced by tamoxifen. B6 x BALB/c F1 mice used |
| | iFABP-OVA | OVA | FRC | Intestinal epithelial cells | Thy1.2 | |
| Prox1-creER$^{T2}$ x EYFP$^{stop\text{-}flox}$ | Prox1 x EYFP | EYFP | LEC | Liver, heart | Thy1.2 | CreER$^{T2}$ induced by tamoxifen |
| Lyve-1-cre x EYFP$^{stop\text{-}flox}$ | Lyve1 x EYFP | | LEC | BEC, DC, macrophages, T cells | Thy1.2 | Constitutively active Lyve-1 cre |

| TCR transgenic mice | | | | | |
|---|---|---|---|---|---|
| **Referred to as** | **Antigen recognized** | **CD4 or CD8** | **Restriction element** | **Thy alelle** | **Notes** |
| Bg1 | β-gal | CD8 | H2-K$^{b}$ | Thy1.1 | |
| Bg2 | | CD4 | I-A$^{b}$ | Thy1.1 | |
| Clone 4 | HA | CD8 | H2-K$^{d}$ | Thy1.1 | B6 x BALB/c F1 mice used |
| TS1 | | CD4 | I-E$^{d}$ | Thy1.1 | B6 x BALB/c F1 mice used |
| AFH | Tyrosinase | CD8 | AAD | Thy1.2 | Albino (tyrosinase$^{neg}$) mice. AAD$^{+}$ |
| TFH | | CD8 | AAD | Thy1.2 | Tyrosinase$^{+}$ mice. AAD$^{+}$ |
| Batf3$^{-/-}$ x TFH | | CD8 | AAD | Thy1.2 | Tyrosinase$^{+}$ BatF3$^{-/-}$ mice - lack CD8a and CD103 DC. AAD$^{+}$ |
| OT-I | OVA | CD8 | H2-K$^{b}$ | Thy1.1 | |

# LYMPHATIC ENDOTHELIAL CELLS SERVE AS A RESERVOIR OF PERIPHERAL TISSUE ANTIGENS FOR THE INDUCTION OF CD4 T CELL ANERGY

## Introduction

Immune tolerance is imposed through multiple processes that begin during T cell development and continue in the periphery. During negative selection in the thymus, medullary thymic epithelial cells (mTEC) and thymic dendritic cells (DC) present self-antigens to tolerize auto-reactive CD4 and CD8 T cells. Intrinsic tolerance mechanisms induce deletion or anergy of high affinity self-reactive T cells, while lower affinity CD4 cells are converted into regulatory T cells that mediate extrinsic tolerance[71,171]. DC can acquire antigen in the periphery and migrate into the thymus[68], or thymic resident DC can capture circulating antigen[69]. In addition to presenting ubiquitous antigens, mTEC also transcribe and present a variety of peripheral tissue antigens (PTA) under the control of the autoimmune regulatory element (Aire)[77,78], increasing the diversity of self-antigens presented in the thymus.

Thymic tolerance does not eliminate all self-reactive T cells, necessitating mechanisms of peripheral tolerance that principally occur in lymph nodes (LN). Immature DC continually survey peripheral tissues to acquire self-antigens, which are then presented in the draining LN to induce T cell deletion, anergy, or regulatory T cell ($T_{reg}$) formation[172]. In contrast to DC, which are specialized for acquiring antigens from other tissues, several subsets of LN cells transcribe and express PTA, analogous to mTEC in the thymus. Extrathymic Aire-expressing cells (eTAC) transcribe and present PTA in an Aire-dependent manner, leading to CD8 T cell deletional tolerance and CD4 T

cell anergy[79,96]. eTAC are developmentally related to DC, as they express the transcription factor zbtb46 and are $CD45^{lo}CD11c^{lo}$ [96]. PTA are also transcriptionally expressed independently of Aire by several subsets of radioresistant LN stromal cells (LNSC), including lymphatic endothelial cells (LEC), fibroblastic reticular cells (FRC), and blood endothelial cells (BEC)[80,81]. While the effects of PTA expressed in BEC has not yet been directly tested, LEC and FRC have both been shown to induce deletional tolerance of CD8 T cells[21,80,81,112,173].

We previously showed that LEC transcribe and present an epitope from the melanocyte differentiation protein tyrosinase on chimeric MHC I molecules termed AAD, which contain the peptide binding domain from HLA-A*0201 and the CD8-binding domain from $H\text{-}2D^{d}$ [156]. This leads to the proliferation and deletion of tyrosinase-specific CD8 T cells[80,174]. Proliferating tyrosinase-specific CD8 T cells activated by LEC in the absence of 4-1BB costimulation upregulate PD-1, which binds to PD-L1 on a radioresistant stromal cell, inhibits the upregulation of the IL-2 receptor, and leads to death[21]. LEC express the highest level of PD-L1 among the LNSC. In addition, LEC also express HVEM and MHC II[21], which are ligands for the BTLA / CD160 and LAG-3 inhibitory pathways[175–177]. Tyrosinase and PD-L1 are expressed at higher levels by LEC in the LN (LN-LEC) compared to LEC from tissue lymphatics in the diaphragm or colon[120], suggesting that the LN microenvironment endows LN-LEC with tolerogenic properties not found in tissue LEC. In this study, we investigated whether the expression of MHC II by LN-LEC is related to their tolerogenic role, and whether MHC II is used to induce CD4 T cell tolerance.

The MHC II antigen presentation pathway has been extensively studied in professional antigen presenting cells (APC) and in cell lines. MHC II molecules are synthesized in the ER, where they form a complex with invariant chain (Ii). This complex can either go directly to the late endosomal MHC II loading compartment (MIIC) from the trans-Golgi network, or it can be initially transported to the plasma membrane[22–25]. Ii contains AP-2 binding domains in its cytoplasmic tail, which cause the MHC:Ii complex to be internalized from the plasma membrane by clathrin-mediated endocytosis[26,27]. MHC II:Ii complexes are delivered to the MIIC, and Ii is cleaved by cathepsins, leaving the class II Ii-associated peptide (CLIP) in the peptide-binding groove. CLIP is then exchanged for antigenic peptides by the non-classical MHC II molecule H2-M. H2-M can be inhibited by H2-O, altering the representation of peptides presented[34,35]. The MHC II presentation pathway is under the master control of class II transactivator (CIITA), which induces transcription of MHC II molecules as well as Ii and H2-M[178]. LEC express MHC II[21], but the functionality of the MHC II processing pathway and the ability of LEC to load self-peptides onto MHC II molecules has not been investigated. Additionally, it is unknown whether PTA expression in LEC leads to CD4 T cell tolerance.

To investigate whether LEC present endogenous MHC II antigens and induce tolerance of PTA-specific CD4 T cells, we created transgenic systems where the model antigens β-galactosidase (β-gal) or hemagglutinin (HA) were expressed in LEC under the control of LEC-specific Lyve-1 or Prox1 promoters. Using these complementary models, we demonstrate that LEC do not directly present these PTA on MHC II molecules, but instead provide antigen to DC to induce CD4 T cell anergy.

## Results

*LEC in the LN but not the diaphragm express intermediate levels of MHC II*

We previously showed that LN-LEC express MHC II molecules[21]. To determine whether this was a specialized property of LN LEC, we compared the level of MHC II molecules on LN-LEC with those on tissue lymphatic LEC, other LNSC subsets and hematopoietically-derived APC. MHC II molecules were expressed on LN-LEC, but not on tissue LEC from the diaphragm (D-LEC) (Figure 1). LN-LEC express similar levels of MHC II molecules as LN-BEC and LN-FRC. Although the geometric mean fluorescence intensity (gMFI) of the entire LEC population is approximately 10% of the gMFI of the entire macrophage population, the highest-expressing population of LEC expresses similar levels of MHC II as some of the macrophages. This suggests that some LEC express intermediate levels of MHC II sufficient for antigen presentation.

A recent paper has shown that LEC can acquire some of their surface MHC II molecules from hematopoietically-derived cells[179]. To determine the extent to which LEC either endogenously synthesize their own MHC II or acquire it from hematopoietically-derived cells, we used reciprocal MHC $II^{-/-}$ bone marrow chimeras to restrict genetic MHC II expression to either the radiosensitive hematopoietically-derived cells or radioresistant stromal cells. We found that the MHC $II^{-/-}$ LEC in CD45.1 → MHC $II^{-/-}$ bone marrow chimeras continued to display MHC II molecules, suggesting they can acquire MHC II from hematopoietically-derived cells (Figure 2). However, LEC still express MHC II in the reciprocal MHC $II^{-/-}$ → CD45.1 chimeras, indicating that LEC can also endogenously synthesize their own MHC II molecules. The level of MHC II and percent of MHC $II^{+}$ LEC was similar between the two groups of chimeras (Figure 2B, C)

suggesting that the absence of one source of MHC II can be compensated for by another source to maintain a relatively constant level of MHC II on LEC. Additionally, mRNA for the C57Bl/6 (B6) I-$A^b$ MHC II molecules was detected in LEC (Figure 3), demonstrating that LEC transcribe MHC II molecules. These results demonstrate LN-LEC endogenously synthesize MHC II molecules in addition to acquiring MHC II molecules from hematopoietically-derived cells.

*Complementary models to evaluate antigen expression in LEC*

There are no CD4 TCR transgenic models available that are directed against endogenous PTA known to be selectively expressed in LEC. Therefore, to investigate the role of LEC in CD4 T cell tolerance, we created models in which the cytosolic protein β-galactosidase (β-gal) is transgenically expressed as a PTA in LEC. Rosa26$^{stop\text{-}LacZ}$ [161]mice express β-gal after cre-mediated excision of a floxed stop codon. These mice were crossed with mice expressing either Lyve-1-cre[139] or Prox1-creER$^{T2}$ [165], and the resulting mice are referred to as Lyve-1 x β-gal or Prox1 x β-gal, respectively. Lyve-1 is commonly used as a specific marker for LEC[180,181]. The Lyve-1-cre is constitutively active in all LEC, as well as in subsets of BEC, lymphoid and myeloid cells, possibly due to recombination in Lyve-$1^+$ precursor cells during development[139] (Figure 4). Prox1 is the master transcriptional regulator inducing LEC differentiation[180]. The Prox1-creER$^{T2}$ is induced by tamoxifen administration, and mediates high-efficiency recombination in LEC but not in BEC, FRC, DC, macrophages, T or B cells (Figure 4). Since Prox1-creER$^{T2}$ can also lead to recombination in Prox$1^+$ cells in the liver and heart[165], we used skin-draining LN in our analysis to eliminate any potential effect of antigen draining from these peripheral sites. Thus, Lyve-1-cre and Prox1-creER$^{T2}$ provide two complementary

models to investigating LEC-induced tolerance, as they both lead to expression in LEC and ectopic expression in other cell types differs between the two.

*LEC present endogenous β-gal epitopes on MHC I and induce deletion of β-gal-specific CD8 T cells via the PD-1/PD-L1 and LAG-3/MHC II pathways*

To test whether LEC from Lyve-1 x β-gal or Prox1 x β-gal mice express the β-gal protein, we first tested whether they induced the in vitro proliferation of Bg1 CD8 T cells, which express a transgenic TCR specific for the β-gal$_{96-103}$ epitope presented by H-2K$^{b}$ [163]. CFSE-labeled Bg1 cells proliferated when co-cultured with purified LN-LEC from both models (Figure 5). No significant proliferation was induced by FRC or BEC from the same mice (Figure 5), although they do express H-2K$^{b}$ (Figure 6). Thus, LEC are the only stromal cell in LN of either Lyve-1 x β-gal or Prox1 x β-gal mice that presents β-gal$_{96-103}$ at immunologically relevant levels.

To determine whether presentation of β-gal by LEC leads to CD8 tolerance, we transferred congenic Thy1.1 Cell-Trace Violet (CTV)-labeled Bg1 cells into Lyve-1 x β-gal or Prox-1 x β-gal mice. A substantial fraction of Bg1 cells proliferated by day 3 (Figure 7A). The majority of the proliferating cells died by day 7, and were almost completely gone by day 14. In all mice, a population of Bg1 cells remained undivided. While this could indicate that these cells had become anergic, it has also been shown that not all CD8 T cells from Bg1 mice stain with β-gal$_{96-103}$ tetramer[182], suggesting they are not β-gal-specific. Since Lyve-1-cre has been reported to induce recombination in hematopoietic cells[139], we used MHC I$^{-/-}$ → Lyve-1 x β-gal bone marrow chimeras to eliminate any potential direct antigen expression and presentation in hematopoietically-derived cells. This restricts β-gal expression and presentation to the radioresistant

LNSC. Bg1 cells transferred into these chimeras proliferated (Figure 7B) and upregulated Annexin V (Figure 7C). These results demonstrate that, as previously observed with tyrosinase, LEC present the MHC I restricted β-gal$_{96-103}$ epitope and induce deletional tolerance of β-gal specific CD8 T cells.

We previously showed that LEC-induced deletion of tyrosinase-specific CD8 T cells is driven by the PD-1/PD-L1 pathway[21]. Upon proliferation, Bg1 cells transferred into MHC I$^{-/-}$ → Lyve-1 x β-gal mice upregulated PD-1 and LAG-3 (Figure 7C), which are the ligands for PD-L1 and MHC II, respectively, and many cells co-expressed both (Figure 7D). In vivo administration of anti-PD-L1 or anti-LAG-3 antibodies individually did not inhibit deletion of Bg1 cells, but when PD-L1 and LAG-3 blockades were combined the Bg1 cells accumulated rather than deleted (Figure 8). These results confirm the importance of the PD-1/PD-L1 pathway for LEC-induced CD8 T cell tolerance[21], and additionally implicate the LAG-3/MHC II pathway. This suggests that one role for MHC II on LEC is as an inhibitory ligand for the induction of CD8 T cell tolerance.

*LEC do not present endogenous β-gal derived epitopes on MHC II*

The above results demonstrate that LEC from both Prox-1 x β-gal and Lyve-1 x β-gal mice present an MHC I restricted β-gal derived epitope. To test whether the same LEC present β-gal epitopes on MHC II molecules, we used Bg2 CD4 T cells[164], which recognize β-gal$_{721-739}$ presented by I-A$^{b}$. Bg2 cells transferred into Lyve-1 x β-gal or Prox-1 x β-gal mice proliferated by day 3 and continued to proliferate and accumulate by day 7 (Figure 9). However, Bg2 cells did not substantially proliferate in MHC II$^{-/-}$ → Lyve-1 x β-gal or MHC II$^{-/-}$ → Prox1 x β-gal bone marrow chimeras, in which the radioresistant

**Figure 1: LN-LEC express intermediate levels of MHC II.**

Pooled LN and diaphragms from B6 or MHC $II^{-/-}$ mice were enzymatically digested, stained, and analyzed by flow cytometry. LEC ($DAPI^{neg}CD45^{neg}CD31^{+}gp38^{+}$), BEC ($DAPI^{neg}CD45^{neg}CD31^{+}gp38^{neg}$), FRC ($DAPI^{neg}CD45^{neg}CD31^{neg}gp38^{+}$), macrophages ($DAPI^{neg}CD11c^{neg/low}CD11b^{+}F4/80^{+}$), and B cells ($DAPI^{neg}CD19^{+}CD11c^{neg}CD11b^{neg}$) were stained extracellularly for MHC II, and gMFI was calculated.

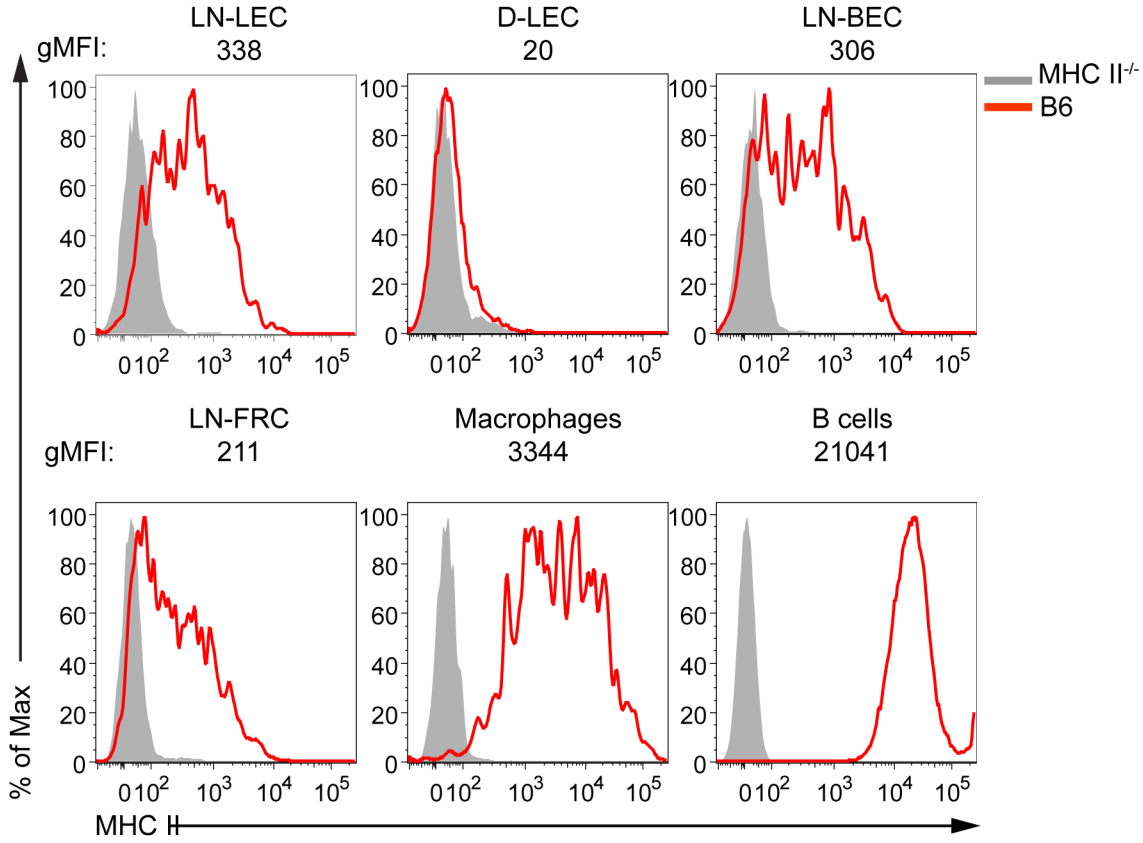
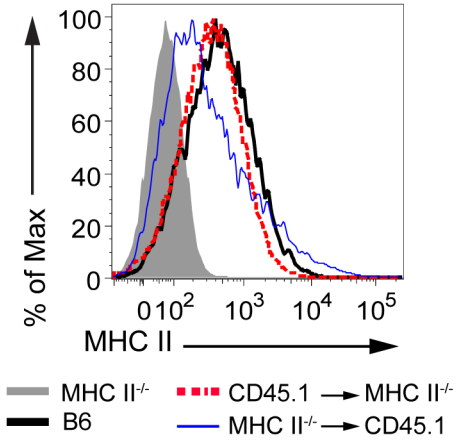

LN-LEC
gMFI:
338
D-LEC
20
LN-BEC
306
MHC II-/-
B6
LN-FRC
gMFI:
211
Macrophages
3344
B cells
21041
% of Max
MHC II
100
80
60
40
20
0
0
10^2
10^3
10^4
10^5

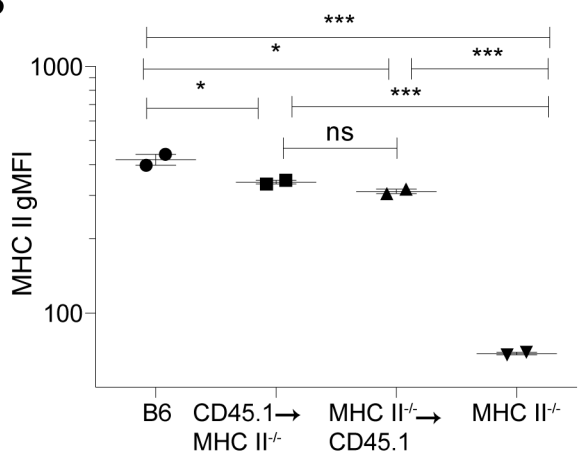
**Figure 2: LEC express endogenous MHC II in addition to acquiring MHC II from hematopoietically-derived cells.**

Pooled LN from B6, MHC $II^{-/-}$, CD45.1 → MHC $II^{-/-}$ or MHC $II^{-/-}$ → CD45.1 mice were enzymatically digested, and stained extracellularly for MHC II expression on LEC. A representative plot (A), gMFI (B), and percent of MHC $II^{+}$ LEC (C) is shown.

A


100
80
60
40
20
0
% of Max
0 10^2 10^3 10^4 10^5
MHC II
MHC II-/-
B6
CD45.1 → MHC II-/-
MHC II-/- → CD45.1

B


***
*
***
*
***
ns
1000
100
MHC II gMFI
B6
CD45.1→ MHC II-/-
MHC II-/- → CD45.1
MHC II-/-

C

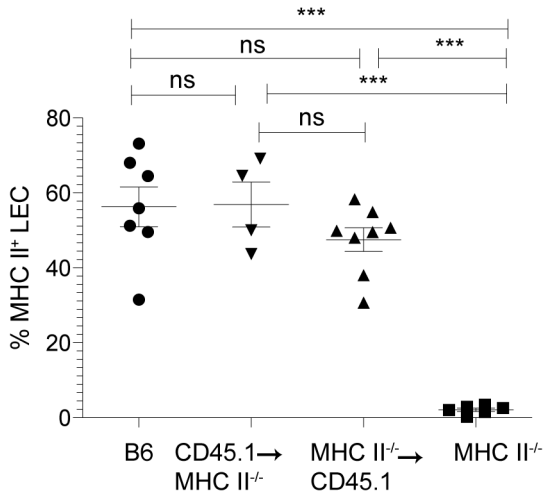

***
ns
***
ns
***
ns
80
60
40
20
0
% MHC II+ LEC
B6
CD45.1→ MHC II-/-
MHC II-/- → CD45.1
MHC II-/-

**Figure 3: LEC transcribe I-A$^{b}$.**

B cells (DAPI$^{neg}$CD19$^{+}$), macrophages (DAPI$^{neg}$CD11c$^{neg/low}$CD11b$^{+}$), LEC (DAPI$^{neg}$CD45$^{neg}$CD31$^{+}$gp38$^{+}$), BEC (DAPI$^{neg}$CD45$^{neg}$CD31$^{+}$gp38$^{neg}$), and FRC (DAPI$^{neg}$CD45$^{neg}$CD31$^{neg}$gp38$^{+}$) from B6 mice were electronically sorted, and cultured 3T3 cells were harvested. mRNA was purified and qPCR was performed as described in Methods.

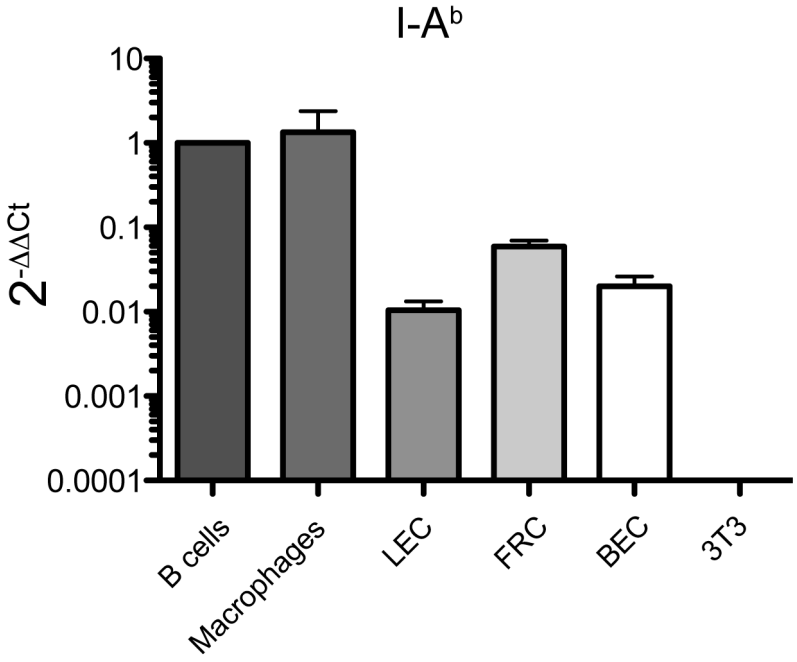

I-A^b
10
1
0.1
0.01
0.001
0.0001
2^-ΔΔCt
B cells
Macrophages
LEC
FRC
BEC
3T3

**Figure 4: Lyve-1-cre and Prox1-creER$^{T2}$ induce recombination in LEC.**

Prox1-CreER$^{T2}$ x EYFP$^{stop\text{-}flox}$ mice were maintained on tamoxifen chow for 2 weeks. Pooled inguinal, axillary, and brachial LN from B6, Lyve-1-cre x EYFP$^{stop\text{-}flox}$ (Lyve-1 x EYFP), or Prox1-CreER$^{T2}$ x EYFP$^{stop\text{-}flox}$ (Prox1 x EYFP) mice were enzymatically digested and EYFP expression in LEC (DAPI$^{neg}$CD45$^{neg}$CD31$^{+}$gp38$^{+}$), BEC (DAPI$^{neg}$CD45$^{neg}$CD31$^{+}$gp38$^{neg}$), FRC (DAPI$^{neg}$CD45$^{neg}$CD31$^{neg}$gp38$^{+}$), DC (DAPI$^{neg}$CD11c$^{high}$), macrophages (DAPI$^{neg}$CD11c$^{neg/low}$CD11b$^{+}$), B cells (DAPI$^{neg}$CD19$^{+}$), CD4 T cells (DAPI$^{neg}$CD4$^{+}$CD19$^{neg}$) and CD8 T cells (DAPI$^{neg}$CD8$^{+}$ CD19$^{neg}$) was analyzed by flow cytometry.

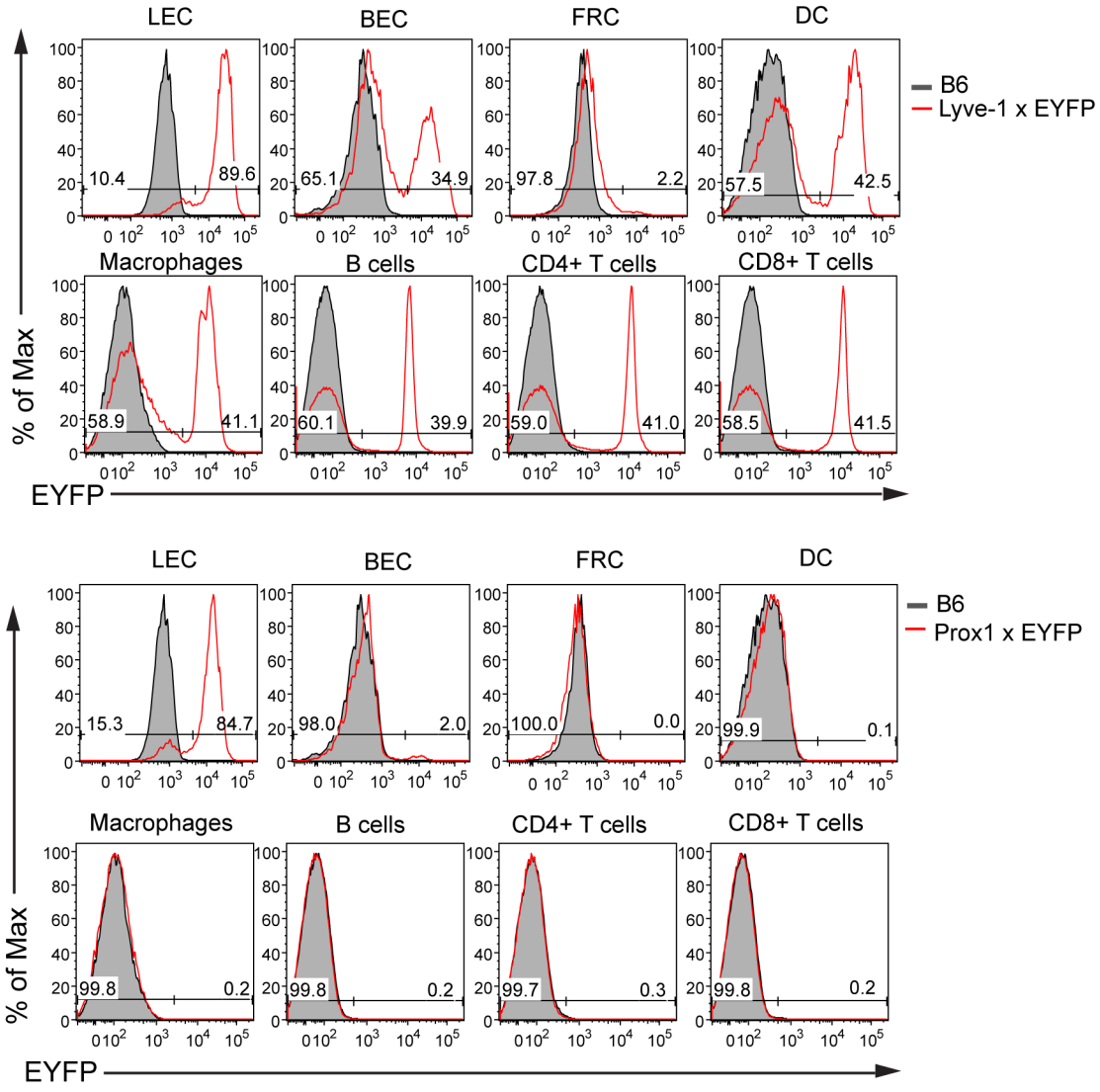
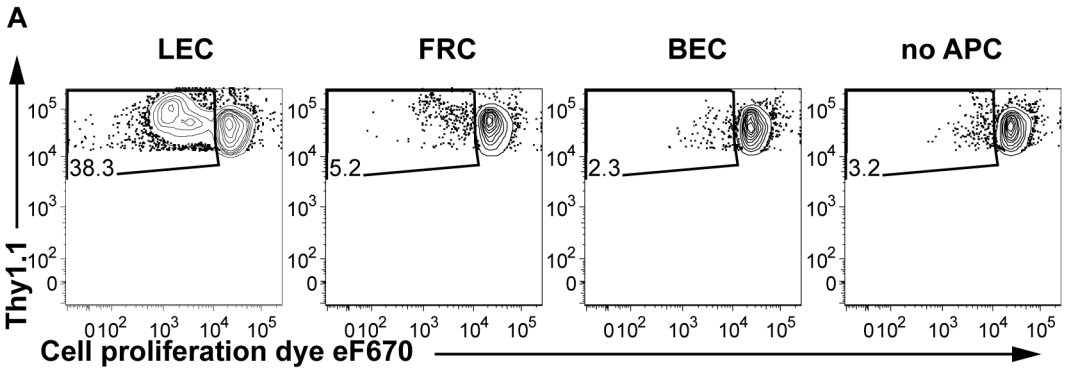

LEC
BEC
FRC
DC
Macrophages
B cells
CD4+ T cells
CD8+ T cells
10.4
89.6
65.1
34.9
97.8
2.2
57.5
42.5
58.9
41.1
60.1
39.9
59.0
41.0
58.5
41.5
B6
Lyve-1 x EYFP
% of Max
EYFP
LEC
BEC
FRC
DC
Macrophages
B cells
CD4+ T cells
CD8+ T cells
15.3
84.7
98.0
2.0
100.0
0.0
99.9
0.1
99.8
0.2
99.8
0.2
99.7
0.3
99.8
0.2
B6
Prox1 x EYFP
% of Max
EYFP

**Figure 5: LEC from Prox1 x β-gal and Lyve-1 x β-gal mice induce Bg1 proliferation.**

LNSC from Prox1 x β-gal (A) or Lyve-1 x β-gal (B) mice were electronically sorted and co-cultured with CFSE labeled Thy1.1 Bg1 T cells for 4 days. Plots are gated on $CD8^+$ $Thy1.1^+$ cells, and numbers represent divided Bg1 cells out of total Bg1 cells.


A
LEC
FRC
BEC
no APC
38.3
5.2
2.3
3.2
Thy1.1
Cell proliferation dye eF670

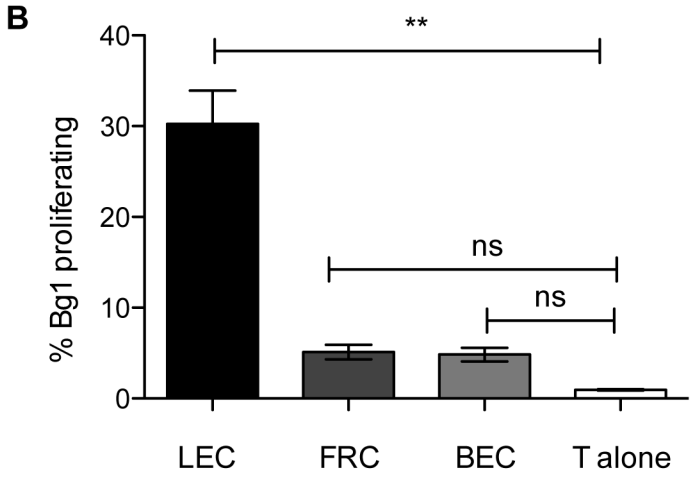

B
**
ns
ns
% Bg1 proliferating
LEC
FRC
BEC
T alone

**Figure 6: FRC and BEC express MHC I.**

Pooled LN from B6 mice were enzymatically digested, stained, and analyzed by flow cytometry. LEC ($DAPI^{neg}CD45^{neg}CD31^{+}gp38^{+}$), BEC ($DAPI^{neg}CD45^{neg}CD31^{+}gp38^{neg}$), FRC ($DAPI^{neg}CD45^{neg}CD31^{neg}gp38^{+}$) and DC ($DAPI^{neg}CD11c^{high}CD11b^{+}$) were stained extracellularly for MHC I.

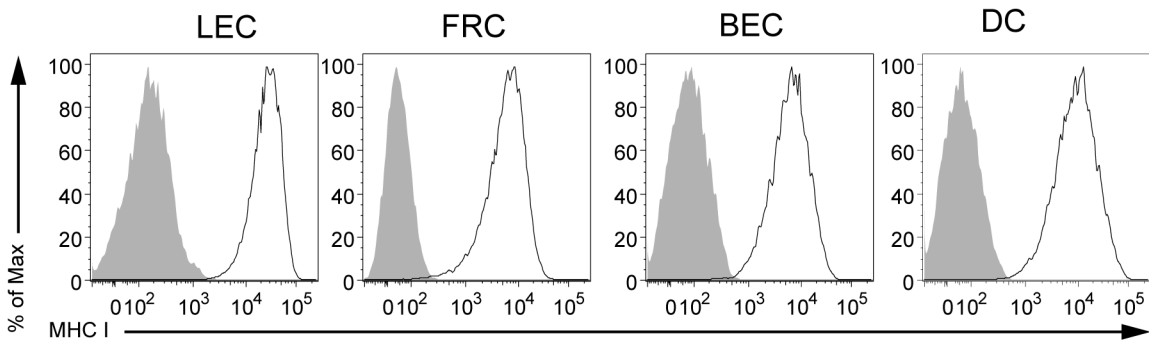

LEC
FRC
BEC
DC
% of Max
MHC I
100
80
60
40
20
0
0 10^2 10^3 10^4 10^5

**Figure 7: Bg1 T cells proliferate and delete in Lyve-1 x β-gal and Prox1 x β-gal mice.**

A, B) CTV-labeled Thy1.1 Bg1 cells were adoptively transferred into the indicated recipients, and skin-draining LNs were analyzed for Bg1 proliferation 3, 7 or 14 days later. Plots are gated on total $CD8^+$ T cells. (C) Bg1 cells adoptively transferred into MHC $I^{-/-}$ → Lyve-1 x β-gal mice were harvested after 3 days and stained for Annexin V, PD-1, or LAG-3. Plots are gated on $CD8^+Thy1.1^+$ Bg1 cells, and numbers represent percent of proliferating (left box) or undivided (right box) Bg1 cells expressing the given marker. (D) Proliferating Bg1 cells transferred into MHC $I^{-/-}$ → Lyve-1 x β-gal mice as in (C) were examined for co-expression of LAG-3 and PD-1. Plot is gated on $CD8^+Thy1.1^+$ cells that had undergone at least 1 division.

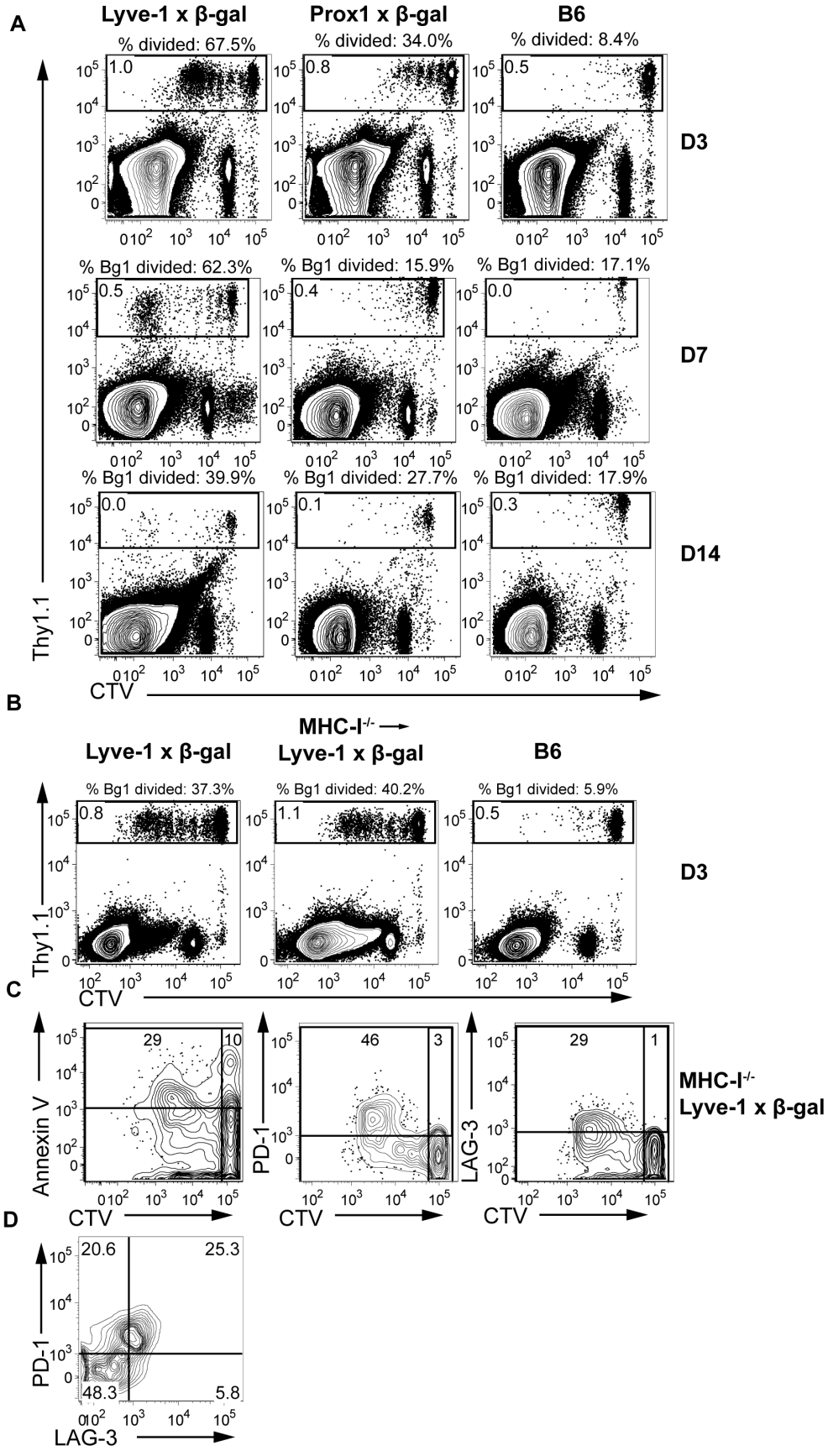
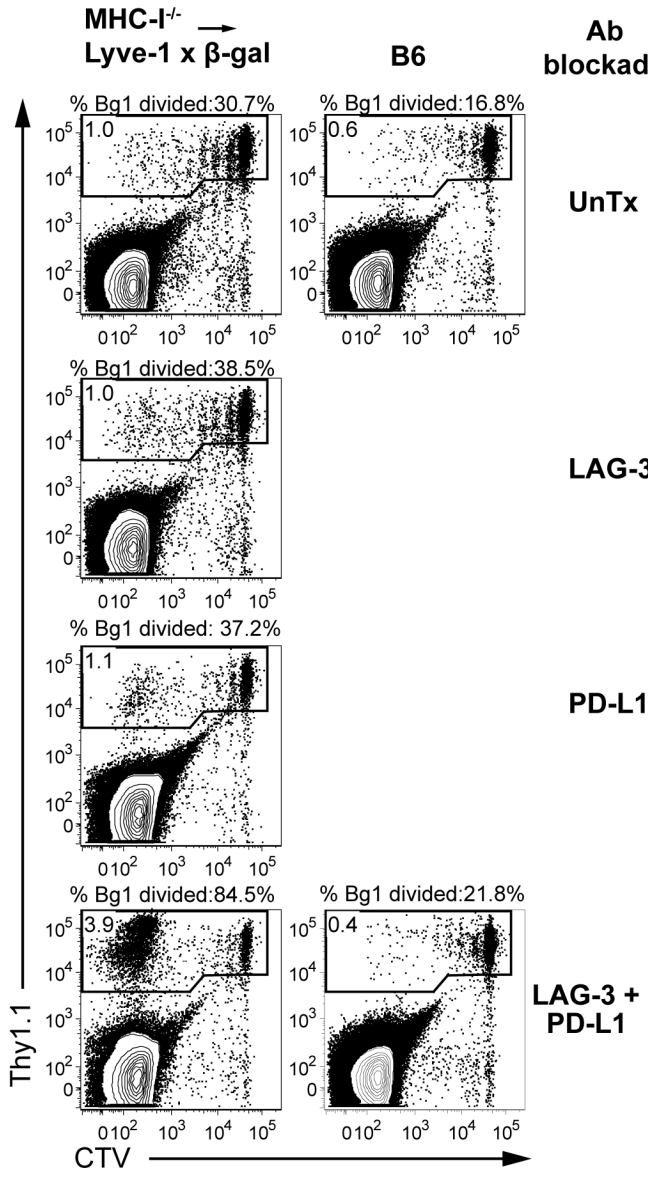

A
Lyve-1 x β-gal
Prox1 x β-gal
B6
% divided: 67.5%
% divided: 34.0%
% divided: 8.4%
1.0
0.8
0.5
D3
% Bg1 divided: 62.3%
% Bg1 divided: 15.9%
% Bg1 divided: 17.1%
0.5
0.4
0.0
D7
% Bg1 divided: 39.9%
% Bg1 divided: 27.7%
% Bg1 divided: 17.9%
0.0
0.1
0.3
D14
Thy1.1
CTV
B
MHC-I-/- →
Lyve-1 x β-gal
Lyve-1 x β-gal
B6
% Bg1 divided: 37.3%
% Bg1 divided: 40.2%
% Bg1 divided: 5.9%
0.8
1.1
0.5
D3
Thy1.1
CTV
C
29
10
46
3
29
1
Annexin V
PD-1
LAG-3
CTV
MHC-I-/-
Lyve-1 x β-gal
D
20.6
25.3
48.3
5.8
PD-1
LAG-3

**Figure 8: Bg1 CD8 T cells delete through both the PD-1/PD-L1 and LAG-3/MHC II pathways.**

Thy1.1$^{+}$ Bg1 cells were transferred into MHC I$^{-/-}$ → Lyve-1 x β-gal or B6 mice treated with blocking antibodies as indicated, and accumulation was analyzed 7 days later. Cumulative data (bottom) is shown for cells transferred into MHC I$^{-/-}$ → Lyve-1 x β-gal chimeras.


MHC-I-/-
Lyve-1 x β-gal
B6
Ab blockade:
% Bg1 divided:30.7%
1.0
% Bg1 divided:16.8%
0.6
UnTx
% Bg1 divided:38.5%
1.0
LAG-3
% Bg1 divided: 37.2%
1.1
PD-L1
% Bg1 divided:84.5%
3.9
% Bg1 divided:21.8%
0.4
LAG-3 + PD-L1
Thy1.1
CTV

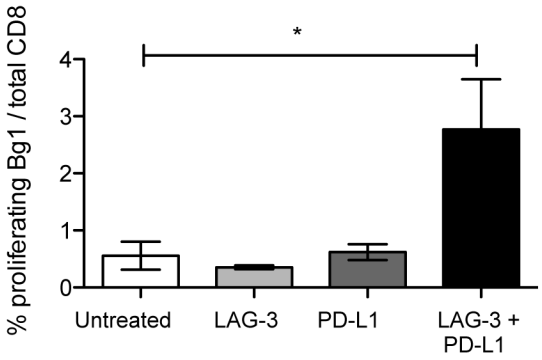
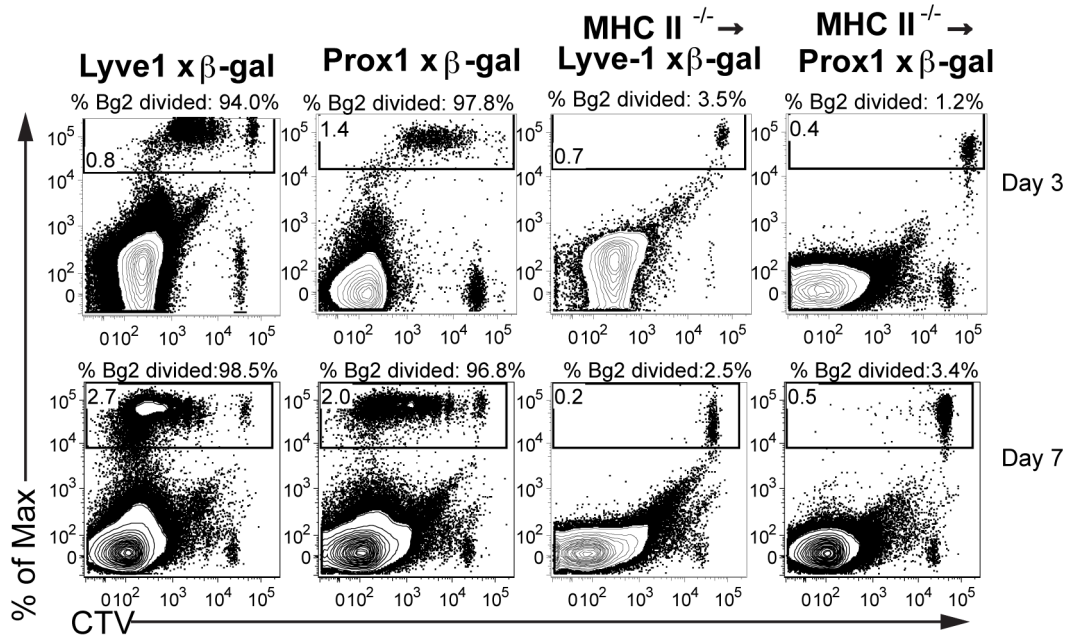

% proliferating Bg1 / total CD8
*
Untreated
LAG-3
PD-L1
LAG-3 + PD-L1

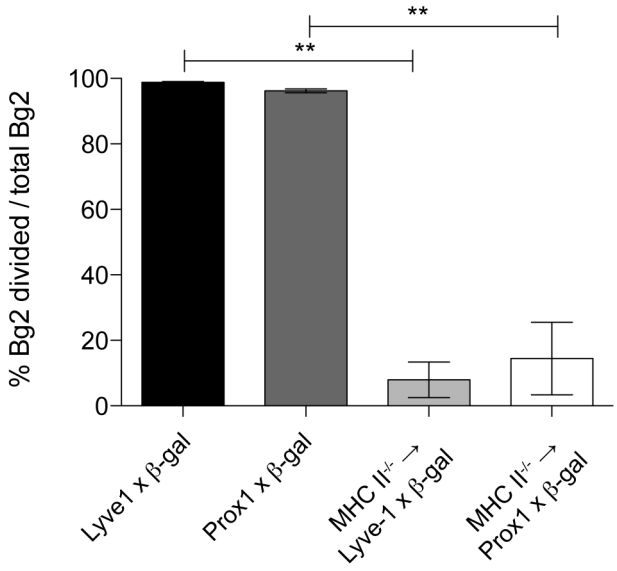
**Figure 9: LEC do not present endogenous β-gal on MHC II in vivo.**

(A) Representative and (B) cumulative data of CTV-labeled Thy1.1$^+$ Bg2 cells adoptively transferred into the indicated recipients. Skin-draining LNs were analyzed 3 or 7 days later. Plots are gated on total CD4$^+$ T cells, and numbers represent percent of Bg2 cells out of total CD4 cells.

A


Lyve1 x β-gal
Prox1 x β-gal
MHC II -/- → Lyve-1 xβ-gal
MHC II -/- → Prox1 x β-gal
% Bg2 divided: 94.0%
% Bg2 divided: 97.8%
% Bg2 divided: 3.5%
% Bg2 divided: 1.2%
0.8
1.4
0.7
0.4
Day 3
% Bg2 divided:98.5%
% Bg2 divided: 96.8%
% Bg2 divided:2.5%
% Bg2 divided:3.4%
2.7
2.0
0.2
0.5
Day 7
% of Max
CTV

B


**
**
% Bg2 divided / total Bg2
100
80
60
40
20
0
Lyve1 x β-gal
Prox1 x β-gal
MHC II-/- → Lyve-1 x β-gal
MHC II-/- → Prox1 x β-gal

LEC express MHC II but bone marrow derived cells do not. This indicates that hematopoietically-derived cells, not LEC, induced the proliferation seen in non-chimeric mice. LEC do not express the costimulatory ligands CD80, CD86, CD70, 4-1BBL or OX40L[21]. Therefore, it was possible that Bg2 T cells recognized β-gal presented by MHC II on LEC, but did not proliferate due to lack of costimulation. However, Bg2 cells adoptively transferred into MHC $II^{-/-}$ → Prox1 x β-gal or MHC $II^{-/-}$ → Lyve-1 x β-gal mice treated with αCD28, αCD40 or α41BB/OX40 agonistic antibodies did not proliferate (Figure 10A, C).

We considered whether the level of MHC II on the surface of LEC might be too low to induce CD4 proliferation. To address this, we treated MHC $II^{-/-}$ → Prox1 x β-gal chimeras with IFNγ, which upregulated the level of MHC II more than 6-fold (Figure 10B). However, Bg2 cells transferred into these mice also did not proliferate (Figure 10A). Finally, we questioned whether LEC might present β-gal, but rapidly induce anergy or suppress T cell proliferation through nitric oxide[151,152]. Therefore, we examined Bg2 cells for upregulation of the early activation markers CD69, CD25, and CD44, and downregulation of CD62L, one day after adoptive transfer. After transfer into Prox1 x β-gal or Lyve1 x β-gal mice, CD69, CD25, and CD44 were all upregulated and CD62L was downregulated (Figure 11). However, expression of these markers on Bg2 cells transferred into MHC $II^{-/-}$ → Prox1 x β-gal or MHC $II^{-/-}$ → Lyve-1 x β-gal bone marrow chimeras was identical to that of Bg2 cells transferred into antigen-free B6 mice (Figure 11), indicating the Bg2 cells were not activated. We conclude that LEC do not present MHC II restricted β-gal epitopes to Bg2 cells in vivo, even though they express the source protein and the restriction element.

To determine whether the level of MHC II on the surface of LEC is sufficient to activate Bg2 T cells, we pulsed B6 LEC with β-gal$_{721-739}$ peptide prior to co-culture with Bg2 T cells. Peptide-pulsed LEC induced Bg2 proliferation (Figure 12A), as did peptide-pulsed FRC, BEC, DC and macrophages. The level of proliferation induced by the LNSC was lower than that induced by DC, which could reflect lower levels of MHC II molecules on these cells and/or lack of costimulatory molecules. However, the results demonstrate that the level of MHC II on LEC is sufficient to present antigen to Bg2 T cells.

Since Figure 9 showed that Bg2 cells did proliferate in non-chimeric Prox1 x β-gal mice and LEC are not responsible for this proliferation, we used in vitro co-cultures to determine which cells from the Prox1 x β-gal mice are presenting β-gal$_{721-739.}$ Electronically sorted LEC, FRC and BEC did not induce Bg2 proliferation (Figure 12B), confirming the in vivo results with MHC II$^{-/-}$ bone marrow chimeras. In contrast, Bg2 cells co-cultured with DC from Prox1 x β-gal mice proliferated strongly, and macrophages from the same mice induced a less robust, but still highly significant, proliferative response. The basis for antigen presentation by these cells is explored further below.

*Lack of antigen presentation on MHC II molecules by LEC is independent of antigen localization*

Presentation of epitopes from cytoplasmic proteins, such as β-gal, by MHC II molecules depends on autophagy[183]. Therefore, we investigated whether LEC could present an I-E$^{d}$ restricted epitope from the membrane protein influenza hemagglutinin (HA), which is generated in an autophagy independent manner[184]. We expressed HA in

**Figure 10: Exogenous costimulation and IFNγ do not induce Bg2 proliferation in vivo.**

(A) CTV-labeled Bg2 cells were adoptively transferred into B6 and MHC $II^{-/-}$ → Prox1 x β-gal mice treated with PBS, αCD28 or IFNγ, and proliferation was analyzed 3 days later. Plots are gated on $Thy1.1^{+}CD4^{+}$ Bg2 cells. (B) LN from MHC $II^{-/-}$ and PBS or IFNγ treated B6 mice were enzymatically digested 24 hours after treatment, and MHC II on LEC was analyzed by flow cytometry. (C) CTV-labeled Bg2 cells were adoptively transferred into B6 and MHC $II^{-/-}$ → Lyve-1 x β-gal mice treated with PBS, α4-1BB and αOX40, αCD28 or αCD40, and proliferation was analyzed 7 days later. Plots are gated on $Thy1.1^{+}CD4^{+}$ Bg2 cells.

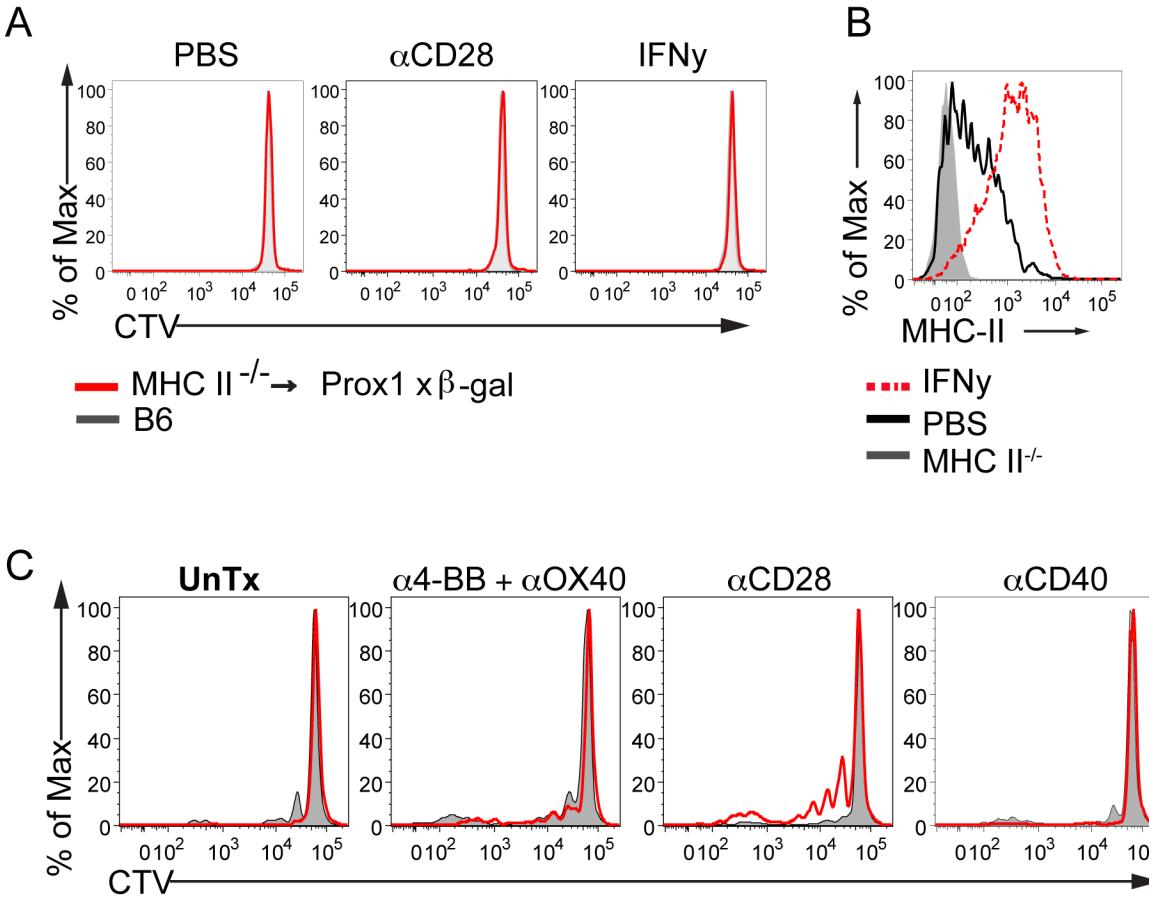

A
PBS
αCD28
IFNy
% of Max
CTV
MHC II -/-→ Prox1 xβ-gal
B6
B
% of Max
MHC-II
IFNy
PBS
MHC II-/-
C
UnTx
α4-BB + αOX40
αCD28
αCD40
% of Max
CTV

**Figure 11: Bg2 cells do not upregulate activation markers in MHC II$^{-/-}$ bone marrow chimeras.**

CTV-labeled Thy1.1$^{+}$ Bg2 cells were transferred into the indicated recipients and activation markers were analyzed 16 hours later. Plots are gated on Thy1.1$^{+}$CD4$^{+}$ Bg2 cells.

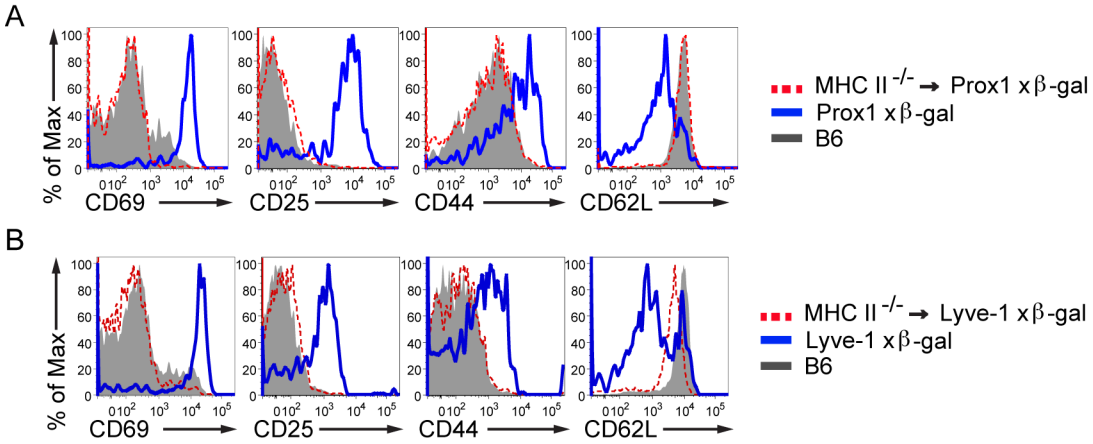
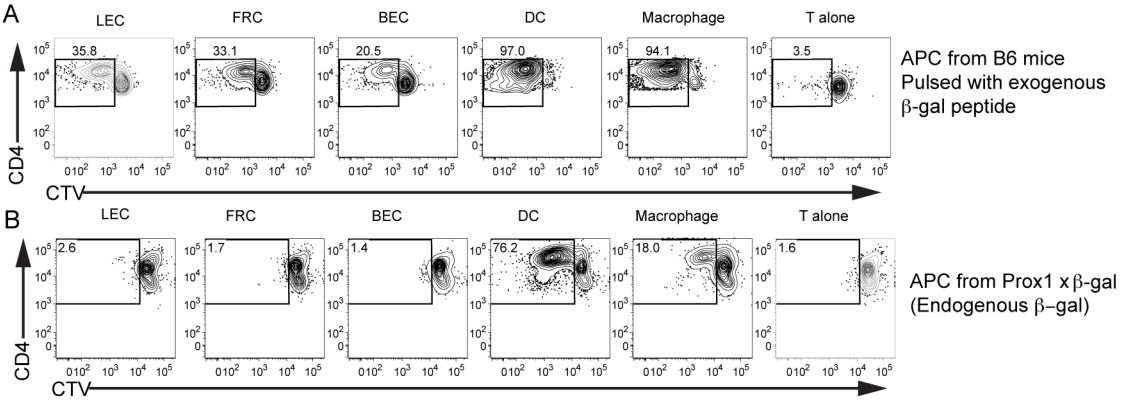

A
% of Max
CD69
CD25
CD44
CD62L
MHC II -/- → Prox1 xβ-gal
Prox1 xβ-gal
B6
B
% of Max
CD69
CD25
CD44
CD62L
MHC II -/- → Lyve-1 xβ-gal
Lyve-1 xβ-gal
B6

**Figure 12: LEC do not present β-gal on MHC II in vitro.**

(A) LNSC and DC from B6 mice were electronically sorted, pulsed with Bg2 peptide for 3 hours, washed and co-cultured with CPD labeled Thy1.1$^+$ Bg2 T cells for 4 days. Plots are gated on CD4$^+$ Thy1.1$^+$ cells, and numbers represent divided Bg2 cells out of total Bg2 cells. (B) LNSC and DC from Prox1 x β-gal mice were electronically sorted and co-cultured with CPD labeled Thy1.1$^+$ Bg2 T cells for 4 days. Plots are gated on CD4$^+$ Thy1.1$^+$ cells, and numbers represent divided Bg2 cells out of total Bg2 cells.


A
LEC
FRC
BEC
DC
Macrophage
T alone
35.8
33.1
20.5
97.0
94.1
3.5
CD4
CTV
APC from B6 mice
Pulsed with exogenous
β-gal peptide
B
LEC
FRC
BEC
DC
Macrophage
T alone
2.6
1.7
1.4
76.2
18.0
1.6
CD4
CTV
APC from Prox1 x β-gal
(Endogenous β–gal)

LEC using Prox1-creER$^{T2}$ x Rosa26$^{stop\text{-}HA}$ (Prox1 x HA) mice and assessed antigen presentation using HA-specific CD4 or CD8 TCR transgenic cells (TS1[167] or Clone 4[162], respectively). LEC from Prox1 x HA mice induced proliferation of Clone 4 CD8 T cells in vitro (Figure 13), indicating that HA is expressed in LEC and presented on H-2K$^d$ MHC I molecules. In contrast, Prox1 x HA LEC did not induce TS1 CD4 T cell proliferation in vitro (Figure 13). TS1 cells transferred into MHC II$^{-/-}$ → Prox1 x HA chimeras did not upregulate CD69 on day 1 or proliferate at day 3 or 7 (Figure 14), indicating that LEC do not present HA on I-E$^d$ MHC II molecules in vivo. In contrast, DC present HA on both MHC I and MHC II to Clone 4 and TS1 cells in vitro (Figure 13). TS1 CD4 T cells transferred into non-chimeric Prox-1 x HA mice upregulated CD69 on day 1 (Figure 14A), and proliferated and accumulated by day 7 after adoptive transfer (Figure 14B). Therefore, like β-gal, HA is expressed in LEC and presented on MHC I to CD8 T cells, but antigen is transferred from LEC to DC for presentation on MHC II to CD4 T cells.

A recent report demonstrated that peptide:MHC II complexes acquired from DC and presented by LEC can lead to CD4 apoptosis without prior proliferation[179]. However, we did not see any differences in T cell survival between TS1 cells co-cultured with or without Prox1 x HA LEC (Figure 15), suggesting that LEC are not directly killing TS1 cells.

We also used a T cell independent assay to directly test whether LEC can form peptide:MHC II complexes. The Y-Ae antibody recognizes I-A$^b$ MHC II molecules presenting an epitope derived from the α chain of I-E MHC II molecules[185]. BALB/c LEC express the I-Eα chain, which is a component of I-E$^d$ MHC II molecules, but B6 LEC do not as the I-Eα chain is deleted in B6 mice[186] (Figure 16A). In B6 x BALB/c mice, both I-

$A^b$ and I-$E^d$ MHC II molecules are co-expressed and will be targeted to the MHC II loading compartment by Ii. In addition, I-$E^d$ molecules localized to endosomes of I-$A^{b+}$ mice lead to the generation of the Y-Ae epitope[187]. DC, macrophages, and B-cells from B6 x BALB/c mice expressed I-$A^b$:I-$E\alpha_{52-68}$ complexes on the cell surface (Figure 16B). In contrast, Y-Ae staining of B6 x BALB/c LEC was not above the background level. Additionally, treating mice with IFNγ to upregulate I-$A^b$ and I-$E^d$ on B6 x BALB/c LEC did not induce Y-Ae staining on LEC, indicating that LEC do not have significant levels of I-$A^b$:I-$E\alpha_{52-68}$ complexes on the cell surface. These results demonstrate that LEC do not present 3 separate antigens, including both cytoplasmic and membrane-bound proteins. Both HA and I-Eα can be presented in the absence of autophagy[184,188,189], suggesting these results are not simply be due to an inability to target self-antigens into the MHC II loading compartment. Instead, the lack of presentation of these antigens suggests a fundamental deficiency in the MHC II processing pathway.

*LEC do not express the peptide editor H2-M*

To determine why LEC do not present these three antigens, we investigated whether they were deficient in any components of the MHC II processing pathway. Ii binds to MHC II molecules in the ER, and targets MHC II to the MIIC[19]. LEC express high levels of Ii (Figure 17), suggesting that MHC II can be correctly targeted within LEC. Ii is subsequently degraded by cathepsins S or L[30,31,190], leaving CLIP, which blocks the MHC II peptide-binding groove. LEC identified by the specific marker 10.1.1[80] contained punctate cathepsin L staining by immunofluorescence microscopy (Figure 18A). We used the cathepsin L substrate $(CBZ\text{-}Phe\text{-}Arg)_2$-Rhodamine110, which fluoresces when cleaved, to test whether LEC express active cathepsin L. LN-LEC cleave this substrate

to generate fluorescent Rhodamine110, and the fluorescence is reduced in the presence of the cathepsin L inhibitor 1-naphthalenesulfonyl-IW-CHO, indicating that LN-LEC express active cathepsin L (Figure 18B). In contrast, D-LEC do not generate a rhodamine110 signal above the background level in cells pretreated with 1-naphthalenesulfonyl-IW-CHO, suggesting that the MHC $II^{neg}$ D-LEC do not express active cathepsin L. The presence of active cathepsin L suggests that LN-LEC can digest Ii into CLIP.

The peptide editor H2-M exchanges CLIP for high affinity antigenic peptides, and this process can be inhibited by H2-O[34,35]. Therefore, low levels of H2-M or high levels of H2-O could explain the inability of LEC to present antigenic peptides. We did not detect H2-M or H2-O expression in LEC by flow cytometry (Figure 19A, B). Confirming this, we also found that LEC also do not express significant levels of H2-Mα or H2-Mβ mRNA (Figure 19C). This suggests that LEC cannot exchange CLIP for antigenic peptides. We attempted to measure the level of CLIP bound to $I\text{-}A^b$ MHC II molecules using the 15G4 antibody that specifically recognizes $I\text{-}A^b$:CLIP complexes. However, LEC exhibited an extremely high level of background staining in the MHC $II^{-/-}$ negative control, compromising the ability to detect a positive CLIP signal in B6 mice (Figure 19D). With this limitation, our data nonetheless supports a model in which MHC II molecules are correctly targeted into the MIIC by Ii before Ii is degraded, but the lack of H2-M prevents the exchange of CLIP for antigenic peptides.

*DC acquire antigen from LEC and induce anergy*

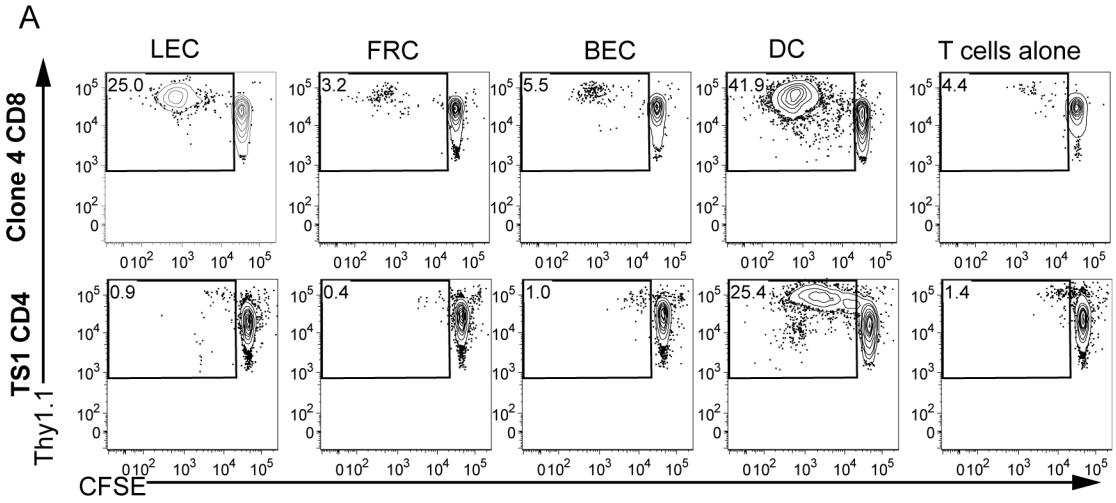
While LEC do not directly present β-gal or HA on MHC II, Figure 12 and 13 demonstrate that these antigens are presented by DC and macrophages, leading to the proliferation

of β-gal or HA-specific CD4 T cells. Since the Prox1-creER$^{T2}$ does not lead to genetic recombination in DC or macrophages (Figure 4), this indicates that these cells acquired antigen transcribed in LEC. Since this presentation did not occur in MHC II$^{-/-}$ → Prox1 x β-gal (Figure 9) or MHC II$^{-/-}$ → Prox1 x HA (Figure 14) bone marrow chimeras, it also indicates that the antigen is loaded onto MHC II in the hematopoietically derived cells, rather than transferred through cross-dressing of already formed MHC II- β-gal peptide complexes acquired from LEC. To formally demonstrate that antigen is acquired from LEC rather than synthesized in hematopoietically-derived cells, we used bone marrow chimeras where genetic expression of β-gal is restricted to either the hematopoietically-derived or radioresistant cells. Bg2 CD4 T cells transferred into Prox-1 x β-gal → CD45.1 chimeras did not proliferate (Figure 20), demonstrating that β-gal is not genetically expressed in hematopoietically-derived cells. In contrast, Bg2 T cells transferred into the reciprocal CD45.1 → Prox1 x β-gal chimeras did proliferate, indicating that β-gal genetically expressed in LEC can be transferred to hematopoietically-derived cells. This demonstrates that DC and macrophages acquire antigens to present from LEC.

The presentation of β-gal or HA by hematopoietically-derived cells in vivo led to proliferation and accumulation of CD4 T cells over 7 days (Figure 9, 14, 20). To determine the fate of these cells, we tested whether they developed into either $T_{reg}$ or anergic cells. After 3 days, Bg2 cells transferred into Prox1 x β-gal, MHC II$^{-/-}$ → Prox1 x β-gal, and B6 mice all had equivalently low levels of FoxP3 staining (Figure 21), indicating that they were not differentiating into $T_{reg}$. To test whether Bg2 cells became anergic, we adoptively transferred CD25$^{neg}$ CTV-labeled Bg2 cells into recipient mice

**Figure 13: LEC present the MHC I but not the MHC II epitope from HA in vitro.**

LNSC and DC from Prox1 x HA mice were electronically sorted and co-cultured with CFSE-labeled Thy1.1$^{+}$ Clone 4 CD8 cells (top) or TS1 CD4 cells (bottom) for 4 days. Plots are gated on CD8$^{+}$Thy1.1$^{+}$ (top) or CD4$^{+}$Thy1.1$^{+}$ (bottom) cells, and numbers represent percent of dividing cells. Representative (A) and cumulative (B) data is shown.


A
LEC
FRC
BEC
DC
T cells alone
Clone 4 CD8
TS1 CD4
Thy1.1
CFSE
25.0
3.2
5.5
41.9
4.4
0.9
0.4
1.0
25.4
1.4

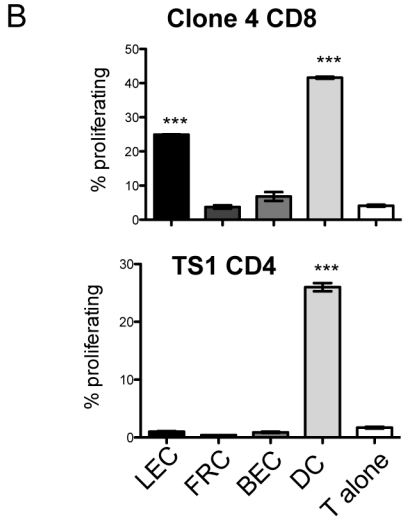

B
Clone 4 CD8
% proliferating
***
TS1 CD4
LEC
FRC
BEC
DC
T alone

**Figure 14: LEC do not present HA on MHC II in vivo.**

(A) CTV-labeled Thy1.1$^+$ TS1 cells were transferred into the indicated recipients, and CD69 was analyzed 16 hours after transfer. Plot is gated on Thy1.1$^+$ CD4$^+$ cells. (B) CTV-labeled Thy1.1$^+$ TS1 cells were transferred into the indicated recipients, and proliferation was measured 3 or 7 days later. Plot is gated on CD4$^+$ cells.

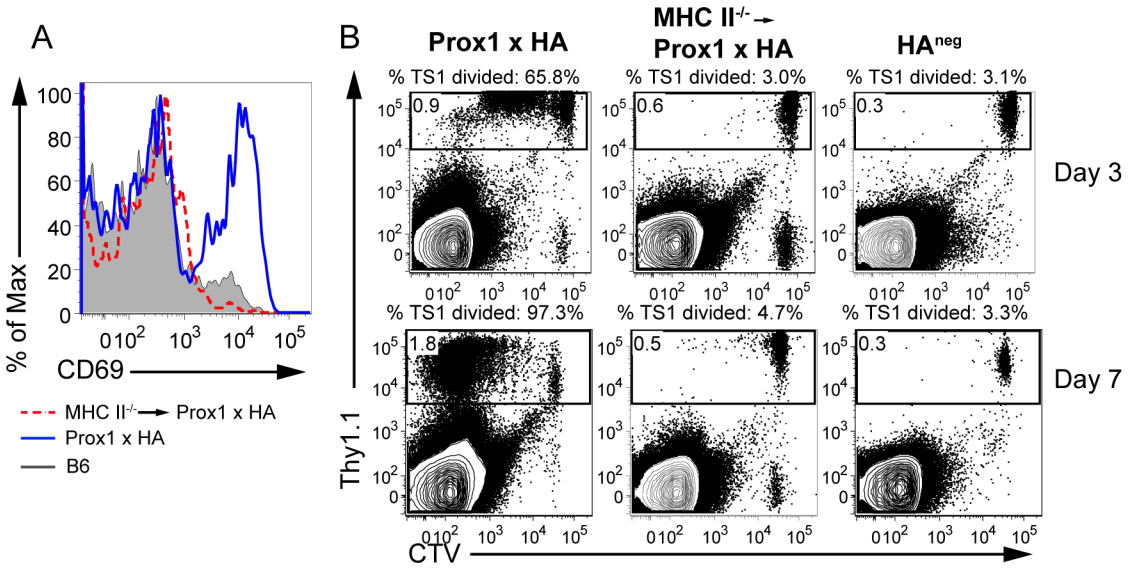
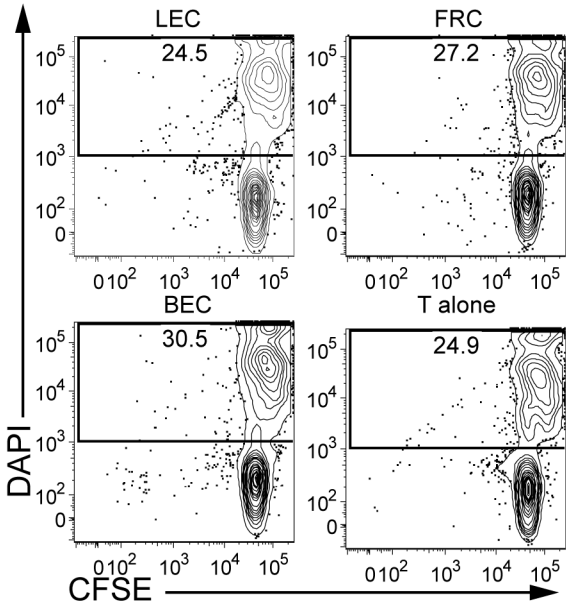

A
% of Max
CD69
MHC II-/- → Prox1 x HA
Prox1 x HA
B6
B
Prox1 x HA
MHC II-/- → Prox1 x HA
HAneg
% TS1 divided: 65.8%
% TS1 divided: 3.0%
% TS1 divided: 3.1%
% TS1 divided: 97.3%
% TS1 divided: 4.7%
% TS1 divided: 3.3%
Day 3
Day 7
Thy1.1
CTV

**Figure 15: LEC do not induce TS1 death in vitro.**

LNSC from Prox1 x HA mice were electronically sorted and co-cultured with CFSE-labeled Thy1.1$^{+}$ TS1 CD4 cells for 4 days. Plots are gated on CD4$^{+}$Thy1.1$^{+}$ cells, and numbers represent percent of DAPI$^{pos}$ Bg2 cells.


LEC
24.5
FRC
27.2
BEC
30.5
T alone
24.9
DAPI
CFSE

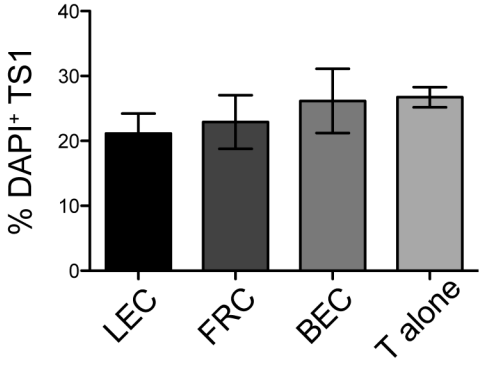

% DAPI+ TS1
LEC
FRC
BEC
T alone

**Figure 16: LEC do not present I-Eα on MHC II.**

(A) LEC ($DAPI^{neg}CD45^{neg}CD31^{+}gp38^{+}$), DC ($DAPI^{neg}CD11c^{high}CD11b^{+}$) and B cells ($DAPI^{neg}CD19^{+}$) from Balb/c or B6 mice were flow sorted, and cultured 3T3 cells were harvested. mRNA was purified and qPCR was performed for I-Eα. (B) LN from B6 or B6 x BALB/c mice treated with PBS or IFNγ were enzymatically digested, and Y-Ae expression was analyzed on the indicated populations by flow cytometry.

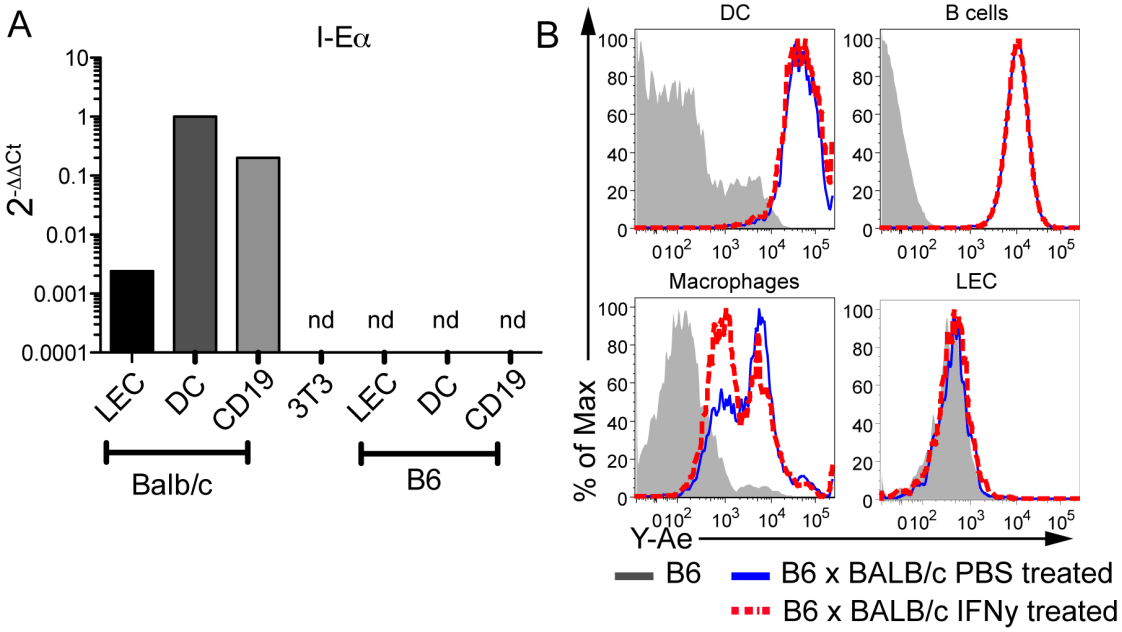

A
I-Eα
2-ΔΔCt
10
1
0.1
0.01
0.001
0.0001
nd
nd
nd
nd
LEC
DC
CD19
3T3
LEC
DC
CD19
Balb/c
B6
B
DC
B cells
Macrophages
LEC
% of Max
Y-Ae
B6
B6 x BALB/c PBS treated
B6 x BALB/c IFNy treated

**Figure 17: LEC express Ii.**

B6 LEC (live $CD45^{neg}$ $CD31^{+}10.1.1^{+}$) and B cells (live $CD19^{+}$) were stained intracellularly for Ii or isotype control.

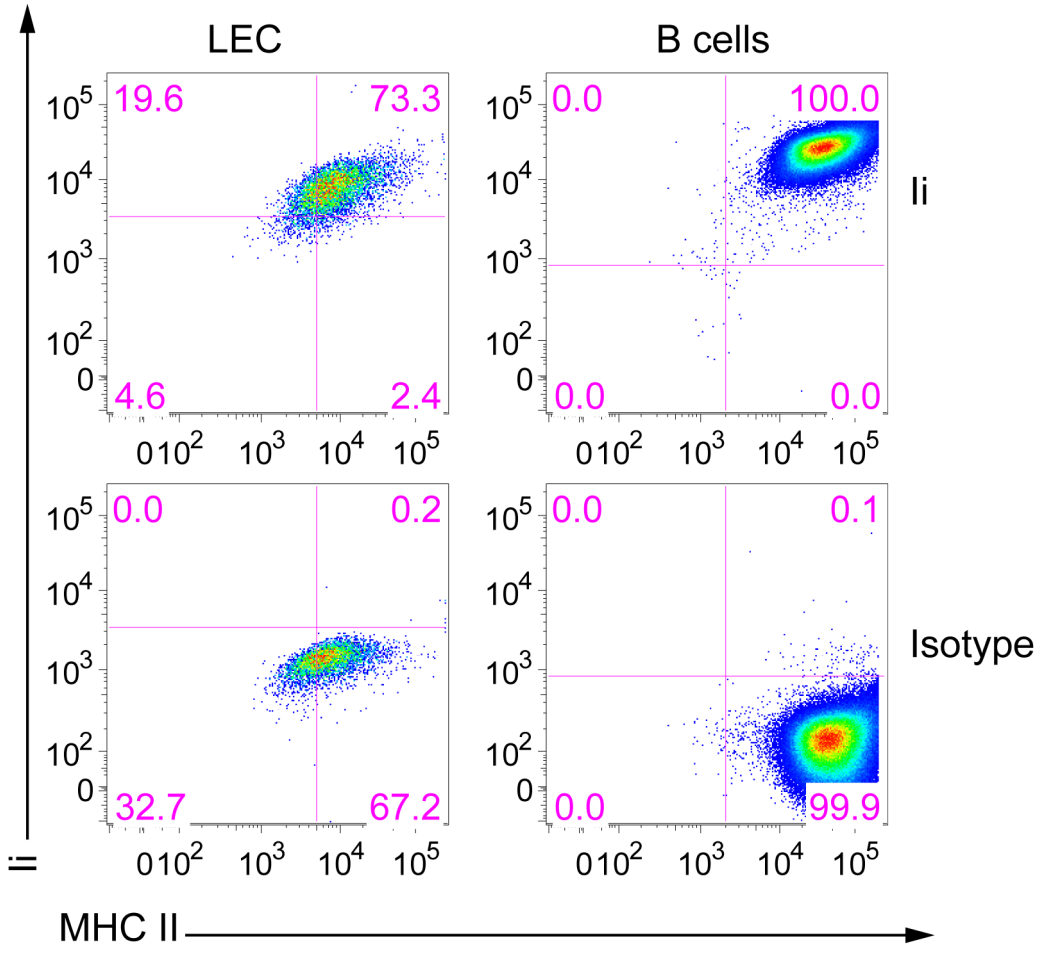
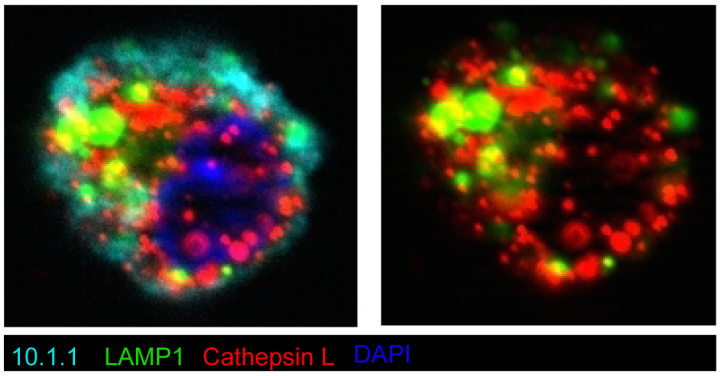

LEC
B cells
19.6
73.3
4.6
2.4
0.0
100.0
0.0
0.0
0.0
0.2
32.7
67.2
0.0
0.1
0.0
99.9
Ii
Isotype
MHC II

**Figure 18: LEC express active Cathepsin L.**

(A) LNs were enzymatically digested, depleted of $CD45^+$ cells, stained with 10.1.1 extracellularly and LAMP1 and cathepsin L intracellularly prior to being cytospun onto slides. Images are shown with (left) and without (right) 10.1.1 and DAPI staining. (B) LN-LEC or D-LEC were preincubated with the cathepsin L inhibitor 1-naphthalenesulfonyl-IW-CHO or DMSO vehicle control for 20 minutes at 37°C, followed by a 20 minute incubation with the cathepsin L substrate $(CBZ\text{-}Phe\text{-}Arg)_2$-rhodamine 110 at 37°C.

A


10.1.1
LAMP1
Cathepsin L
DAPI

B

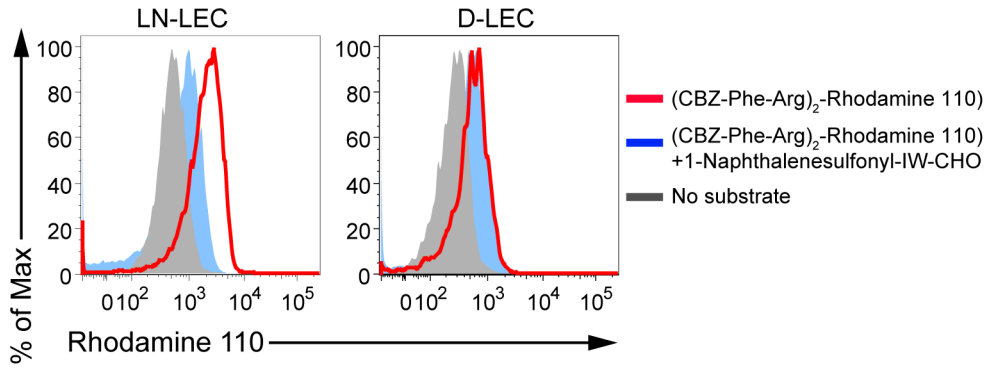

LN-LEC
D-LEC
% of Max
Rhodamine 110
(CBZ-Phe-Arg)2-Rhodamine 110)
(CBZ-Phe-Arg)2-Rhodamine 110)
+1-Naphthalenesulfonyl-IW-CHO
No substrate

**Figure 19: LEC do not express H2-M or H2-O.**

LEC (live $CD45^{neg}$ $CD31^{+}10.1.1^{+}$) and B cells (live $CD19^{+}$) were stained for H2-M (A) or H-2O (B) intracellularly. (C) LEC, DC, and B cells were sorted electronically, and 3T3 cells were harvested from culture. mRNA was purified and qPCR was performed for the indicated genes. (D) LEC and B cells from B6 or MHC $II^{-/-}$ (negative control) mice were stained extracellularly for CLIP.

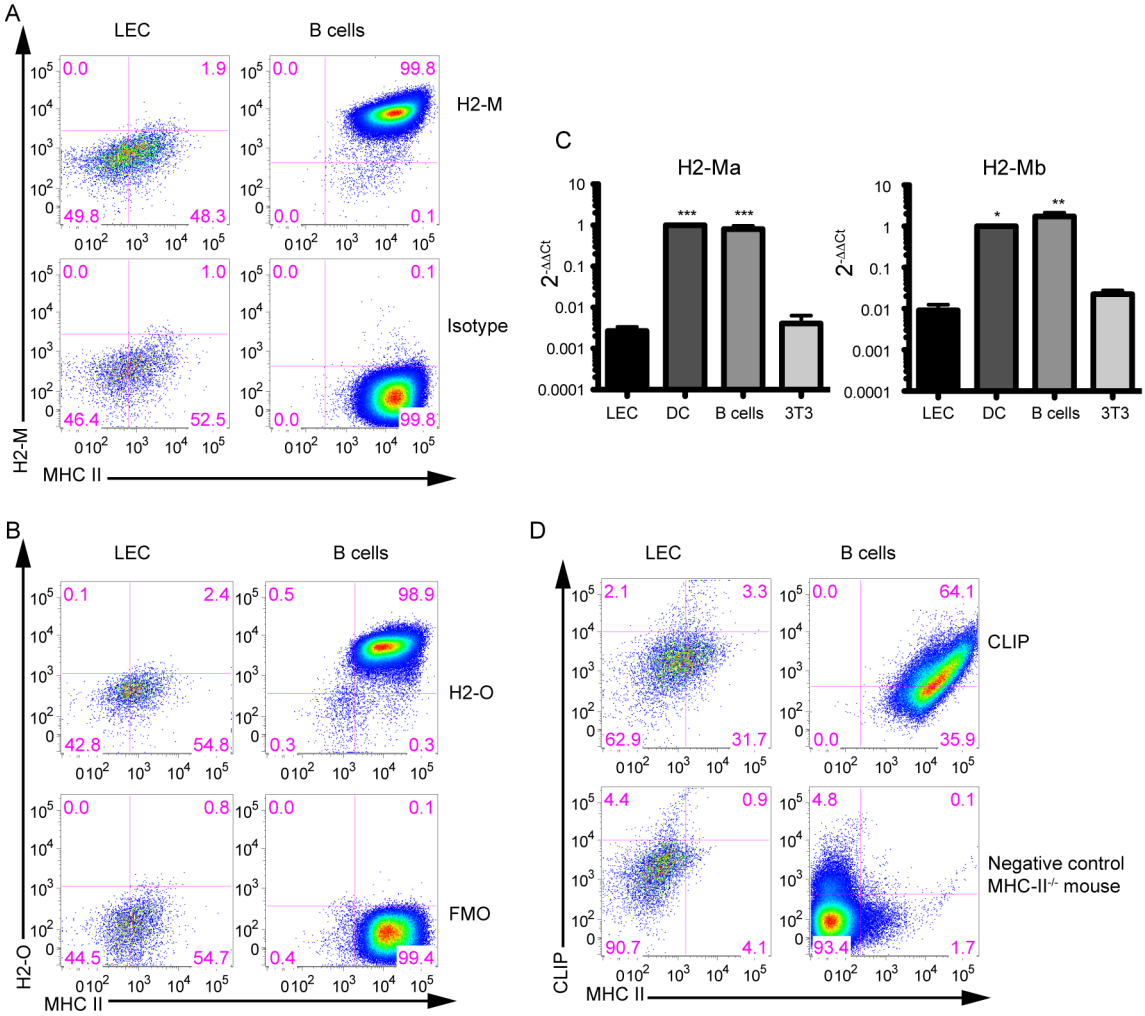

A
LEC
B cells
H2-M
Isotype
H2-M
MHC II
B
H2-O
FMO
H2-O
MHC II
C
H2-Ma
H2-Mb
2-ΔΔCt
LEC
DC
B cells
3T3
D
CLIP
Negative control
MHC-II-/- mouse
CLIP
MHC II

**Figure 20: Hematopoietically-derived cells acquire β-gal from LEC.**

CTV-labeled Thy1.1$^+$ Bg2 cells were transferred into the indicated recipients, and proliferation in skin-draining LNs was measured 7 days later. Plots are gated on CD4$^+$ T cells.

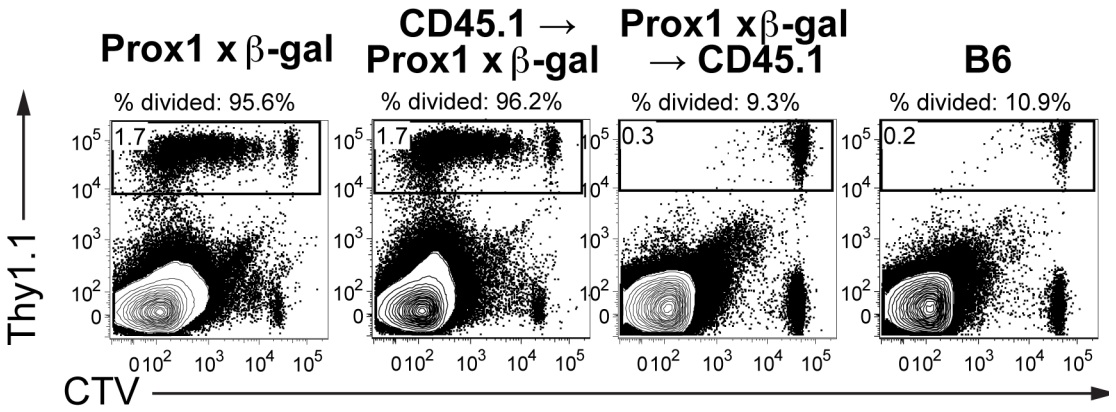
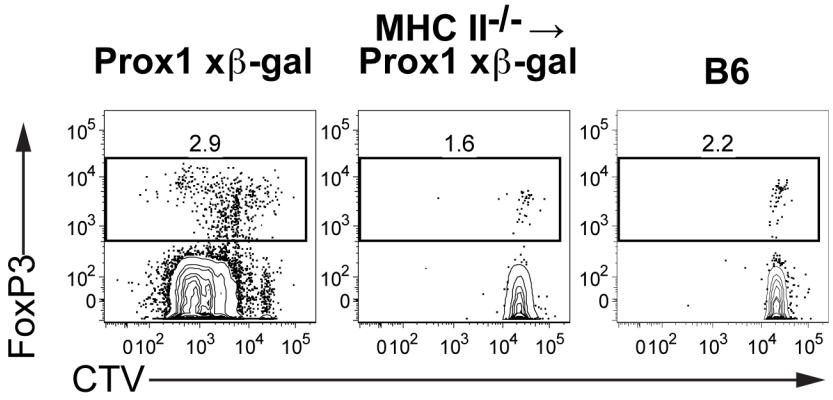

Prox1 x β-gal
CD45.1 → Prox1 x β-gal
Prox1 xβ-gal → CD45.1
B6
% divided: 95.6%
% divided: 96.2%
% divided: 9.3%
% divided: 10.9%
1.7
1.7
0.3
0.2
Thy1.1
CTV

**Figure 21: Bg2 cells do not differentiate into $T_{reg}$ cells.**

CTV-labeled Thy1.1$^+$ Bg2 cells were transferred into the indicated recipients, and $T_{reg}$ development was measured 3 days later. Plots are gated on CD4$^+$Thy1.1$^+$ Bg2 cells.


Prox1 xβ-gal
MHC II-/- → Prox1 xβ-gal
B6
2.9
1.6
2.2
FoxP3
CTV

and re-challenged them 28 days later with β-gal$_{721-739}$-pulsed bone marrow derived DC (BMDC). Proliferation was measured 5 days later by Ki67 upregulation (Figure 22A). In this experimental setup, CTV$^{dilute}$Ki67$^{pos}$ cells are actively proliferating in response to BMDC, whereas CTV$^{dilute}$Ki67$^{neg}$ cells proliferated initially after adoptive transfer but did not respond subsequently to the β-gal pulsed BMDC, and are thus anergic. As expected, Bg2 cells transferred into B6 mice remained naïve and proliferated strongly upon BMDC stimulation, as evidenced by their dilution of CTV and >85% expression of Ki67 (Figure 22B, C). Consistent with previous work[191], Bg2 cells transferred into B6 mice treated with 300 μg β-gal$_{721}$ peptide IV 0 and 3 days after adoptive transfer were CTV$^{dilute}$ and Ki67$^{neg}$, and thus anergic. Bg2 cells transferred into Prox-1 x β-gal mice were also CTV$^{dilute}$Ki67$^{neg}$ after BMDC re-challenge, and therefore were also anergic. Bg2 cells transferred into either MHC II$^{-/-}$ → Prox-1 x β-gal or MHC II$^{-/-}$ → CD45.1 bone marrow chimeras were strongly and equivalently CTV$^{dilute}$Ki67$^{pos}$, indicating that they retained sensitivity to BMDC re-challenge. We also tested whether Bg2 cells differentiate into $T_{reg}$ cells at this later timepoint. We found no increase of FoxP3$^{+}$CD25$^{+}$ Bg2 cells in Prox-1 x β-gal mice compared to B6 mice (Figure 22B), further demonstrating that $T_{reg}$ formation is not a form of tolerance induced in this model. These results confirm that LEC do not present β-gal and do not directly tolerize Bg2 cells. Instead, LEC provide β-gal to DC, which are essential for induction of anergy in Bg2 cells.

*Transfer of β-gal from LEC do DC does not solely occur through phagocytosis*

Antigen transfer from LEC to DC could occur through phagocytosis of apoptotic LEC, secretion of antigen-loaded exosomes or gap junctions. LEC do not load β-gal onto MHC II molecules, so cross-dressing of preloaded peptide:MHC II complexes does

not occur in this model. Phagocytosis depends intracellular signaling through the small GTPase Rac1, and inhibition or deletion of Rac1 inhibits engulfment of apoptotic cells[192,193]. We made bone marrow chimeras using $Rac1^{flox/flox}$ x $CD11c^{cre}$ → Lyve-1 x β-gal mice to ablate Rac1 in dendritic cells. To test whether these DC had an impaired ability to acquire β-gal from LEC, we co-cultured the DC from these chimeric mice with naïve Bg2 CD4 cells, and measured Bg2 proliferation. Bg2 co-cultured with DC from both $Rac1^{flox/flox}$ x $CD11c^{cre}$ → Lyve-1 x β-gal and $Rac1^{WT}$ x $CD11c^{cre}$ → Lyve-1 x β-gal chimeras proliferated (Figure 23), indicating that antigen transfer is not entirely dependent on DC phagocytosis. Recognition of phosphatidylserine is a commonly used signal triggering the engulfment of both apoptotic cells and exosomes[194–196]. Treatment with Annexin V has been used in vivo to block phosphatidylserine recognition[197]. Therefore, we treated Prox1 x β-gal mice with Annexin V beginning at the time tamoxifen chow was started, and adoptively transferred CTV-labeled Bg2 cells 8 days later. Bg2 cells proliferated equivalently in Annexin V and PBS treated mice (Figure 24), indicating that antigen transfer does not solely rely on recognition of phosphatidylserine. Combined, these results suggest that engulfment of apoptotic LEC is not required for transfer of antigens to DC. A recent report has shown that antigens can be transferred from macrophages to DC through connexin 43 gap junctions[198]. LEC in the LN sinuses are in close contact with DC, and both cell types express high levels of connexin 43[199], suggesting gap junctions are an attractive possibility for the mechanism of antigen transfer between LEC and DC. Alternatively, multiple mechanisms of antigen transfer may be used, and blocking a single method of transfer may not be sufficient to completely inhibit uptake of β-gal by DC. Future work is needed to distinguish between these possibilities.

**Figure 22: Presentation of antigen acquired from LEC leads to Bg2 anergy**

(A) Experimental design to measure tolerance induction. CTV-labeled $CD25^{neg}Thy1.1^{+}$ CD4 T cells were adoptively transferred, and recipient mice were challenged 28 days later with peptide-pulsed BMDC. Proliferation was measured 5 days later by Ki67 upregulation, and FoxP3 and CD25 were measured to assess $T_{reg}$ formation. (B) Representative and (C) cumulative data from skin-draining LNs.

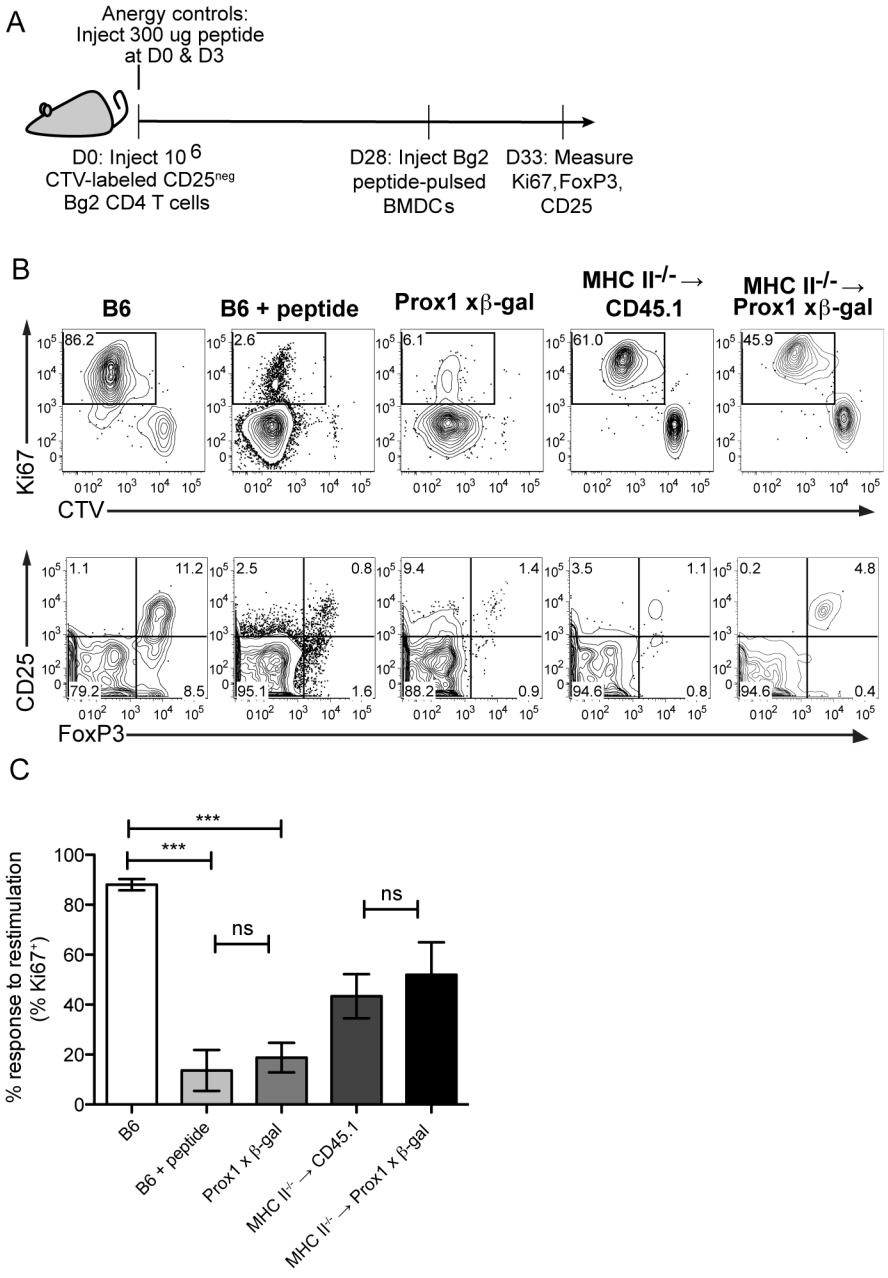
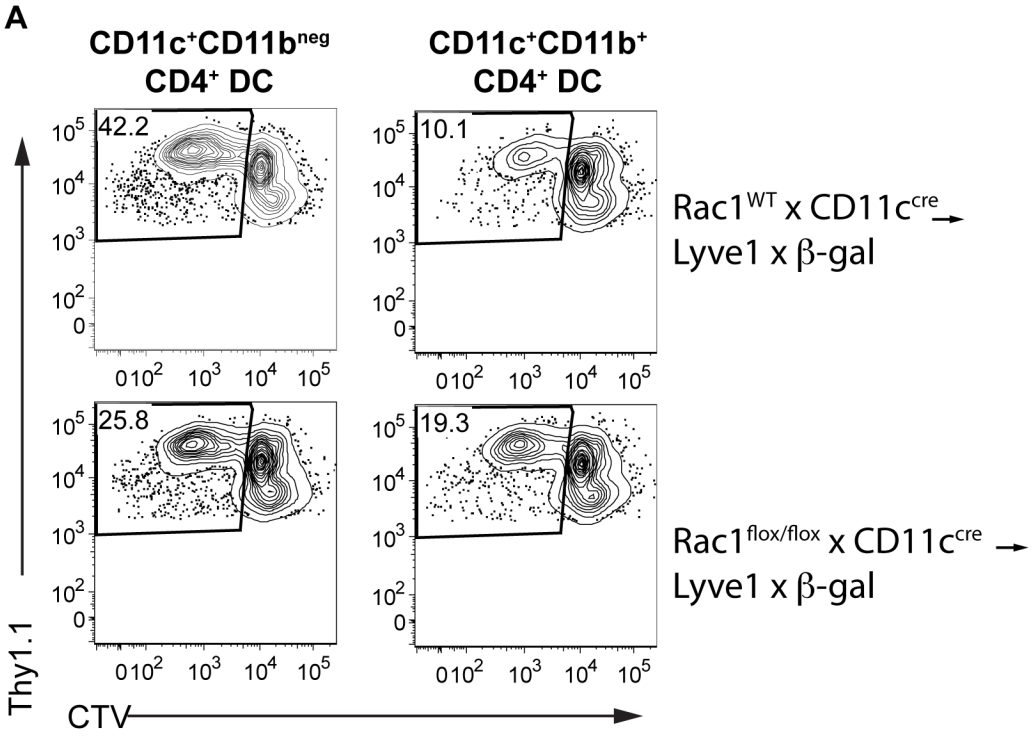

A
Anergy controls:
Inject 300 ug peptide
at D0 & D3
D0: Inject 10 6
CTV-labeled CD25neg
Bg2 CD4 T cells
D28: Inject Bg2
peptide-pulsed
BMDCs
D33: Measure
Ki67, FoxP3,
CD25
B
B6
B6 + peptide
Prox1 xβ-gal
MHC II-/- →
CD45.1
MHC II-/- →
Prox1 xβ-gal
Ki67
CTV
CD25
FoxP3
C
% response to restimulation
(% Ki67+)
***
***
ns
ns
B6
B6 + peptide
Prox1 x β-gal
MHC II-/- → CD45.1
MHC II-/- → Prox1 x β-gal

**Figure 23: Phagocytosis is not required for antigen transfer**.

$CD11c^{+}CD11b^{neg}CD4^{+}$ DC or $CD11c^{+}CD11b^{+}CD4^{+}$ DC were sorted from $Rac1^{WT}$ x $CD11c^{cre}$ → Lyve-1 x β-gal or $Rac1^{flox/flox}$ x $CD11c^{cre}$ → Lyve-1 x β-gal bone marrow chimeras and were co-cultured with CTV-labeled Bg2 cells for 4 days. Representative (A) and cumulative (B) proliferation of the Bg2 cells is shown.


A
CD11c+CD11b^neg CD4+ DC
CD11c+CD11b+ CD4+ DC
42.2
10.1
25.8
19.3
Rac1^WT x CD11c^cre →
Lyve1 x β-gal
Rac1^flox/flox x CD11c^cre →
Lyve1 x β-gal
Thy1.1
CTV

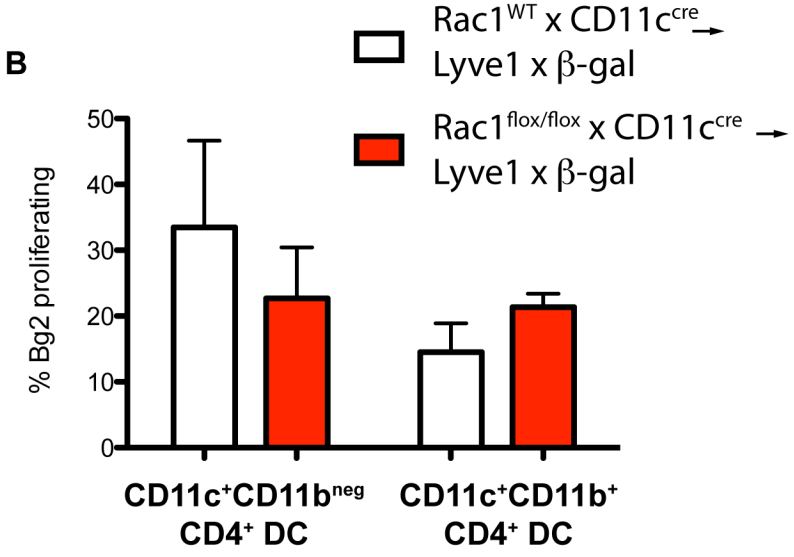
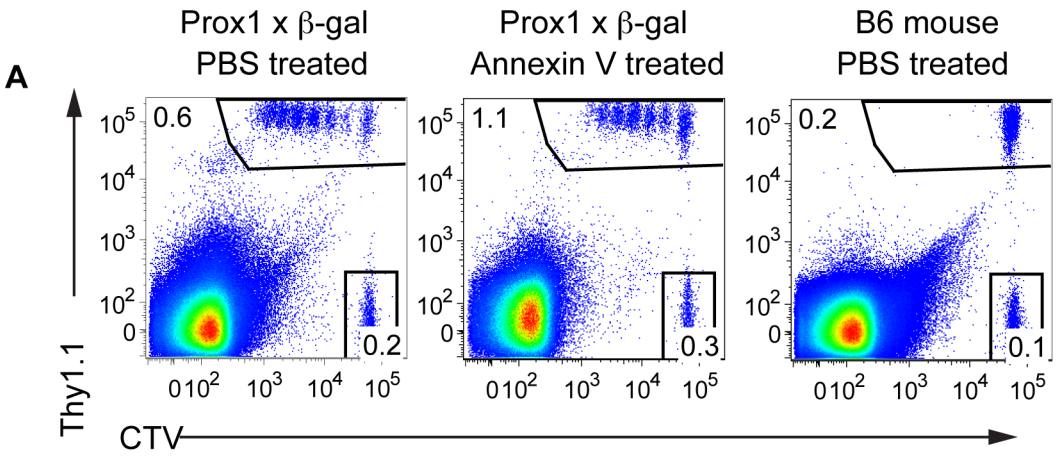
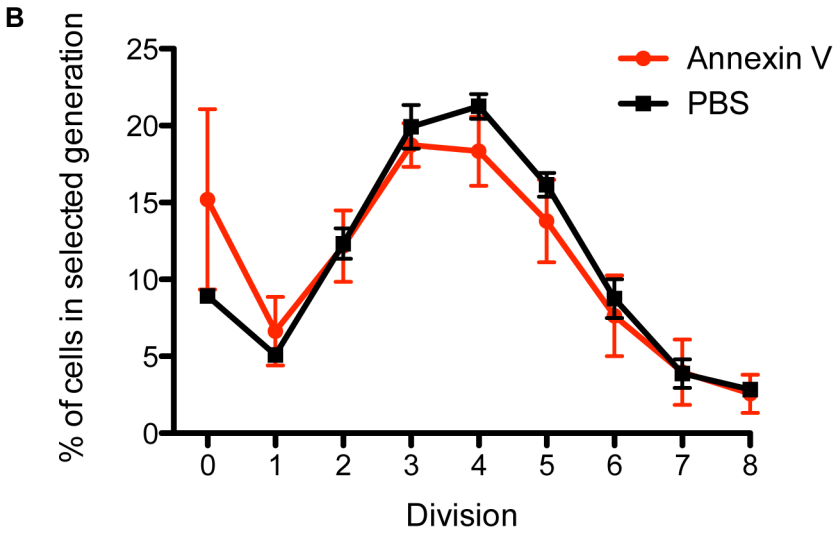

B
Rac1^WT x CD11c^cre →
Lyve1 x β-gal
Rac1^flox/flox x CD11c^cre →
Lyve1 x β-gal
% Bg2 proliferating
CD11c+CD11b^neg CD4+ DC
CD11c+CD11b+ CD4+ DC

**Figure 24: Phosphatidylserine recognition is not required for antigen transfer**.

Prox1 x β-gal mice were treated with 1 mg/kg of Annexin V IP every other day starting when the mice were placed on tamoxifen chow. 1 x $10^6$ CTV-labeled Bg2 cells were adoptively transferred 8 days later, and proliferation in peripheral LN was measured 3 days later. Representative (A) and cumulative (B) data is shown.


A
Prox1 x β-gal
PBS treated
Prox1 x β-gal
Annexin V treated
B6 mouse
PBS treated
0.6
0.2
1.1
0.3
0.2
0.1
Thy1.1
CTV


B
% of cells in selected generation
Annexin V
PBS
Division

**Discussion**

LEC express a variety of PTA and directly induce CD8 T cell tolerance, but their ability to induce CD4 tolerance to PTA has not previously been examined. In this study, we demonstrate that MHC II expression on LEC is sufficient for presentation of exogenously pulsed peptides, but LEC do not present peptides from 3 endogenous proteins, regardless of antigen localization. LEC do not express H2-M, suggesting that they cannot efficiently load antigen peptides onto MHC II molecules. While LEC do not directly present PTA on MHC II, they do provide them to DC, which then induce anergy. Therefore, LEC serve as a reservoir of PTA in the LN, which are acquired by DC for the induction of CD4 T cell tolerance. Additionally, MHC II plays a role in the deletional tolerance of Bg1 CD8 T cells, which delete through either the PD-1/PD-L1 or LAG-3/MHC II pathways after recognizing antigen presented by LEC.

LEC do not present epitopes derived from β-gal, HA, or I-Eα on MHC II. This lack of presentation is independent of antigen source protein localization to cytoplasm, plasma membrane, or endosome. Additionally, HA and I-Eα can be presented in autophagy deficient cells[184,188,189], suggesting this is not due to a specific limitation in autophagic uptake. In particular, the lack of Y-Ae staining suggests that the problem does not lie in loading the source proteins into the MHC II processing pathway, since I-Eα is a component of the I-$E^d$ MHC II molecule in BALB/c mice and will be endogenously targeted to the MIIC by Ii. LEC express high levels of Ii, suggesting that MHC II molecules are appropriately chaperoned from the ER to the plasma membrane and into the MIIC. Additionally, cathepsin L is active in LEC, suggesting that Ii can be cleaved into CLIP. However, LEC do not express H2-M, suggesting that their ability to exchange CLIP for antigenic peptides is severely compromised. Although elevated levels of CLIP

could not be detected in LEC, the high level of non-specific background CLIP staining in LEC substantially limited the ability to detect a positive signal. Together, this data suggests a model where MHC II:Ii complexes are correctly formed in the ER and transit to the MIIC, where Ii is cleaved by cathepsin L into CLIP. However, without H2-M, CLIP cannot be efficiently exchanged for antigen peptides, preventing β-gal, HA, or I-Eα from being loaded on MHC II molecules.

The lack of endogenous antigen presentation by LEC is somewhat surprising, particularly in light of results in two other studies. Onder et al[200] used podoplanin-cre transgenic mice to induce β-gal expression in FRC and LEC, analogous to our models, and demonstrated that Bg2 cells proliferated after adoptive transfer into these mice. However, they did not determine the cell responsible for inducing proliferation. Our results suggest that their observations were a consequence of antigen transfer from LEC to DC and macrophages, rather than direct presentation by either FRC or LEC.

In the second study, Dubrot et al[179] showed that LEC acquired preloaded peptide:MHC II complexes from DC and thereby directly induced a non-proliferation dependent form of apoptosis of antigen-specific T cells in vitro[179]. Our data using CD45.1 → MHC $II^{-/-}$ bone marrow chimeras supports their conclusion that LEC can acquire MHC II molecules from hematopoietic cells in vivo, a process referred to as cross-dressing[201]. However, we also found that LEC express similar levels of MHC II in MHC $II^{-/-}$ → CD45.1 chimeras, and they express mRNA for MHC II molecules and some elements of the MHC II processing and presentation pathway. Thus, LEC synthesize a large fraction of the MHC II molecules that they express. Importantly, while our results establish that LEC provide source proteins to DC for efficient presentation by both MHC I and MHC II

molecules, we saw no evidence that these particular MHC II:peptide complexes were transferred back to the LEC in vivo in sufficient quantities to induce CD4 T cell recognition. Since Dubrot et al[179] did not demonstrate CD4 T cell recognition of antigens displayed on MHC II by LEC in vivo, and the form of apoptotic death demonstrated in vitro was unusual, much remains to be done to establish the relevance of LEC cross-dressing in tolerance induction in vivo.

Conversely, our results provide compelling evidence that LEC indirectly function in peripheral CD4 tolerance by serving as a reservoir of PTA in the LN, which are acquired and presented by DC to induce anergy. Similarly, mTEC and DC share responsibility for CD4 tolerance induction in the thymus. mTEC express high levels of MHC II, and directly present some antigens to CD4 T cells, while others are transferred to DC for MHC II presentation[70–75]. It is not entirely clear what determines whether an antigen is presented by mTEC, DC, or both, but this may be related to the efficiency with which the antigen can access the MHC II presentation pathway. mTEC are not highly phagocytic[202], but do constitutively undergo autophagy[188], allowing cytoplasmic proteins to enter the MHC II processing pathway. Aichinger et al demonstrated that when targeting of a model antigen to autophagosomes is disrupted, mTEC are no longer able to induce negative selection, but DC presentation can compensate and maintain tolerance at high antigen levels[203]. This suggest one possible model where PTA that are not efficiently incorporated into autosomes are not presented on MHC II by mTEC, but are instead passed to DC for the induction of tolerance. Although it is unknown whether LEC constitutively undergo autophagy like mTEC[188], MHC II epitopes from HA and I-Eα can be generated independently of autophagy[184,188,189], suggesting that a lack of constitutive autophagy alone would not preclude presentation. While further work is

needed to determine why particular antigens are transferred to DC, our work demonstrates that PTA that are not presented by LEC on MHC II can be provided to DC for the induction of tolerance, a process analogous to what occurs in the thymus.

A variety of different mechanisms are used to transfer antigens amongst different cell types. In the gut, macrophages take up soluble fed antigens and transfer them to $CD103^+$ DC through gap junctions for the induction of oral tolerance[198]. Antigen transfer can also occur through exosomes[204–206] or phagocytosis of apoptotic cells[207–209]. Antigen transfer between mTEC and DC in the thymus is enhanced by the presence of Aire, but the exact mechanism is unclear[73]. Transfer of peptide:MHC II complexes from DC to LEC occurs through a mechanism involving cell contact and/or exosomes[179]. LEC could potentially transfer antigens to DC through exosomes, gap junctions, or DC phagocytosis of apoptotic LEC. We found that transfer of β-gal from LEC to DC does not solely rely on phagocytosis, as blocking phosphatidylserine recognition or inhibiting DC phagocytosis does not block antigen transfer in vivo. Further work is needed to elucidate the exact mechanism by which PTA are transferred from LEC to DC.

Although MHC II molecules on LEC do not directly present peptides for CD4 T cell tolerance, they play an important role in CD8 T cell tolerance. We previously demonstrated LEC induce deletion of tyrosinase-specific CD8 T cells through PD-1/PD-L1[21]. Here, we show that both the PD-1/PD-L1 and LAG-3/MHC II pathways are used in combination to tolerize β-gal specific CD8 T cells. LAG-3 and PD-1 have previously been demonstrated to act synergistically in other models of tolerance to self and tumor antigens[210,211]. Although we have not specifically shown that MHC II on LEC as opposed to other MHC $II^+$ cells is used as the ligand for LAG-3, this is likely as LEC are the only

cells presenting β-gal$_{96-103}$ in MHC I$^{-/-}$ → Lyve-1 x β-gal bone marrow chimeras. This suggests that LEC are inherently tolerogenic cells, and can engage multiple inhibitory pathways to enforce CD8 T cell tolerance.

In summary, we have demonstrated that while LN-LEC express intermediate levels of MHC II molecules, they do not present MHC II β-gal, HA, or I-Eα peptides. Instead, MHC II on the cell surface is important to enforce CD8 T cell tolerance through the LAG-3 pathway. LEC indirectly induce CD4 anergy by transcribing PTA and transferring them to DC for MHC II presentation, a process analogous to what occurs in the thymus. Since thymic tolerance is incomplete, peripheral transcription of PTA by LEC and other LNSC ensures that self-reactive T cells have multiple opportunities to be tolerized. LEC synergize with cross-tolerizing DC by enhancing the availability of PTA in all LNs, not just draining LN. By directly presenting antigens to CD8 T cells as well as transferring antigens to DC, LEC play multiple tolerogenic roles.

# APPENDIX A: INFLAMMATION DOES NOT ALTER THE ABILITY OF LEC TO INDUCE TOLERANCE

## Introduction

Peripheral tolerance induction is classically ascribed to immature or semi-mature DC cross-presenting endogenous antigens from apoptotic cells in draining LN. We and others have recently shown that LEC, FRC, and eTAC adventitiously express PTA and directly present derived epitopes, leading to abortive proliferation and deletion of CD8 T cells[20,79–81]. LEC, FRC, eTAC, BEC and mTEC express distinct but partially overlapping subsets of PTA[79–81]. LEC endogenously express and present tyrosinase, a melanocyte differentiation protein required for pigment production, leading to the abortive proliferation and deletion of FH T cells[20,80]. In iFABP-OVA mice, ovalbumin (OVA) is expressed in the small intestine under the control of the intestinal fatty acid binding protein (iFABP) promoter and is adventitiously expressed as a PTA in FRC[81,173]. Deletional tolerance of OVA-specific CD8 T cells (OT-I) is induced by FRC, which directly present OVA in all LN, and by $CD8\alpha^+$ DC that cross-present OVA derived from the small intestine in the mesenteric LN[81,173]. Recently, we found that LEC induce deletion of tyrosinase-specific FH T cells through the PD-1:PD-L1 pathway[21]. LEC do not express CD70, CD80, CD86, 4-1BBL or OX40L in the steady-state, and when FH T cells are activated in the absence of these costimulatory molecules, the proliferating T cells rapidly upregulate PD-1 to high levels. PD-1 interacts with PD-L1 on a radioresistant stromal cell, which inhibits T cell upregulation of the IL-2 receptor, depriving the cells of an essential survival signal and leading to their death. However, if the PD-1:PD-L1 pathway is blocked or if FH T cells receive an exogenous costimulatory signal through 4-1BB, they accumulate instead of undergoing deletion. Deletional tolerance in iFABP-

OVA mice is also mediated by the PD-1:PD-L1 pathway[112]. In both of these models of LNSC-induced tolerance, the CD8 T cells cause autoimmunity if tolerance is disrupted[21,112], demonstrating the functional importance of this pathway.

LEC express the highest level of PD-L1 of any cell in the LN[21]. LEC located in medullary and subcapsular sinuses express the highest levels of PD-L1, with lower expression on cortical LEC[21,120]. Medullary sinus LEC are also the only subset that expressed a sufficient level of tyrosinase to activate FH T cells[120]. Since the medullary sinus is the exit from the LN, this suggests that LEC engage and induce deletion of PD-1 expressing FH T cells as they attempt to leave. It is also possible that FH T cells upregulate PD-1 as they exit, and engage PD-L1 expressing subcapsular LEC in downstream LN. Interestingly, FRC express very low-levels of PD-L1 in the steady-state[21,81], suggesting that deletion of OT-I cells in iFABP-OVA mice might involve PD-L1 expressed on a cell other than FRC.

Presentation of antigens by DC can induce either tolerance or immunogenic activation of CD8 T cells, depending on the type and maturation status of the DC. Tolerance occurs in the steady-state when immature or semi-mature DC present self-antigens derived from apoptotic cells[64]. DC matured by CD40 or the TL3 ligand polyinosinic:polycytidylic acid (Poly(I:C)) upregulate costimulatory molecules such as CD80 and CD86, as well as cytokines such as IL-1, IL-6, IL-12, and TNFα, leading to immunogenic CD8 activation[212,213]. We questioned whether TLR3 or CD40 ligation might similarly cause LNSC to mature and induce immunogenic instead of tolerogenic CD8 T cell responses.

Several studies have examined the effects of TLR or CD40 ligation on cultured or primary endothelial cells. Cultured human LEC express TLR1-6 and TLR9, and respond to TLR stimulation by upregulating chemokines and adhesion molecules that recruit lymphocytes, macrophages and DC to lymphatic vessels[145,214]. Primary murine LEC and FRC express TLR3, and treatment with Poly(I:C) upregulates PD-L1 on LEC and FRC but does not change expression of CD80 or CD86[81]. Poly(I:C) downregulates OVA expression in FRC of iFABP-OVA mice, leading to reduced OT-I proliferation in vitro; however, the functional consequences in vivo were not evaluated. Interestingly, TLR3 signaling does not uniformly control PTA expression, as proteolipid protein was downregulated in LEC but upregulated in FRC. LEC and FRC also respond to signaling by the pro-inflammatory cytokines IFNγ and TNFα by secreting nitric oxide (NO), which limits T cell proliferation[151,152]. These studies suggest that inflammatory conditions might act on LEC to reduce antigen recognition, leading to ignorance, which would limit both tolerance and potential immunogenic activation.

LEC and FRC have been reported to express CD40[81], suggesting they might respond to activated $CD40L^+$ CD4 T cells. CD40 stimulation of cultured human umbilical vein endothelial cells (HUVECs) upregulates adhesion molecules, chemokines and cytokines that support lymphocyte adhesion and trafficking, antigen processing and presentation genes, and viral infection sensors such as TLR3 and RIG-I[215]. The consequences of CD40 signaling in primary LEC and FRC have not been evaluated. CD40 signaling provided by $CD40L^+$ CD4 T cells might also alter LNSC-induced tolerance of CD8 T cells. In a model system where β-galactosidase is expressed in LEC and FRC, co-transferring CD4 and CD8 T cells specific for β-gal prevented CD8 T cell deletion[200]. Although the mechanism was not investigated, one possibility is that LEC

develop a more immunogenic phenotype if CD40L on CD4 T cells binds to CD40 on the LEC. Additionally, OT-I cells transferred into iFABP-OVA mice treated with an agonistic CD40 antibody proliferate and accumulate instead of undergoing deletion, leading to lethal autoimmune enteritis[216]. At the time, it was not known that FRC present OVA and induce tolerance in these mice, so it was assumed that the abrogation of tolerance was due to the maturation of cross-tolerizing DC. An alternative possibility is that anti-CD40 induces the maturation of LNSC and leads to immunogenic instead of tolerogenic presentation of PTA. Therefore, we investigated how CD40 or TLR3 ligation changes the phenotype of LNSC, tyrosinase expression levels, and whether these factors alter the ability of LNSC to induce CD8 T cell tolerance.

**Results and Discussion:**

*Lymph node stromal cells respond to inflammatory stimuli by upregulating PD-L1 and MHC II, but not costimulatory molecules*

Freshly isolated LEC and FRC have been reported to express CD40 protein and TLR3 mRNA[81], and TLR4 has been detected on primary cultured LEC[214], suggesting that LEC might undergo maturation similar to that of DC during an immune response. In vivo treatment with the TLR3 agonist Poly(I:C), TLR4 agonist LPS or an agonistic CD40 antibody failed to induce a significant upregulation of the costimulatory molecules CD70, CD80, CD86 or 4-1BBL (Figure 25) on LEC and FRC, but did increase expression of the co-inhibitory molecule PD-L1. LEC and FRC also increased MHC II levels in response to CD40 stimulation, but not after Poly(I:C) or LPS treatment. In contrast, $CD11c^+$ DC upregulated PD-L1, CD80, CD86 and MHC II in response to Poly(I:C) treatment, and additionally upregulated CD70 in response to CD40 treatment (Figure 25). LPS

treatment primarily led to upregulation of PD-L1 and MHC II on DC. Therefore, the response of LNSC to TLR or CD40 signals is distinct from that of professional APC: although TLR and CD40 ligation induces both LNSC and DC to upregulate PD-L1, LNSC do not upregulate costimulatory molecules and show a more selective upregulation of MHC II molecules.

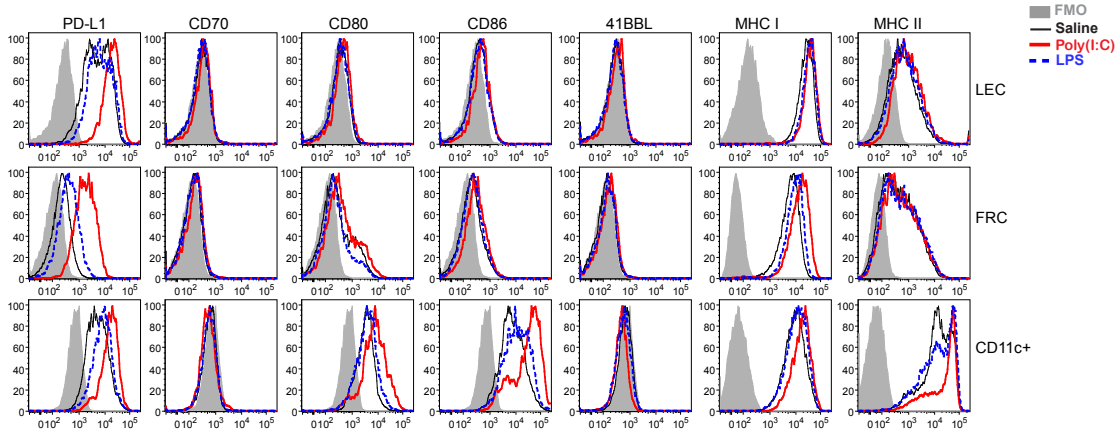
*LEC maintain tolerance after TLR3 stimulation*

A previous report demonstrated that treatment with the TLR3 ligand Poly(I:C) causes LNSC to downregulate some PTAs while maintaining or upregulating others[81]. OVA downregulation in FRC led to reduced OT-I proliferation in vitro, but the in vivo effects were not tested. We found that Poly(I:C) treatment downregulated tyrosinase mRNA by 20-fold in LEC, while CD40 treatment did not substantially affect tyrosinase mRNA expression levels (Figure 26). To determine whether this downregulation diminished or eliminated antigen presentation in vivo, we transferred FH T cells into Poly(I:C) treated tyrosinase$^{+}$ mice. These cells still underwent complete proliferation and deletion (Figure 27A). FH T cells transferred into mice treated with LPS also underwent proliferation and deletion (Figure 27A). In contrast, agonistic anti-4-1BB costimulation rescued FH T cells from deletion (Figure 27B) as previously reported[21]. This indicates that although Poly(I:C) downregulates tyrosinase, it is still presented at sufficient levels to induce CD8 T cell proliferation and deletion instead of T cell ignorance. This also provides additional evidence that Poly(I:C) and LPS do not induce the maturation of LEC to immunogenic APC.

**Figure 25: LEC respond to inflammatory conditions by upregulating PD-L1**

C57Bl/6 mice were treated with saline, 250 µg Poly(I:C) IV, or 250 ng LPS IP (A), or 25 µg anti-CD40 IP (B) 2 days before harvest. Pooled inguinal, axillary, brachial, cervical and mesenteric LN were enzymatically digested and separated into LNSC and CD45+ cells using magnetic beads, and stained for the indicated molecules. All plots are gated on single live cells, and are further gated on lineage markers: LEC are $CD45^{neg}$ $CD31^{+}$ $gp38^{+}$, FRC are $CD45^{neg}$ $CD31^{neg}$ $gp38^{+}$, BEC are $CD45^{neg}$ $CD31^{+}$ $gp38^{neg}$, DC are $CD11c^{high}$.

A


PD-L1
CD70
CD80
CD86
41BBL
MHC I
MHC II
FMO
Saline
Poly(I:C)
LPS
LEC
FRC
CD11c+

B

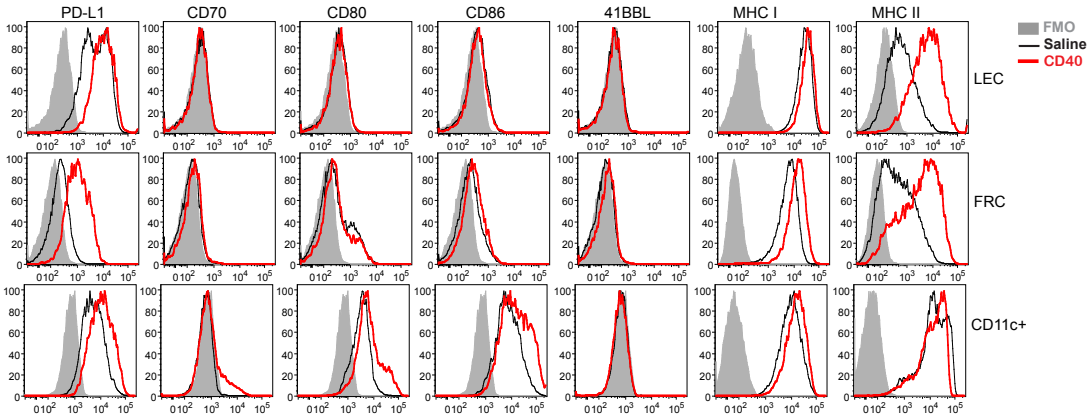

PD-L1
CD70
CD80
CD86
41BBL
MHC I
MHC II
FMO
Saline
CD40
LEC
FRC
CD11c+

**Figure 26: TLR3 ligation downregulates tyrosinase mRNA**

LNSC were isolated and FACS sorted from the skin-draining and mesenteric LN of C57Bl/6 mice treated with Poly(I:C) or anti-CD40 antibody. Tyrosinase mRNA was compared to untreated LEC using the $2^{-\Delta\Delta Ct}$ method.

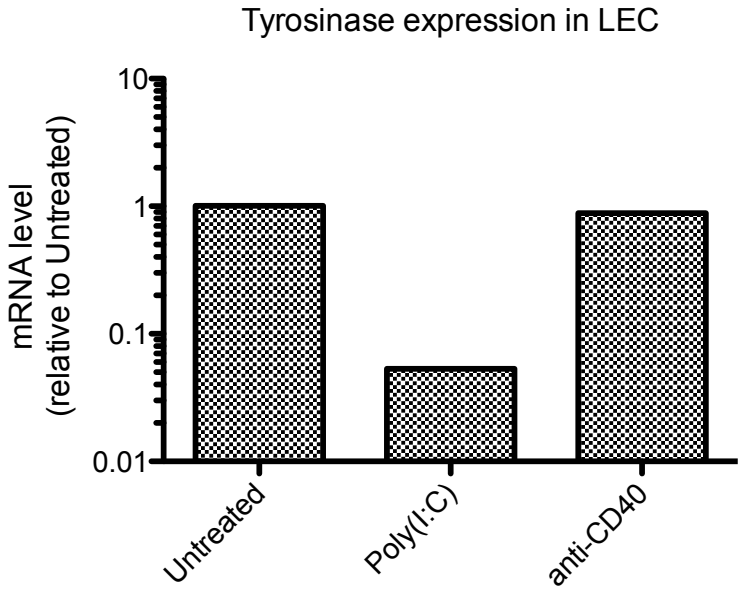
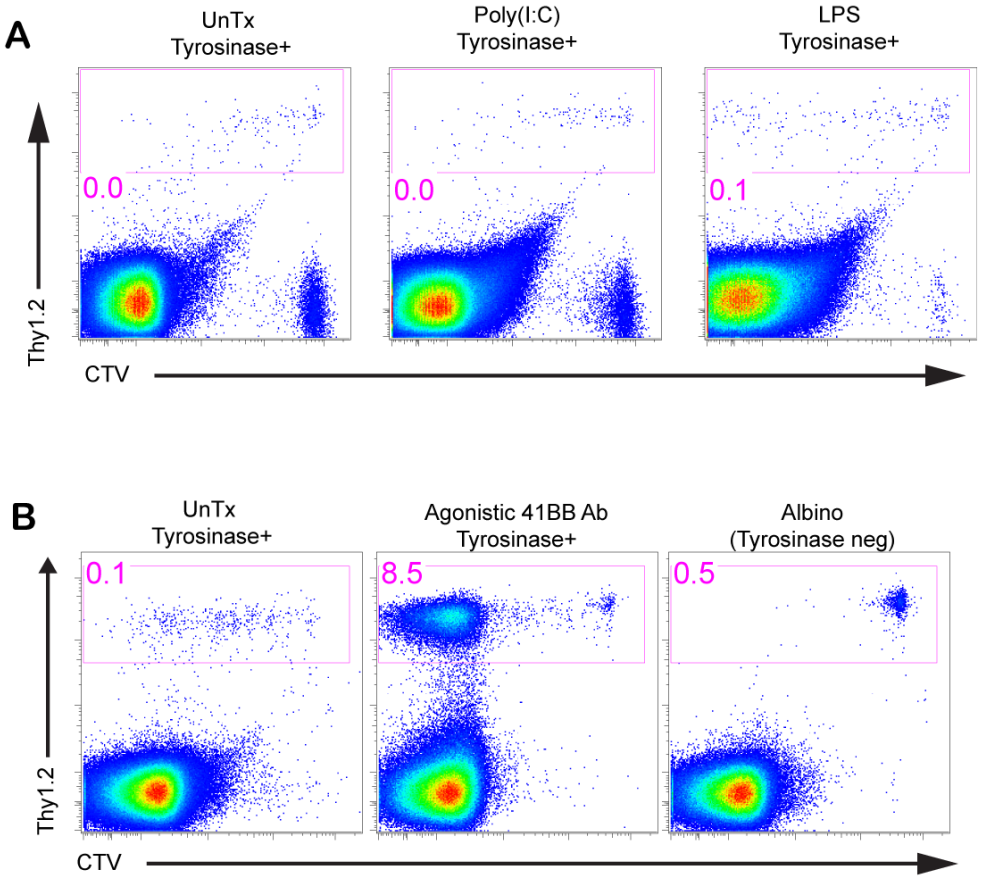

Tyrosinase expression in LEC
mRNA level
(relative to Untreated)
10
1
0.1
0.01
Untreated
Poly(I:C)
anti-CD40

**Figure 27: TLR3 or TLR4 ligation does not inhibit LEC-induced tolerance**

1E6 CTV-labeled Thy1.2 FH T cells were transferred into mice treated as indicated, and proliferation and accumulation was measured in skin-draining LN 7 days after transfer. CTV-labeled Thy1.1 cells were co-transferred as an injection control in A.


A
UnTx
Tyrosinase+
Poly(I:C)
Tyrosinase+
LPS
Tyrosinase+
0.0
0.0
0.1
Thy1.2
CTV
B
UnTx
Tyrosinase+
Agonistic 41BB Ab
Tyrosinase+
Albino
(Tyrosinase neg)
0.1
8.5
0.5
Thy1.2
CTV

*CD40 stimulation acts on hematopoietic cells to inhibit LNSC-mediated tolerance*

Although the ability of LEC to induce tolerance was not altered by Poly(I:C) or LPS, we questioned whether CD40 engagement would have a different effect. Unlike Poly(I:C) treatment, CD40 ligation did not change levels of tyrosinase mRNA expression but did lead to MHC II upregulation (Figure 25, 26), indicating that LEC respond differently to CD40 ligation and TLR3 stimulation. In contrast to the maintenance of tolerance induction after TLR3 stimulation, treatment with agonistic anti-CD40 led to the accumulation of FH T cells (Figure 28). However, this accumulation was substantially less than that seen with agonistic anti-4-1BB (Figure 27B) or when deletion was prevented using PD-$1^{KO}$ FH T cells (Figure 28).

Deletion of FH T cells is induced by the rapid, high-level upregulation of PD-1 on proliferating cells[21]. We found that CD40 stimulation reduced the upregulation of PD-1 on adoptively transferred T cells compared to untreated animals (Figure 29). However, the level of PD-1 was still higher on cells transferred into CD40 treated mice compared to mice treated with agonistic anti-4-1BB and anti-OX40 or mice infected with vaccinia virus expressing tyrosinase, corresponding to the smaller degree of rescue seen in anti-CD40 treated mice.

To determine whether PD-L1 was still inducing partial deletion in anti-CD40-treated mice, we used PD-$L1^{het}$ $tyrosinase^{+}$ mice, which express half the level of PD-L1 of wild-type mice (Figure 30) and normally induce slower but still complete deletion of FH T cells[21]. Interestingly, anti-CD40 treatment led to a greater accumulation of CD8 T cells in PD-$L1^{het}$ $tyrosinase^{+}$ mice than wild-type mice (Figure 28). This suggests that the level of signaling through the PD-1:PD-L1 pathway determines the completeness of deletion

induced by LEC. In keeping with the upregulation of PD-L1 on LEC by anti-CD40, this suggests after anti-CD40 treatment PD-L1 is still involved in the induction of deletional tolerance by LEC, even if it is not complete.

Since LEC do not upregulate costimulatory molecules that might rescue CD8 T cells from deletion but instead upregulate PD-L1 (Figure 25B), we questioned whether another cell type could be responsible for the accumulation of FH T cells. A variety of other cells express CD40, including DC, B cells, and CD8 T cells[213]. We used bone marrow chimeras where CD40 was knocked out on hematopoietic cells to determine if anti-CD40 was acting on radioresistant LEC or radiosensitive hematopoietic cells. Importantly, anti-CD40 treatment did not lead to accumulation of FH T cells in $CD40^{KO} \rightarrow PD\text{-}L1^{het}$ $tyrosinase^{+}$ chimeras (Figure 28). This indicates that anti-CD40 acts on a hematopoietic cell and not radioresistant LEC or adoptively transferred CD8 T cells. Radiosensitive hematopoietic cells do not cross-present tyrosinase from melanocytes in the steady-state[20], so this does not reflect maturation of a cross-presenting tolerogenic DC. Instead, accumulation might be due to anti-CD40 induced cytokines such as IL-6, IL-7 or IL-12 that are known to support T cell expansion and survival[213,217]; however, we have shown that IL-2, IL-7, IL-12 and IL-15 immune complexes do not prevent FH T cells from undergoing deletion (Figure 31). It is also possible that high levels of costimulatory molecules on CD40-activated hematopoietic cells rescue CD8 T cells from deletion by forming a 3-cell cluster with LEC.

In iFABP-OVA mice that express OVA in FRC, anti-CD40 stimulation breaks tolerance and leads to OT-I accumulation[216]; however, it is unknown whether anti-CD40 is acting on FRC, cross-presenting DC, or antigen-free hematopoietic cells. To address

**Figure 28: anti-CD40 inhibits LEC-induced tolerance through its effects on hematopoietic cells**

$1x10^6$ CTV-labeled PD-$1^{WT}$ or PD-$1^{KO}$ FH T cells were adoptively transferred into the indicated mice or chimeras along with non-specific CTV-labeled Thy1.1 cells as an injection control. Antigen specific cells were distinguished using tyrosinase tetramer. Mice were treated with 25 μg anti-CD40 antibody IP every other day starting the day prior to adoptive transfer. Proliferation and accumulation in skin-draining LN was measured 7 days after transfer.

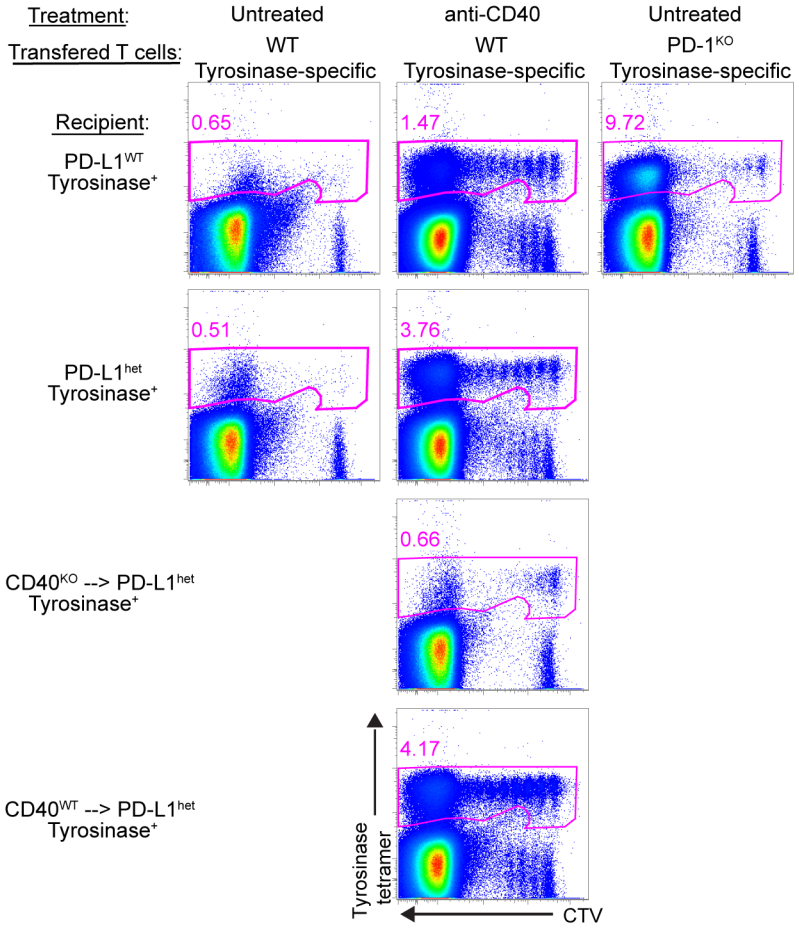
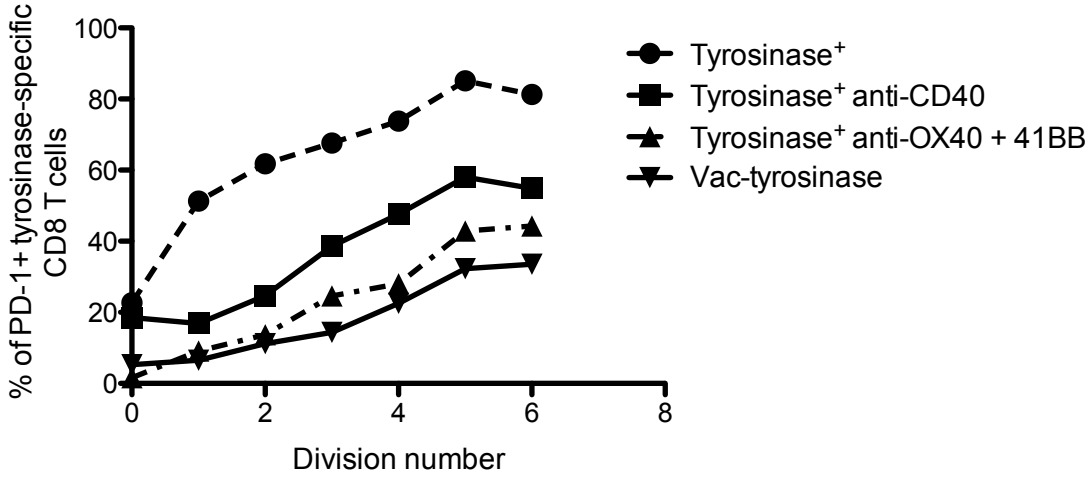

Treatment:
Transfered T cells:
Recipient:
Untreated
WT
Tyrosinase-specific
anti-CD40
WT
Tyrosinase-specific
Untreated
PD-1KO
Tyrosinase-specific
PD-L1WT
Tyrosinase+
0.65
1.47
9.72
PD-L1het
Tyrosinase+
0.51
3.76
CD40KO --> PD-L1het
Tyrosinase+
0.66
CD40WT --> PD-L1het
Tyrosinase+
4.17
Tyrosinase tetramer
CTV

**Figure 29: Anti-CD40 treatment leads to blunted upregulation of PD-1.**

1E6 CTV-labeled FH T cells were transferred into tyrosinase$^{+}$ mice were treated with anti-CD40 or anti-OX40 and 4-1BB, or Tyrosinase$^{neg}$ mice infected with vaccinia virus expressing tyrosinase. Cells were harvested 3 days after adoptive transfer, and the percentage of FH T cells expressing PD-1 (A) and the level of PD-1 by gMFI (B) was measured.

**A**


% of PD-1+ tyrosinase-specific CD8 T cells
100
80
60
40
20
0
0
2
4
6
8
Division number
Tyrosinase+
Tyrosinase+ anti-CD40
Tyrosinase+ anti-OX40 + 41BB
Vac-tyrosinase

**B**

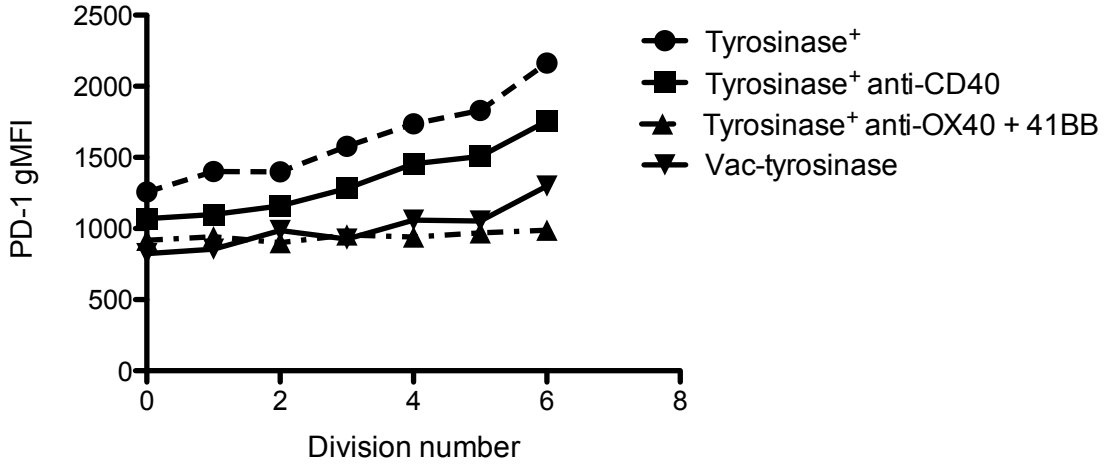

PD-1 gMFI
2500
2000
1500
1000
500
0
0
2
4
6
8
Division number
Tyrosinase+
Tyrosinase+ anti-CD40
Tyrosinase+ anti-OX40 + 41BB
Vac-tyrosinase

**Figure 30: PD-L1$^{het}$ mice express half the level of PD-L1 as WT mice.**

Pooled inguinal, axillary, brachial, cervical and mesenteric LN were enzymatically digested and separated using CD45 magnetic beads. Cells were stained with PD-L1 along with lineage antibodies. LEC are CD45.2$^{neg}$ CD31$^{+}$ gp38$^{+}$, FRC are CD45.2$^{neg}$ CD31$^{neg}$ gp38$^{+}$, BEC are CD45.2$^{neg}$ CD31$^{+}$ gp38$^{neg}$, DC are CD45.2$^{+}$CD11c$^{high}$B220$^{neg}$, and B cells are CD45.2$^{+}$B220$^{+}$CD11c$^{neg}$.

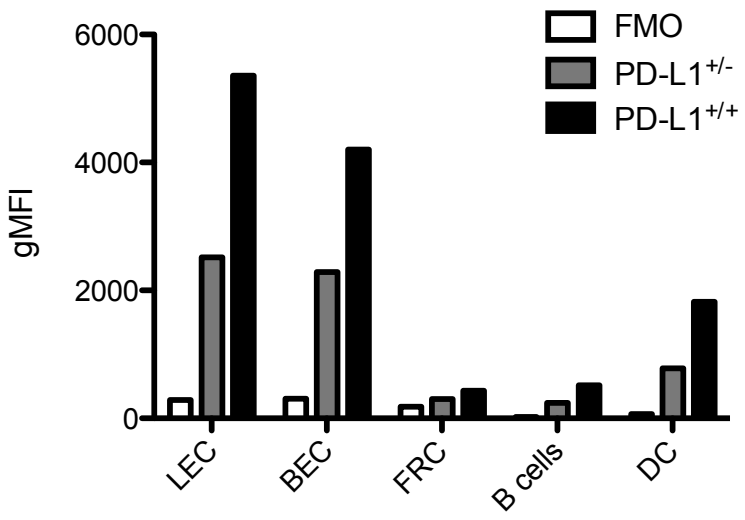
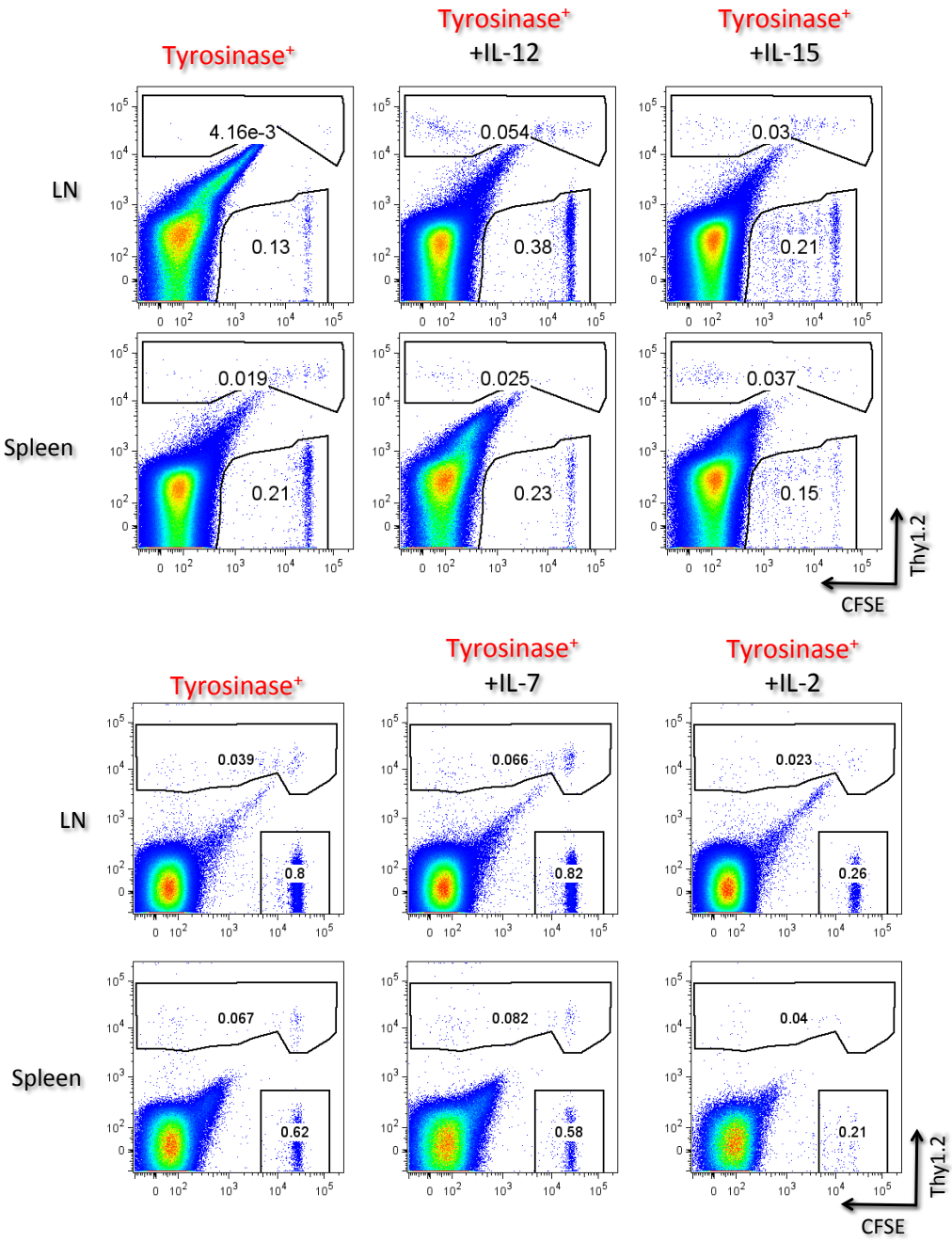

FMO
PD-L1+/-
PD-L1+/+
gMFI
6000
4000
2000
0
LEC
BEC
FRC
B cells
DC

**Figure 31: IL-12, IL-15, IL-7 or IL-2 do not rescue FH cells from undergoing deletion.**

Thy1.2 FH cells were adoptively transferred into tyrosinase$^{+}$ mice were treated with IL-12, IL-15, IL-7 or IL-2 immune complexes. Deletion was measured 7 days later. Plots are gated on CD8$^{+}$ T cells. Data from Cindy Guidi.


Tyrosinase+
Tyrosinase+ +IL-12
Tyrosinase+ +IL-15
LN
Spleen
4.16e-3
0.13
0.054
0.38
0.03
0.21
0.019
0.21
0.025
0.23
0.037
0.15
Thy1.2
CFSE
Tyrosinase+
Tyrosinase+ +IL-7
Tyrosinase+ +IL-2
LN
Spleen
0.039
0.8
0.066
0.82
0.023
0.26
0.067
0.62
0.082
0.58
0.04
0.21
Thy1.2
CFSE

this, we used $CD40^{KO}$→ iFABP-OVA bone marrow chimeras. DC cross-presenting OVA are found in mesenteric but not skin-draining LN[173], while OVA presenting FRC are found in all LN. We used FTY720 to prevent T cell egress from their priming LN[218]. Therefore, any OT-I cells found in the skin draining LN must have been activated by FRC, not DC. OT-I cells transferred into non-chimeric iFABP-OVA mice treated with FTY720 underwent deletion in skin-draining LN as expected, but accumulated dramatically in mice treated with agonistic anti-CD40 + FTY720 (Figure 32A). Similar to the results in $tyrosinase^{+}$ mice, anti-CD40 did not rescue OT-I cells from deletion in $CD40^{KO}$→iFABP-OVA bone marrow chimeras (Figure 32B). Together, these results demonstrate that CD40 does not mature LEC or FRC into immunogenic APC, but instead activates a hematopoietic cell that rescues CD8 T cells from deletional tolerance induced by these two LNSC populations.

Although CD40 expression on primary LEC and FRC has been previously reported by another group[81], after determining that anti-CD40 is not directly affecting the tolerogenic properties of LEC or FRC, we re-investigated this issue using $CD40^{-/-}$ mice as a biological negative control. We found that the substantial amount of HM40-3 CD40 staining previously reported on LEC[81] was in fact non-specific, as an equivalent level was detected on $CD40^{-/-}$ LEC. Interestingly, anti-CD40 acting on hematopoietic cells can still prevent tolerance induction when the hematopoietic cells are not directly presenting antigen, as tyrosinase is not cross-presented in the steady-state[20] and OVA is not cross-presented in skin-draining LNs[173]. This appears to be unique to anti-CD40, as FH T cells still delete in mice infected with vaccinia virus expressing the irrelevant antigen OVA (Figure 34). Further work would be needed to determine how anti-CD40 is uniquely able to prevent deletion. This demonstrates that the inflammatory environment in the LN

indirectly affects LNSC-induced tolerance, and suggests that strong immune responses may prevent recent thymic emigrants from undergoing tolerance even if the antigen is not cross-presented.

**Figure 32: anti-CD40 inhibits FRC-induced tolerance through its effects on hematopoietically derived cells**

$5x10^5$ CTV-labeled Thy1.1 OT-I cells were adoptively transferred into Thy1.2 iFABP-OVA mice (a) or $CD40^{-/-}$ → iFABP-OVA chimeras (b) along with CTV-labeled Thy1.2 cells as an injection control. Mice received 1 mg/kg FTY720 IP daily and 25 μg anti-CD40 IP as indicated every other day beginning the day before adoptive transfer, and skin-draining LN were harvested 6 days after T cell transfer.

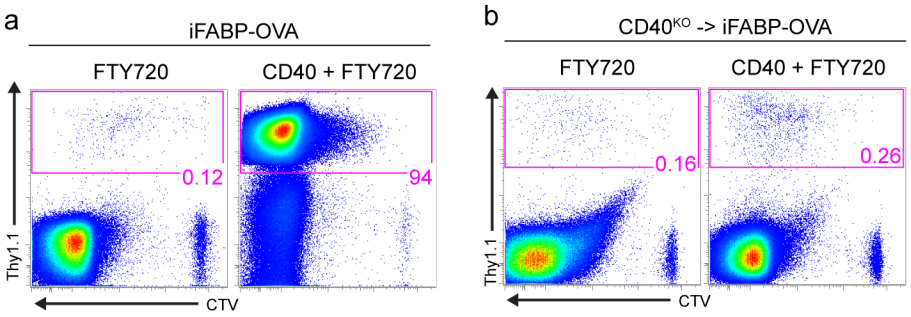

a
iFABP-OVA
FTY720
CD40 + FTY720
0.12
94
Thy1.1
CTV
b
CD40KO -> iFABP-OVA
FTY720
CD40 + FTY720
0.16
0.26
Thy1.1
CTV

**Figure 33: LEC, FRC, and BEC do not express CD40.**

Pooled inguinal, axillary, brachial, cervical and mesenteric LN were enzymatically digested and separated into LNSC and CD45+ cells using magnetic beads. Cells were stained for CD40 along with either CD31, gp38, and CD45 or CD11c, CD11b, CD19, and CD8. All plots are gated on single live cells, and are further gated on lineage markers: LEC are $CD45^{neg}$ $CD31^{+}$ $gp38^{+}$, FRC are $CD45^{neg}$ $CD31^{neg}$ $gp38^{+}$, BEC are $CD45^{neg}$ $CD31^{+}$ $gp38^{neg}$, DC are $CD11c^{high}CD19^{neg}$, B cells are $CD19^{+}CD11c^{neg}$, and CD8 T cells are $CD8^{+}CD19^{neg}CD11c^{neg}$.

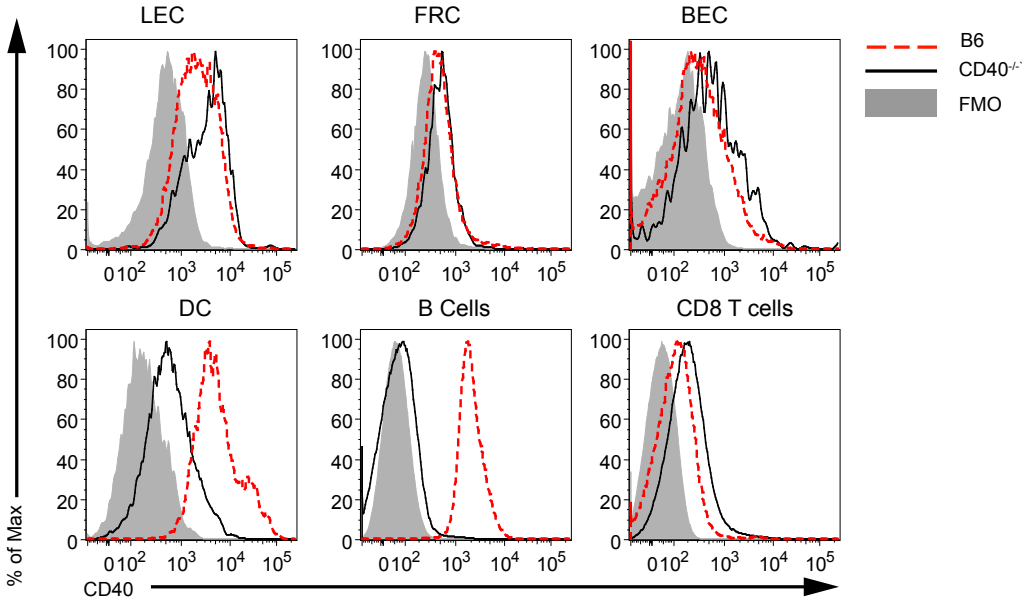
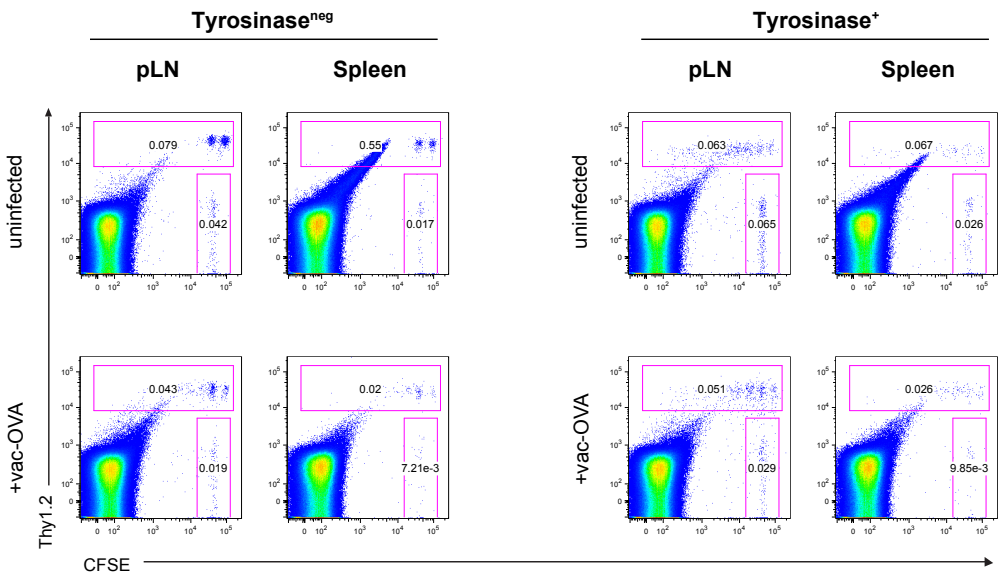

LEC
FRC
BEC
DC
B Cells
CD8 T cells
B6
CD40-/-
FMO
% of Max
CD40
100
80
60
40
20
0
0
10^2
10^3
10^4
10^5

**Figure 34: FH T cells still undergo deletion in mice infected with vaccinia-ova.**

Thy1.2$^{+}$ FH cells were adoptively transferred into Tyrosinase$^{neg}$ or Tyrosinase$^{+}$ mice that were either uninfected or infected 24 hours previously with vaccinia virus expressing OVA. Deletion was measured 7 days later. Plots are gated on CD8$^{+}$ T cells. Data from Cindy Guidi.


Tyrosinase^neg
Tyrosinase+
pLN
Spleen
pLN
Spleen
uninfected
+vac-OVA
Thy1.2
CFSE
0.079
0.042
0.55
0.017
0.063
0.065
0.067
0.026
0.043
0.019
0.02
7.21e-3
0.051
0.029
0.026
9.85e-3

**Conclusions:**

LEC express TLR3 and TLR4 molecules[81,214]. However, these studies demonstrate that the effects of ligating these molecules on the function of LEC and FRC are quite different from their effects on DC. Ligating TLR3 or TLR4 does not upregulate costimulatory molecules on LEC or FRC. TLR3 and TLR4 ligation does upregulate PD-L1 on LEC and FRC, although this could be due to direct effects on LNSC or indirect effects due to inflammatory mediators secreted by activated hematopoietic cells, because it was done in vivo. Since LNSC do not upregulate costimulatory molecules, it appears that TLR3 and TLR4 stimulation activates different transcription factors in DC compared to LNSC. Although CD40 expression on LEC and FRC has previously been reported[81], we found that this expression is actually non-specific antibody staining, as equivalent staining is seen in $CD40^{-/-}$ mice. However, anti-CD40 treatment does lead to upregulation of PD-L1 and MHC II on LEC and FRC. This is likely due to IFNγ or other inflammatory cytokines secreted by DC after anti-CD40 treatment, as both PD-L1 and MHC II are upregulated by IFNγ.

TLR3 ligation has variable effects on the level of expression of different PTAs. Poly(I:C) downregulates expression of several PTAs including tyrosinase and OVA, but upregulates others[81]. Although tyrosinase downregulation does not affect activation of FH T cells in vivo, it is possible that lower affinity T cells might remain ignorant under these conditions. The reported upregulation of some PTA after TLR3 ligation is intriguing, as it suggests that LNSC are not simply reducing PTA expression to prevent potential immunogenic activation. The true significance of this phenomenon is still unclear.

Since TLR3 or TLR4 ligation on LNSC does not directly change their tolerogenic phenotype, we speculate that the role of these sensors may be to allow LNSC to support an ongoing immune response. TLR ligation induces changes in chemokine and cytokine secretion[145], which allows LEC to influence cell migration. TLR4 ligation in LEC is essential for recruiting macrophages that induce lymphangiogenesis[214], increasing the density of tissue lymphatics and allowing the LN to expand in order to accommodate an increased number of cells. TLR ligation does not cause LEC or FRC to become immunogenic APC; in contrast, the upregulation of PD-L1 suggests that they might be more effective at inducing tolerance. Alternatively, the upregulation of PD-L1 may be important to protect LNSC from immune-mediated destruction, as has been reported for other tissues[117].

Interestingly, CD40 ligation on a hematopoietic cell prevents both FH and OVA-specific CD8 T cells from undergoing deletional tolerance. This occurs in situations where the hematopoietically-derived cells are not presenting tyrosinase or OVA. Although the precise mechanism of T cell accumulation remains to be defined, this suggests that inflammatory mediators induced by anti-CD40 can act in a bystander fashion to activate self-reactive T cells. This is unique to anti-CD40 treatment, as treatment with inflammatory cytokines or infection with vaccinia virus expressing an irrelevant antigen did not prevent FH deletion. Our results suggest that during a strong immune response LNSC-induced tolerance can be compromised, allowing bystander self-reactive T cells that have recently emigrated from the thymus to become immunogenically activated and induce autoimmune disease.

# APPENDIX B: TYROSINASE CROSS-PRESENTATION BY CD8α$^+$ OR CD103$^+$ DC IS REQUIRED FOR THE INDUCTION OF ADULT BUT NOT JUVENILE VITILIGO

## Introduction

Vitiligo affects 0.5 – 2% of the world's population independently of gender[219,220], and is characterized by progressive depigmentation resulting from a loss of melanocytes in the cutaneous epidermis and hair follicles[221]. Vitiligo is often associated with other autoimmune diseases[220], and an autoimmune etiology has been proposed based on perilesional infiltration of activated CD4 and CD8 T cells[222–225], and the ability of immunosuppressive treatments to modulate the disease[226–229].

Tyrosinase has been identified as an autoantigen in human vitiligo, and both tyrosinase-specific antibodies and CD8 T cells have been identified from vitiligo patients[230–234]. Tyrosinase does not efficiently bind to murine MHC I molecules. Therefore, we created a murine model using a chimeric MHC I molecule, termed AAD, which contains the peptide-binding domain of human HLA*0201 and the CD8 binding domain of murine H-2D$^d$. All mice used in this study express AAD. These mice efficiently load and present the murine tyrosinase peptide $Tyr_{369\text{-}377}$, which differs from the human epitope by a single amino acid substitution which does not affect its binding to HLA*0201. $C^{38R145L}$ albino mice have a complete deletion in the tyrosinase gene, and therefore are not tolerant to tyrosinase. We used these albino mice to created a transgenic $Tyr_{369}$-specific CD8 T cell receptor, termed FH, which recognizes $Tyr_{369}$ in the context of AAD. FH cells do not undergo central tolerance. Additionally, experiments using bone marrow chimeras and Langerhans-DTR mice demonstrated that under steady-state conditions, tyrosinase is not cross-presented by hematopoietically-derived

cells or by radioresistant Langerhans cells. Instead, in adult animals radioresistant lymphatic endothelial cells (LEC) in both skin-draining and mesenteric LN constitutively transcribe and present tyrosinase[20,21,80]. LEC do not constitutively express CD80, CD86, CD70, 4-1BBL or OX40L, and presentation of tyrosinase by LEC in the absence of a costimulatory signal leads to the rapid, high-level upregulation of PD-1 on the FH cells[21]. PD-1 binds to PD-L1 on a radioresistant cell, preventing upregulation of the high affinity IL-2R and leading to T cell death. However, when FH mice are bred to tyrosinase$^{+}$ mice, the resulting Tyrosinase$^{+}$FH (referred to here as TFH) mice spontaneously develop vitiligo beginning during the juvenile period and continuing into adulthood[97]. Juvenile vitiligo begins by postnatal day 3 with bilateral depigmentation of the ear epidermis, and continues with rings of depigmentation around each eye and the muzzle between days 5 – 21. Juvenile vitiligo is fully developed by 6 weeks of age. Adult vitiligo is characterized by depigmentation of hair follicles, which begins before 7 weeks and continues though 21 weeks of age. Adult and juvenile vitiligo are both dependent on CD8 T cells recognizing $Tyr_{369}$ presented by AAD, and are inhibited by the presence of regulatory T cells ($T_{reg}$)[20,97]. This correlates with human studies that have shown increased CD8 T cell and decreased $T_{reg}$ infiltration into human vitiligo lesions[235]. CCR4 is important for the trafficking of CD8 T cells to the skin[236], and CCR5 and CXCR3 are important for trafficking to sites of inflammation[237–240]. Juvenile and adult vitiligo were both substantially reduced in CXCR3$^{-/-}$ mice, and CCR5$^{-/-}$ mice had diminished juvenile vitiligo but normal development of adult vitiligo[97]. CCR4$^{-/-}$ mice developed both juvenile and adult vitiligo normally. This suggests that the inflammatory environment is important for trafficking of TFH T cells to the skin, and that different constellations of receptors are required for juvenile and adult vitiligo, perhaps dependent on the location of vitiligo.

Additionally, IFNγ but not perforin is required for vitiligo induction[97]. IFNγ could be involved either in directly killing melanocytes, or in upregulating adhesion molecules and chemokine expression to increase infiltration of FH T cells into the sites of vitiligo[241,242]. This previous work demonstrates that autoimmune vitiligo is a complex process with spatially and temporally distinct mechanisms during the juvenile and adult phases.

The development of vitiligo in TFH mice indicates that LEC-induced tolerance is either incomplete or abrogated in these animals, leading to the immunogenic activation of FH cells. LEC in the LN begin developing by postnatal day 3, but do not begin presenting tyrosinase until after postnatal day 7[120]. Juvenile vitiligo is apparent at postnatal day 3, prior to the induction of LEC-induced tolerance, suggesting cross-presenting dendritic cells (DC) or other antigen presenting cells (APC) may be responsible for the initial activation of FH cells. Under steady-state conditions in adult mice, tyrosinase is not cross-presented at detectable levels by hematopoietically-derived cells[20]. However, presentation of peripheral tissue antigens like tyrosinase by LEC is not known to induce immunogenic activation of T cells[243,244]. Therefore, we asked whether cross-presentation may occur in neonatal animals, inducing destruction of melanocytes may could enable continued cross-presentation of tyrosinase. To test whether cross-presentation of tyrosinase is responsible for vitiligo induction in FH mice, we crossed Batf3$^{-/-}$ mice[10] with TFH mice (Batf3$^{-/-}$ x TFH). Batf3$^{-/-}$ mice lack CD8α$^{+}$ and CD103$^{+}$ DC, which are the most efficient cross-presenting subsets of DC[9–13]. In this chapter, we investigated whether cross-presentation by CD8α$^{+}$ or CD103$^{+}$ DC is responsible for immunogenic FH T cell activation leading to vitiligo in neonates and adults.

**Results**

*CD8α$^+$ and CD103$^+$ DC are not required for juvenile vitiligo induction.*

We evaluated the development of vitiligo in neonatal TFH and Batf3$^{-/-}$ x TFH mice to determine whether cross-presentation by CD8α$^+$ and CD103$^+$ DC is required for juvenile vitiligo. Juvenile vitiligo is characterized by epidermal depigmentation of the ears, followed by depigmentation around the eyes, muzzle and tail. Using a semi-quantitative scoring system[97] (Table 2), we measured the development of juvenile vitiligo twice a week for 4 weeks. The initial depigmentation of the ears, eyes, and muzzle occurred 3.5 days earlier in TFH mice compared to Batf3$^{-/-}$ x TFH mice, and depigmentation of the tail was slightly more severe in TFH mice (Figure 35A-D). However, after the first week, the overall development and severity of juvenile vitiligo was not significantly different between Batf3$^{-/-}$ x TFH and TFH mice (Figure 35E). This suggests that the professional CD8α$^+$ or CD103$^+$ DC are not required for juvenile vitiligo induction. We have previously shown that CD45$^{neg}$ LEC do not present tyrosinase until after day 7 of life[120], suggesting that antigen presentation by LEC is also not responsible for juvenile vitiligo induction. In vitro co-cultures using APC isolated from day 7 neonates demonstrated that tyrosinase is primarily being presented by CD45$^+$ cells, not CD45$^{neg}$ cells, in both Batf3$^{-/-}$ x TFH and TFH mice (Figure 36). These results suggests that another subset of CD45$^+$ cells is capable of cross-presenting tyrosinase, and that CD8α$^+$ or CD103$^+$ DC are not necessary for the induction of juvenile vitiligo.

Since vitiligo induction was similar in TFH and Batf3$^{-/-}$ x TFH mice, we hypothesized that T cell activation and skin infiltration should be similar in these groups of mice. Since juvenile vitiligo is primarily localized to the head, we digested the skin

from the heads of day 7 postnatal TFH and Batf3$^{-/-}$ x TFH pups and found equal CD8 infiltration in both cases (Figure 37A). We also digested back skin from these pups, and found that there was a trend towards more CD8 T cells infiltrating the areas of eventual adult vitiligo in TFH compared to Batf3$^{-/-}$ x TFH mice (Figure 37B). This suggests a model where CD8 T cells activated via juvenile vitiligo can infiltrate the back and destroy additional melanocytes. Although CD8α$^{+}$ or CD103$^{+}$ DC are not required for juvenile vitiligo, enhanced cross-presentation by these subsets in inguinal, axillary or brachial LN could be responsible for the trend towards increased CD8 T cell infiltration into the back skin.

*Cross-presentation by CD8α or CD103+ DC is responsible for severe adult vitiligo*

Although CD8α$^{+}$ or CD103$^{+}$ DC are not required for juvenile vitiligo, we tested whether these DC subsets are required for adult vitiligo, which is characterized by destruction of melanocytes in the hair follicles. By 8 weeks of age, all TFH mice have substantial patches of “salt & pepper” depigmentation, and many have extensive grey or white areas (Figure 38A). In contrast, the Batf3$^{-/-}$ x TFH mice remain black with a few very small areas of depigmentation on their backs. These areas do not increase in size or number by 10 weeks (Figure 38B). Depigmentation of the ears, eyes, muzzle and tail, which occurred equivalently in both mouse strains during the juvenile phase of the disease, is apparent in both Batf3$^{-/-}$ x TFH and TFH mice (Figure 38A-B). Without FH cells, tyrosinase$^{+}$ mice do not develop vitiligo (Figure 38). Using a semi-quantitative scoring system (Table 3), we show that TFH mice develop severe vitiligo, while in Batf3$^{-/-}$ x TFH mice vitiligo is substantially reduced and is not significantly different from tyrosinase$^{+}$FH$^{neg}$ mice (Figure 38C). This demonstrates that cross-presentation of

tyrosinase by CD8α$^{+}$ or CD103$^{+}$ DC is responsible for severe adult vitiligo. To confirm that myeloid cells are acquiring and cross-presenting tyrosinase in TFH mice, we used an in vitro co-culture system. B cells and myeloid cells were flow sorted from TFH, Batf3$^{-/-}$ x TFH, and tyrosinase$^{+}$FH$^{neg}$ mice and co-cultured with naïve CFSE-labeled FH cells isolated from an albino mouse. Myeloid cells from TFH and Batf3$^{-/-}$ x TFH mice both induced proliferation of naïve FH cells (Figure 39). However, the myeloid cells from the tyrosinase$^{+}$ FH$^{neg}$ mouse did not, confirming that cross-presentation requires prior melanocyte destruction and does not occur at substantial levels in the steady-state. Proliferation of the FH cells co-cultured with myeloid cells from the Batf3$^{-/-}$ x TFH mice indicates that other DC or macrophage subsets can acquire tyrosinase to present in the absence of CD8α or CD103$^{+}$ DCs. However, the substantial reduction of vitiligo induction in vivo suggests that cross-presentation by other subsets of DC or macrophages in vivo does not induce robust immunogenic activation of FH cells. B cells did not induce proliferation, regardless of the mouse of origin.

The lack of severe vitiligo in Batf3$^{-/-}$ x TFH mice despite in vitro antigen presentation by myeloid cells suggests that FH cells are not being immunogenically activated to the same extent as in TFH mice. We examined the activation markers CD69 and CD25 on CD8 T cells in the LN. Batf3$^{-/-}$ x TFH CD8 T cells had a trend towards increased levels of CD69 relative to tyrosinase$^{+}$FH$^{neg}$ mice (Figure 40A), indicating that tyrosinase is presented to some cells in these LN. CD69 was significantly upregulated in TFH mice, indicating that CD8α$^{+}$ or CD103$^{+}$ DC are more efficient at activating FH cells. Interestingly, CD25 was upregulated on CD8 T cells from TFH but not Batf3$^{-/-}$ x TFH mice (Figure 40B). We have previously shown that FH cells undergoing tolerogenic activation do not upregulate CD25, leading to their death [21]. This

**Table 2: Evaluation of juvenile vitiligo.**

Scoring system from reference [97]

| Location | Score |
|---|---|
| Ears | 0 = normal (equivalent to C57Bl/6) |
| | 0.5 = remnant spots with pigmentation |
| | 1 = complete depigmentation |
| Eye rings | 0 = normal (equivalent to C57Bl/6) |
| | 0.5 = partial depigmentation of epidermis |
| | 1 = complete depigmentation |
| Muzzle | 0 = normal (equivalent to C57Bl/6) |
| | 0.5 = depigmented whiskers only |
| | 1 = depigmented whiskers and adjacent hair |
| Tail | 0 = normal (equivalent to C57Bl/6) |
| | 0.5 = incomplete depigmentation; spotted |
| | 1 = complete depigmentation |

**Figure 35: TFH and Batf3$^{-/-}$ x TFH mice both develop equivalently severe juvenile vitiligo.**

Vitiligo development in Batf3$^{-/-}$ x TFH (n=12), TFH (n=9), and tyrosinase$^{+}$FH$^{neg}$ (n=5) was assessed twice a week by two independent observers, according to the criteria in Table 2, and statistical differences between TFH and Batf3$^{-/-}$ x TFH mice were calculated using a two-way ANOVA with Bonferroni post-test. Data collected by Eric Tewalt and Holly Davis.

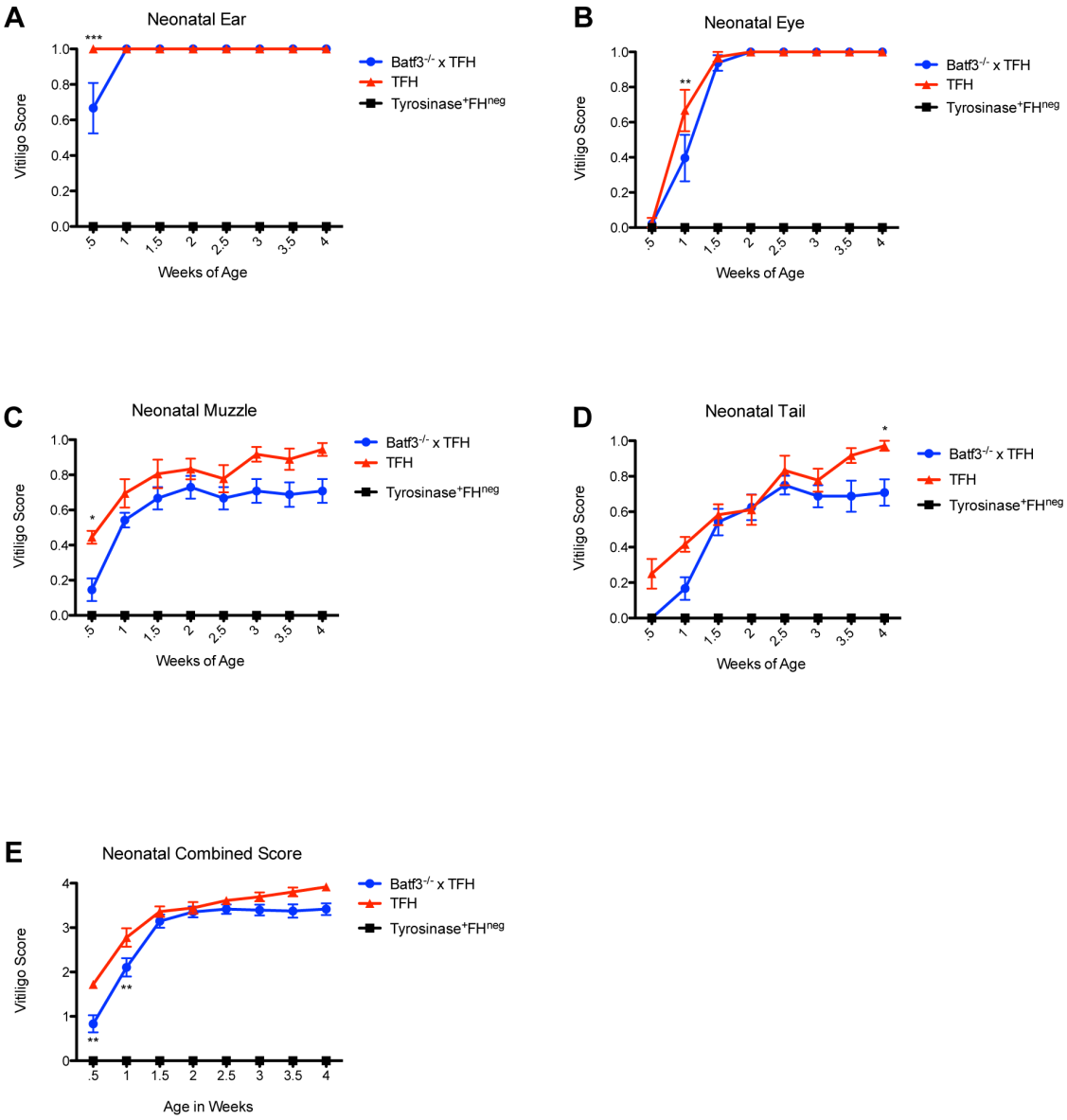

A
Neonatal Ear
Vitiligo Score
Weeks of Age
Batf3-/- x TFH
TFH
Tyrosinase+FHneg
B
Neonatal Eye
C
Neonatal Muzzle
D
Neonatal Tail
E
Neonatal Combined Score
Age in Weeks

**Figure 36: CD45$^{+}$ cells from neonatal Batf3$^{-/-}$ x TFH and TFH both cross-present tyrosinase in vitro**.

CD45$^{+}$ and CD45$^{neg}$ cells were flow sorted from postnatal day 7 Batf3$^{-/-}$ x TFH and TFH mice and co-cultured with naïve CFSE-labeled FH cells from an albino mouse. Proliferation of the FH cells was measured 4 days later. Representative (A) and summary (B) data is shown. Statistical differences between CD45$^{neg}$ and CD45$^{+}$ cells from TFH or Batf3$^{-/-}$ x TFH mice were calculated using a two-way ANOVA with Bonferroni post-test.

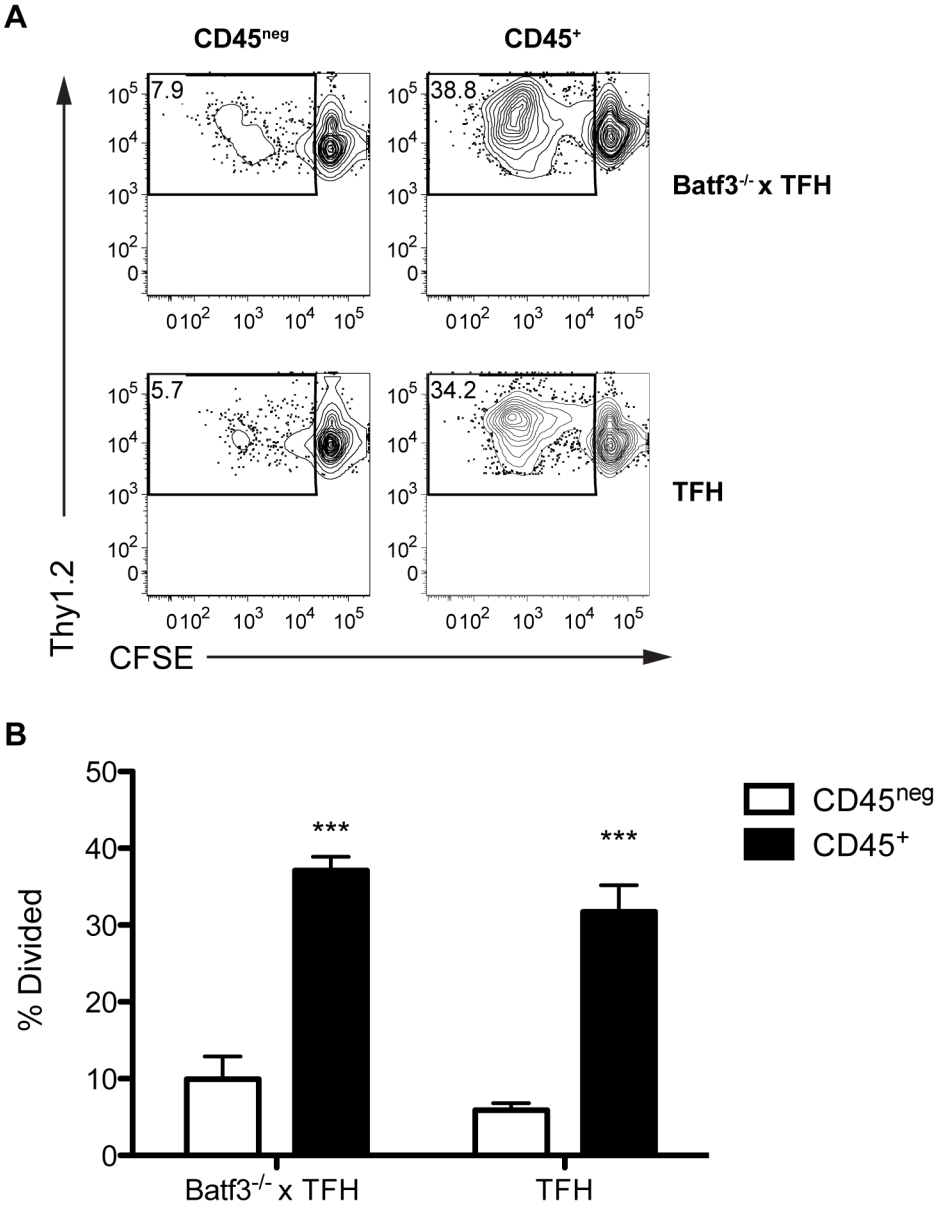
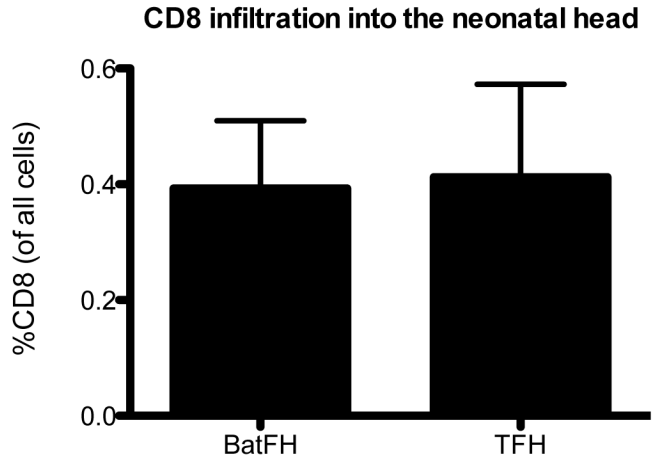

A
CD45neg
CD45+
7.9
38.8
Batf3-/- x TFH
5.7
34.2
TFH
Thy1.2
CFSE
B
% Divided
50
40
30
20
10
0
***
***
CD45neg
CD45+
Batf3-/- x TFH
TFH

**Figure 37: CD8 T cell infiltration in the skin in the head and backs of neonates.**

Skin from the head (A) and backs (B) of postnatal day 7 neonates was enzymatically digested, and the percentage of $CD3^{+}CD8^{+}Thy1.2^{+}$ cells out of total cells was determined by flow cytometry. Differences were not significant using an unpaired T test.

A


CD8 infiltration into the neonatal head
%CD8 (of all cells)
0.6
0.4
0.2
0.0
BatFH
TFH

B

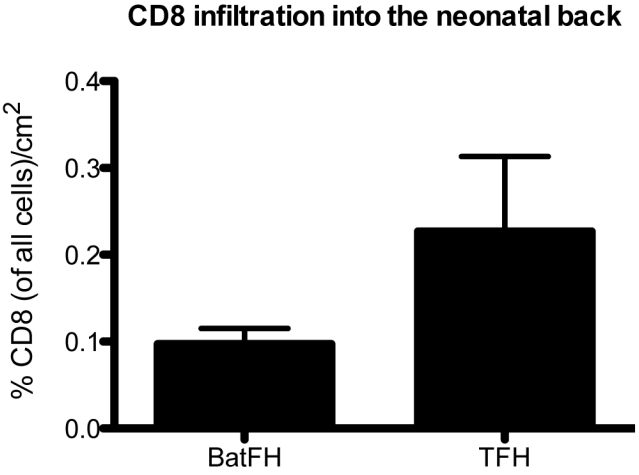
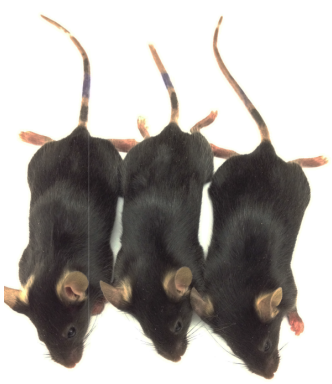

CD8 infiltration into the neonatal back
% CD8 (of all cells)/cm2
0.4
0.3
0.2
0.1
0.0
BatFH
TFH

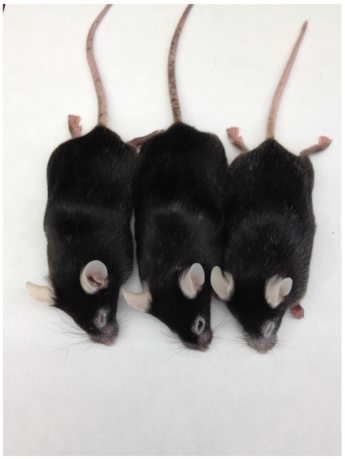
**Figure 38: TFH but not Batf3$^{-/-}$ x TFH mice develop severe adult vitiligo.**

Pictures of representative tyrosinase$^{+}$FH$^{neg}$ , Batf3$^{-/-}$ x TFH, and TFH mice were taken at 8 (A) and 10 weeks (B) of age. The tails of the mice in the left panel of A had been marked for identification purposes. Vitiligo development in Batf3$^{-/-}$ x TFH (n=12), TFH (n=9), and tyrosinase$^{+}$FH$^{neg}$ (n=5) was assessed twice a week by two independent observers, according to the criteria in Table 3, and statistical differences between TFH and Batf3$^{-/-}$ x TFH mice were calculated using a two-way ANOVA with Bonferroni post-test. Vitiligo scoring performed by Eric Tewalt and Holly Davis.

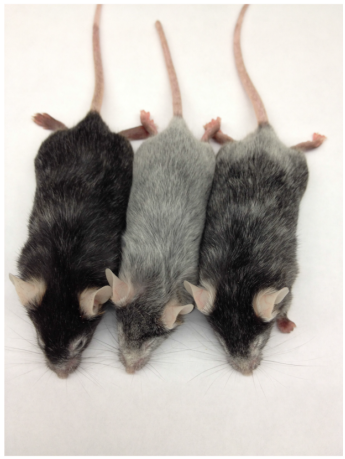
A 8 weeks:



Tyrosinase$^{+}$FH$^{neg}$

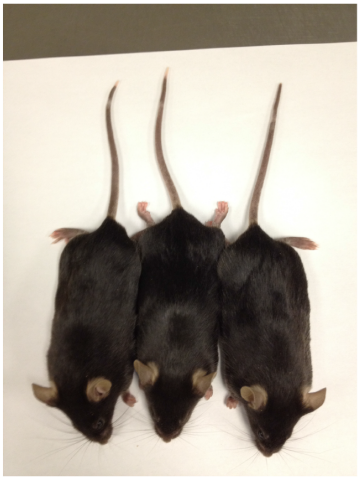


Batf3$^{-/-}$ x TFH

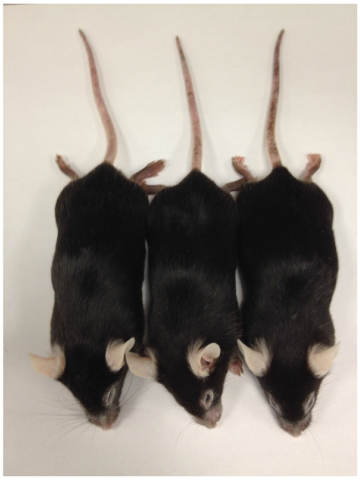


TFH

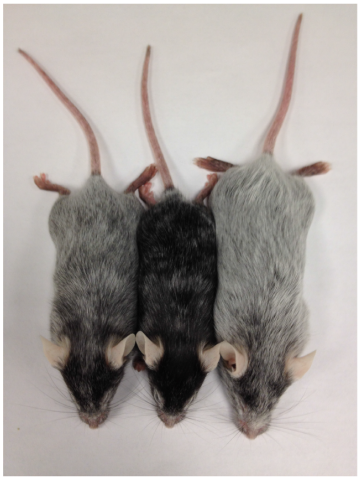
B 10 weeks:



Tyrosinase$^{+}$FH$^{neg}$



Batf3$^{-/-}$ x TFH



TFH

C

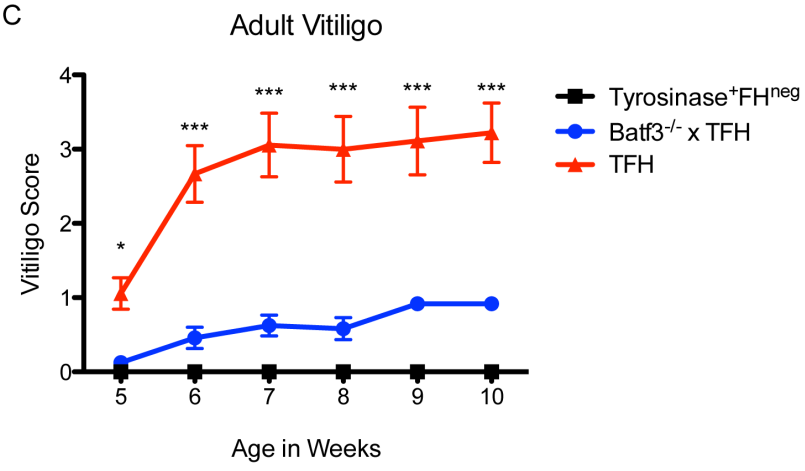


**Table 3: Evaluation of adult vitiligo. Scoring system modified from reference [97]**

**Adult Vitiligo Scoring**

0 = Wild Type (C57Bl/6)

1 = <25% salt/pepper

2 = 25-50% salt/pepper or <25% gray

3 = >50% salt/pepper or 25-50% gray

4 = >50% gray or <50% complete depigmentation

5 = >50% complete depigmentation

suggests that the T cells being activated in Batf3$^{-/-}$ x TFH mice might be activated in a tolerogenic manner and could be more likely die without upregulation of the high affinity IL-2R. Similar results were also seen in the spleen, where cells from Batf3$^{-/-}$ x TFH mice upregulated CD69 (Figure 40C) but did not upregulate CD25 (Figure 40D). These results suggest that in the absence of the professional cross-presenting CD8α$^{+}$ and CD103$^{+}$ DC, tyrosinase presentation in Batf3$^{-/-}$ x TFH mice leads to more tolerogenic activation of the FH T cells.

We hypothesized that decreased immunogenic activation in the Batf3$^{-/-}$ x TFH mice might lead to decreased infiltration of FH T cells into the skin. We digested a 1 cm$^2$ perilesional patch of back skin from TFH, Batf3$^{-/-}$ x TFH, and tyrosinase$^{+}$FH$^{neg}$ mice, and counted the number of CD8$^{+}$ cell by flow cytometry. There was increased infiltration into the back skin of TFH mice (Figure 41), consistent with the increased activation seen in the LN. This suggests that decreased infiltration may be one factor leading to decreased induction of vitiligo in Batf3$^{-/-}$ x TFH compared to TFH mice. Combined, these results demonstrate that cross-presentation by CD8α$^{+}$ or CD103$^{+}$ DCs is required for strong immunogenic activation of FH T cells and severe adult vitiligo.

**Figure 39: CD45$^{+}$ cells from adult Batf3$^{-/-}$ x TFH and TFH both cross-present tyrosinase in vitro**.

CD19$^{+}$ B cells and CD45$^{+}$CD19$^{neg}$CD3$^{neg}$ myeloid cells were flow sorted from adult Batf3$^{-/-}$ x TFH and TFH mice and co-cultured with naïve CFSE-labeled FH cells from an albino mouse. Proliferation of the FH cells was measured 4 days later. Statistical differences between B cells and myeloid cells from TFH or Batf3$^{-/-}$ x TFH mice were calculated using a two-way ANOVA with Bonferroni post-test.

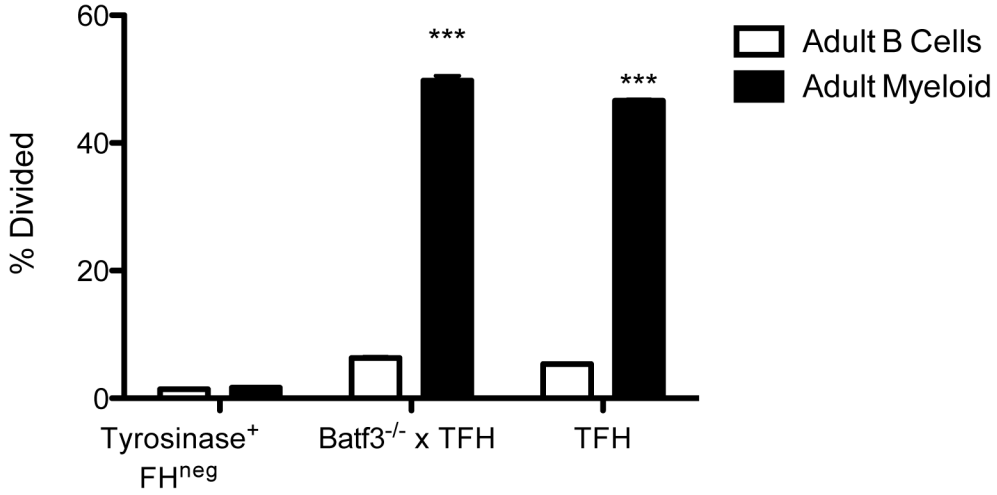

60
40
20
0
% Divided
***
***
Adult B Cells
Adult Myeloid
Tyrosinase+ FHneg
Batf3-/- x TFH
TFH

**Figure 40: CD8 T cells in the LN and spleen of TFH mice are immunogenically activated.**

Percentage of CD8 T cells expressing CD69 (A, C) and CD25 (B, D) in the LN (A,B) and spleen (C,D) of adult tyrosinase$^{+}$FH$^{neg}$ , Batf3$^{-/-}$ x TFH, and TFH mice were calculated by flow cytometry. Means were compared using a one-way ANOVA with Tukey post-test.

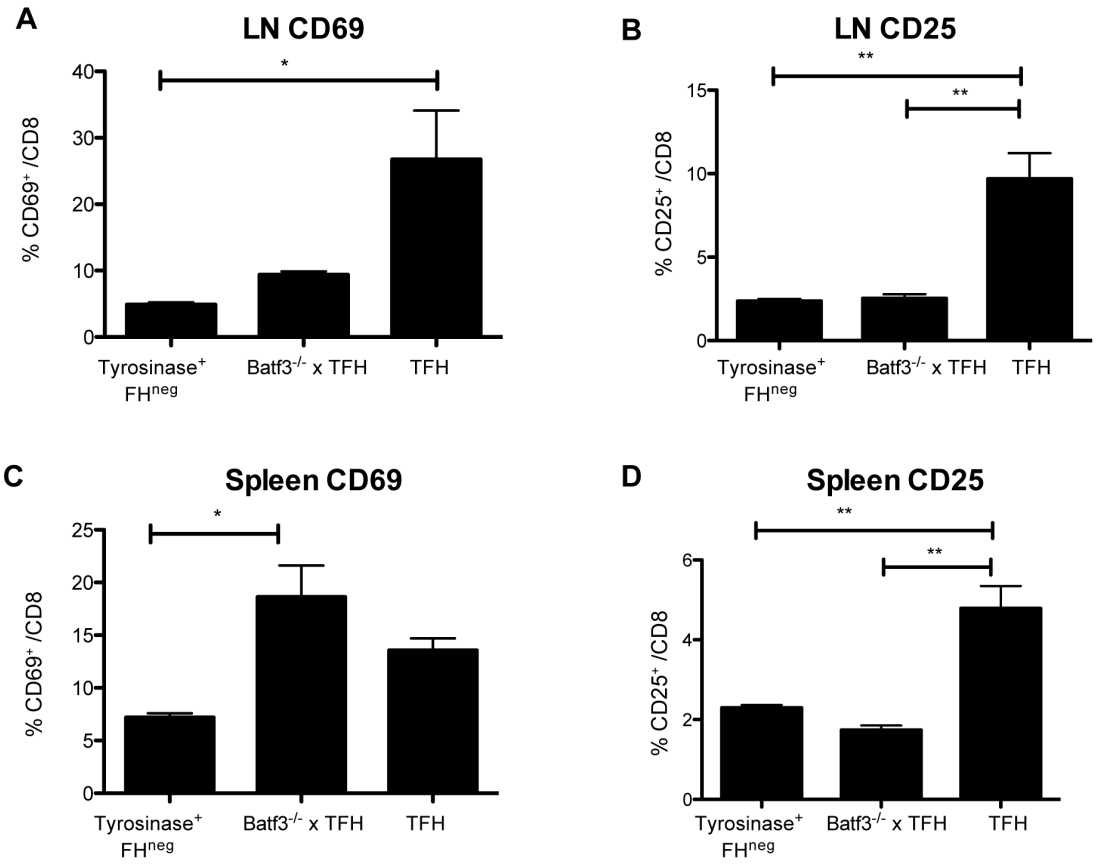
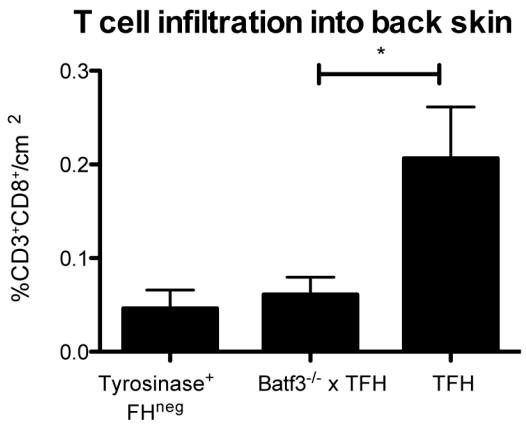

A
LN CD69
% CD69+ /CD8
40
30
20
10
0
*
Tyrosinase+ FHneg
Batf3-/- x TFH
TFH
B
LN CD25
% CD25+ /CD8
15
10
5
0
**
**
Tyrosinase+ FHneg
Batf3-/- x TFH
TFH
C
Spleen CD69
% CD69+ /CD8
25
20
15
10
5
0
*
Tyrosinase+ FHneg
Batf3-/- x TFH
TFH
D
Spleen CD25
% CD25+ /CD8
6
4
2
0
**
**
Tyrosinase+ FHneg
Batf3-/- x TFH
TFH

**Figure 41: Adult TFH mice have increased CD8 T cell infiltration into the back skin.**

Skin from the backs of adult mice was enzymatically digested, and the percentage of $CD3^{+}CD8^{+}Thy1.2^{+}$ cells out of total cells was determined by flow cytometry. Means were compared using a one-way ANOVA with Tukey post-test.


T cell infiltration into back skin
*
0.3
0.2
0.1
0.0
%CD3+CD8+/cm 2
Tyrosinase+ FHneg
Batf3-/- x TFH
TFH

**Discussion:**

In this chapter we demonstrate that severe adult vitiligo is dependent on cross-presentation of tyrosinase by $CD8\alpha^+$ or $CD103^+$ DCs, while juvenile vitiligo does not require cross-presentation by these DC subsets. The development of juvenile but not adult vitiligo in $Batf3^{-/-}$ x TFH mice suggests that T cells are activated by a different APC or in a different location during the neonatal phase of the disease. In vitro co-cultures demonstrated that $CD45^+$ cells can cross-present tyrosinase in both cases. Our in vitro co-culture system measures antigen presentation, but cannot distinguish between immunogenic and tolerogenic T cell activation. Therefore, cross-presentation by non-$CD8\alpha^+$ or $CD103^+$ subsets of DC or macrophages in adult $Batf3^{-/-}$ x TFH mice may partially induce tolerogenic instead of immunogenic activation of the FH T cells. This is supported by the very low level of vitiligo induced and by the lack of upregulation of CD25 in these mice. The co-culture system also normalizes the numbers of DC from both types of mice. If the cross-presenting APC from $Batf3^{-/-}$ x TFH mice is present in low numbers in vivo or is localized outside the T cell zone of the LN, antigen presentation might be seen in vitro but may not be relevant in vivo. While $CD8\alpha^+$ and $CD103^+$ DC are considered to be the most efficient DC subsets for antigen cross-presentation, other reports have demonstrated that plasmacytoid DC[245,246] or $CD11b^+$ DC can also cross-present antigens to CD8 T cells[247]. Further studies will elucidate exactly which subsets of myeloid cells are cross-presenting tyrosinase to induce vitiligo in the absence of $CD8\alpha^+$ or $CD103^+$ DCs.

Cross-presentation by $CD8\alpha^+$ or $CD103^+$ DC is not required during the neonatal period. This could be explained by increased representation or access of the other cross-presenting $CD45^+$ cells to naïve CD8 T cells in the LN, or could be explained by

direct activation of naïve CD8 T cells in the skin. Neonatal LN are disorganized, as there are not distinct T and B cell zones until after postnatal day 4[120,248]. It is possible that the non-CD8α$^{+}$ or CD103$^{+}$ cross-presenting DC have better access to the naïve T cells during this early time period, enabling sufficient FH activation to induce neonatal vitiligo. Alternatively, naïve T cells can infiltrate the skin and be directly tolerized in this peripheral site during the neonatal period[129]. Naïve T cells from adult animals have a much lower ability to infiltrate the skin and are not tolerized in the skin. It is possible that naïve neonatal FH T cells from TFH and Batf3$^{-/-}$ x TFH mice are infiltrating the skin and becoming directly activated by tyrosinase presented in the skin. This mechanism would not require tyrosinase cross-presentation, and T cells from both TFH and Batf3$^{-/-}$ x TFH mice should be equivalently activated by direct antigen presentation in the skin.

Previous work from our lab has shown that activation of CD8 T cells during the neonatal period is required for the induction of adult vitiligo[249]. Juvenile CD8 T cells were ablated by injecting αCD8 antibody from postnatal day 1 to postnatal day 15, and then allowed to reconstitute so adult mice had normal CD8 T cell compartments. These mice did not develop juvenile vitiligo, and only 1/5 mice went on to develop adult vitiligo. Interestingly, a different litter that was not treated with αCD8 antibody until postnatal day 2 developed ear depigmentation, suggesting that auto-reactive T cells emerge and damage epidermal melanocytes within the first 2 days of life. However, mice that were maintained on αCD8 treatment from day 2 – 15 had substantially reduced depigmentation of the back skin through 110 days of age, indicating that temporary elimination of CD8 T cells during the juvenile period partially abrogates adult vitiligo. This indicates that destruction of tyrosinase$^{+}$ cells during the juvenile period is not sufficient for the induction of adult vitiligo without the continued presence of CD8 T cells.

Instead, one possibility is that activated FH cells must seed the areas of eventual adult vitiligo during the juvenile period. In the present study, we have found a trend towards increased CD8 T cell infiltration into the back of day 7 TFH neonates, despite the lack of apparent depigmentation of hair follicles at this time point. The presence of activated effector FH cells could induce inflammation, which could upregulate adhesion molecules and allow effectors activated at a later time point to infiltrate the back and induce severe adult vitiligo. These results suggest that juvenile vitiligo likely initiates adult vitiligo through two mechanisms: destruction of melanocytes leading to tyrosinase cross-presentation, and by seeding sites of adult vitiligo with activated FH T cells during the neonatal period. This study points to distinct mechanisms of antigen presentation and vitiligo induction during the juvenile and adult phases of the disease, which differ in their requirements for cross-presentation by the professional $CD8\alpha^+$ or $CD103^+$ DC.

# APPENDIX C: LEC-INDUCED TOLERANCE INHIBITS MELANOMA IMMUNOTHERAPY

## Introduction

Tyrosinase is a melanocyte differentiation protein that is expressed in melanomas in addition to normal melanocytes[250]. Tyrosinase-specific T cells have been identified in the peripheral blood of melanoma patients, suggesting that it is a suitable target for melanoma immunotherapy. Indeed, it is being used as a target for active vaccination of melanoma patients[102]. However, since tyrosinase is also expressed in normal melanocytes, tolerance mechanisms may hinder the success of these approaches[156–158]. We have shown that LEC induce deletional tolerance of tyrosinase-specific CD8 T cells through the PD-1/PD-L1 pathway[20,21,80]. PD-1 blockades are currently being used to revitalize exhausted T cells with great success in anti-melanoma clinical trials[119]. Therefore, we hypothesized that blocking LEC-induced tolerance by blocking the PD-1 pathway would enhance melanoma adoptive T cell therapy.

## Results and Discussion

To test whether blocking LEC-induced tolerance enhances melanoma immunotherapy, we implanted 1 x $10^5$ B16 melanoma cells expressing AAD (B16-AAD) subcutaneously in either tyrosinase$^+$, tyrosinase$^{neg}$, or tyrosinase$^+$PD-L1$^{-/-}$ mice. LEC-induced tolerance will proceed normally in tyrosinase$^+$ mice[20]. In contrast, tyrosinase$^{neg}$ mice have a complete deletion in the tyrosinase-coding gene, and therefore tyrosinase-specific T cells are not tolerized in these mice. Tyrosinase$^+$PD-L1$^{-/-}$ mice express tyrosinase as a PTA in LEC, but without PD-L1 LEC cannot induce tolerance of FH cells. In mice that did not receive an adoptive transfer, tumors became palpable on average between days 11 – 14 post-implementation, and there were no significant differences

between groups (Figure 42A). Tumors were allowed to grow until they ulcerated or reached a maximal size of 16 mm in any dimension. Tyrosinase$^{+}$ mice that did not receive an adoptive transfer were the first to reach these endpoints with a median survival of 18 days, followed by tyrosinase$^{+}$PD-L1$^{-/-}$ mice with a median survival of 23 days and finally tyrosinase$^{neg}$ mice with a median survival of 28.5 days (Figure 42B). This significant increase in survival time in tyrosinase$^{+}$PD-L1$^{-/-}$ and tyrosinase$^{neg}$ mice suggests that endogenous tyrosinase-specific cells which are spared from tolerance have some anti-tumor efficacy, even though they are present at low endogenous levels. Additionally, there was no elongation of survival time in tyrosinase$^{+}$PD-L1$^{-/-}$ compared to tyrosinase$^{neg}$ mice, suggesting that the lack of PD-L1 in these mice does not affect tumor growth kinetics independently of its effects on tolerance in this model.

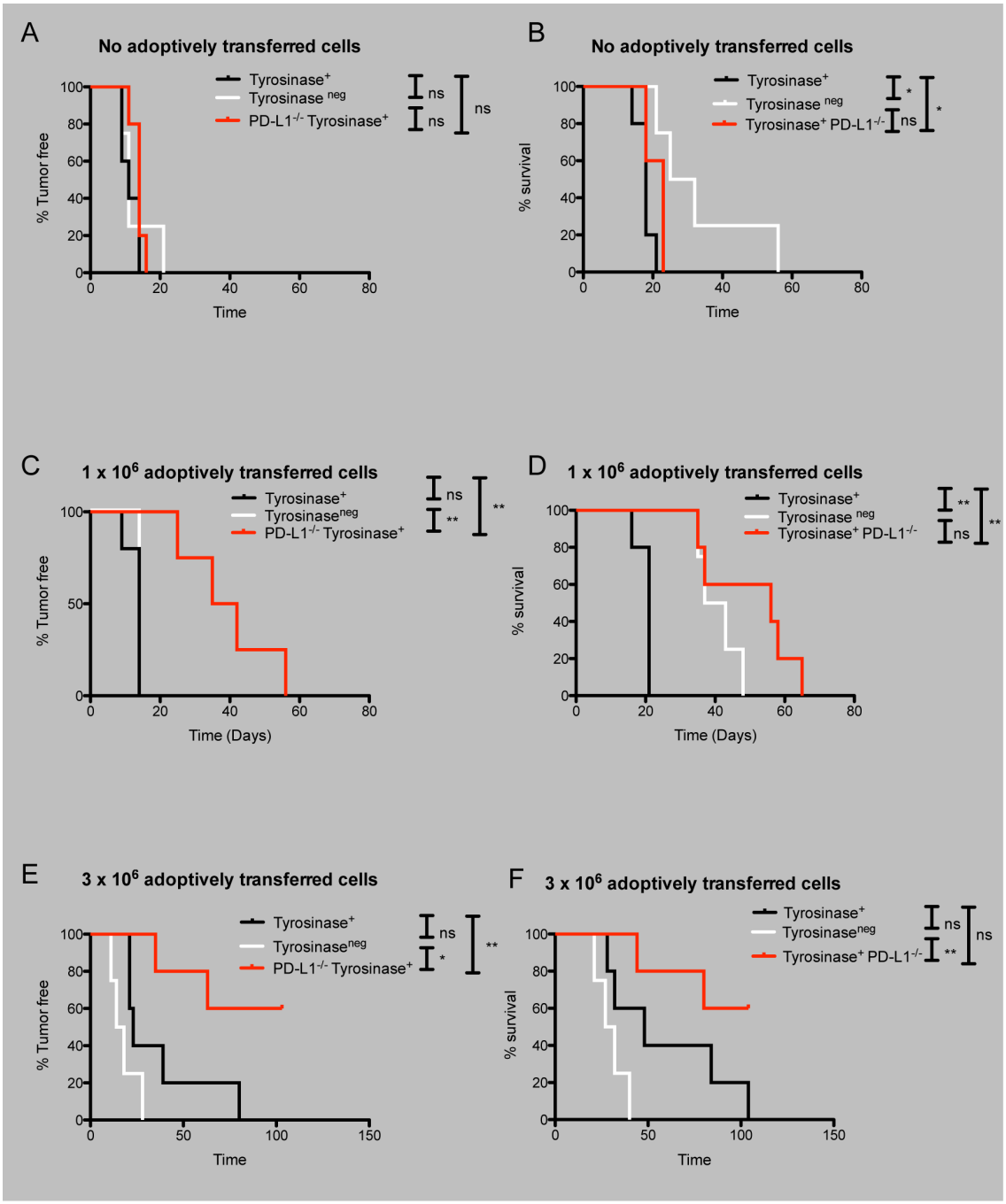
Next, we tested whether adoptively transferring tyrosinase-specific FH cells 3 days prior to tumor implantation would inhibit melanoma outgrowth. Outgrowth of tumors in tyrosinase$^{+}$ mice that received 1 x $10^{6}$ FH cells was similar to mice that did not receive an adoptive transfer, indicating that induction of tolerance by LEC prevented these cells from mounting an effective anti-tumor response (Figure 42A-D). In contrast, tyrosinase$^{neg}$ and tyrosinase$^{+}$PD-L1$^{-/-}$ mice that received 1 x $10^{6}$ FH cells had significantly enhanced median survival times of 40 and 56 days, respectively (Figure 42D). Additionally, 2/5 tumors that grew out in the tyrosinase$^{+}$PD-L1$^{-/-}$ recipients were depigmented, indicating that the tumor downregulated or mutated tyrosinase as a mechanism of immune escape. This demonstrates that inhibiting LEC-induced tolerance can substantially enhance the efficacy of adoptive T cell therapy and enhance the anti-melanoma immune response.

Finally, we adoptively transferred 3 x $10^6$ FH cells to test whether larger numbers of FH T cells could overwhelm the ability of LEC to induce tolerance. In these mice, tyrosinase$^+$PD-L1$^{-/-}$ mice remained tumor-free for longer than tyrosinase$^+$ and tyrosinase$^{neg}$ mice, and 3/5 tyrosinase$^+$PD-L1$^{-/-}$ mice remained tumor free for the duration of the experiment (Figure 42E). Median survival of the tyrosinase$^+$ mice increased to 48 days (Figure 42F), suggesting adoptively transferring large numbers of FH cells can delay the completion of tolerance and allow some of the FH cells to be immunogenically activated instead. Further studies could help elucidate the mechanism by which these cells are immunogenically activated. Given the limited number of LEC in the LN, it is likely that 3 x $10^6$ cells cannot all be activated by LEC prior to tumor implantation. Instead, some of these cells are likely activated by professional APC carrying antigen from the tumor. It is possible that immunogenic activation of FH cells in the LN could even lead to destruction of tyrosinase$^+$ LEC, further dampening LEC-induced tolerance. This enables effector FH cells to develop and inhibit tumor outgrowth, leading to increased survival times in the tyrosinase$^+$ mice.

These results demonstrate that inhibiting LEC-induced tolerance can enhance the anti-melanoma immune response. Approaches using vaccination or adoptive T cell therapies designed to enhance an anti-tyrosinase immune response in particular may synergistically benefit from being combined with an anti-PD-1 blockade. Alternatively, maximizing the number of adoptively transferred T cells could provide an additional approach to increase the number of cells that are immunogenically instead of tolerogenically activated. Although further work is needed to elucidate the mechanism of tumor control, these preliminary results suggest that inhibiting LEC-induced tolerance is an exciting new approach to enhancing anti-melanoma immunotherapy.

**Figure 42: LEC-induced tolerance inhibits melanoma immunotherapy.**

Mice received either 0 (A,B), 1 x $10^6$ (C,D), or 3 x $10^6$ (E,F) FH T cells 3 days prior to subcutaneous implantation of 1 x $10^5$ B16-AAD melanoma cells. Time until a palpable tumor was detected (A, C, E) or until the tumor exceeded 16 mm in any dimension or ulcerated (B, D, F) was measured. Survival curves were compared using the Log-rank (Mantel-Cox) test.


A
No adoptively transferred cells
Tyrosinase+
Tyrosinase neg
PD-L1-/- Tyrosinase+
% Tumor free
Time
ns
ns
ns
B
No adoptively transferred cells
Tyrosinase+
Tyrosinase neg
Tyrosinase+ PD-L1-/-
% survival
Time
*
ns
*
C
1 x 10^6 adoptively transferred cells
Tyrosinase+
Tyrosinaseneg
PD-L1-/- Tyrosinase+
% Tumor free
Time (Days)
ns
**
**
D
1 x 10^6 adoptively transferred cells
Tyrosinase+
Tyrosinase neg
Tyrosinase+ PD-L1-/-
% survival
Time (Days)
**
ns
**
E
3 x 10^6 adoptively transferred cells
Tyrosinase+
Tyrosinaseneg
PD-L1-/- Tyrosinase+
% Tumor free
Time
ns
*
**
F
3 x 10^6 adoptively transferred cells
Tyrosinase+
Tyrosinaseneg
Tyrosinase+ PD-L1-/-
% survival
Time
ns
**
ns

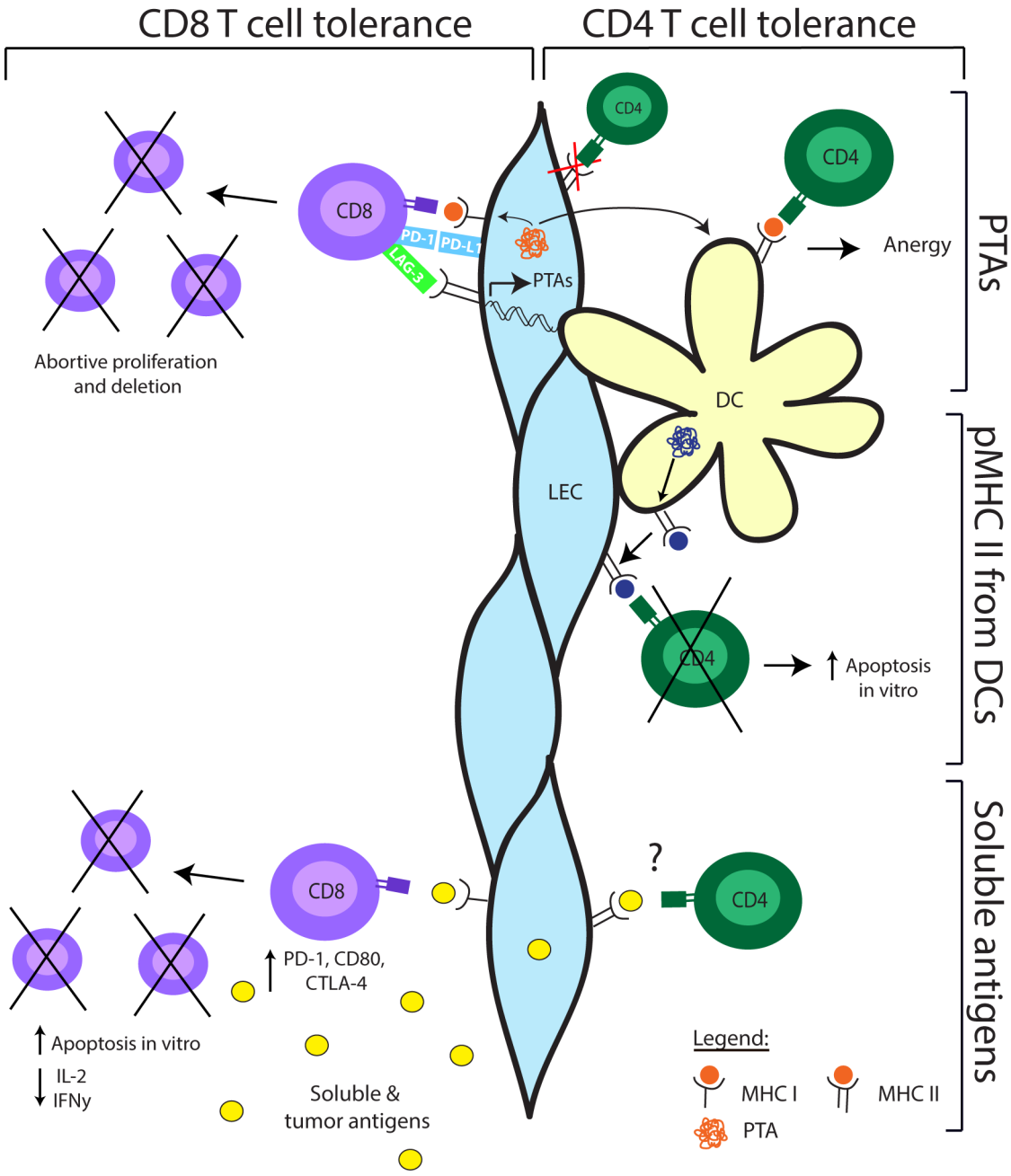
# CONCLUSIONS AND FUTURE DIRECTIONS

LEC have been shown to induce CD8 T cell tolerance, but their role in CD4 T cell tolerance has not been previously examined. To determine whether LEC present antigens on MHC II or are involved in CD4 T cell tolerance, we created model systems where β-gal or HA are expressed as PTA in LEC. We found that LEC present epitopes from both proteins on MHC I molecules but not on MHC II molecules. Instead, LEC provide these PTA to DC, which then induce CD4 T cell anergy (Figure 43).

LEC did not present β-gal or HA on MHC II molecules to CD4 T cells. Additionally, LEC did not stain with the Y-Ae antibody, which detects the I-E$\alpha_{52\text{-}68}$ peptide bound to I-$A^b$ MHC II molecules. The use of the Y-Ae antibody allowed us to directly test the ability of LEC to load the I-E$\alpha_{52\text{-}68}$ peptide onto I-$A^b$ independently of T cell activation. Since LEC do not load I-E$\alpha_{52\text{-}68}$ or present β-gal or HA, it suggests LEC have a global deficit in the MHC II processing and presentation pathway. Therefore, we used flow cytometry and qPCR to measure expression of Ii, Cathepsin L, H2-M and H2-O. We found that LEC express Ii and cathepsin L, but not H2-M or H2-O. The absence of H2-M suggests that LEC are unable to exchange CLIP for antigenic peptides on their MHC II molecules. We have attempted to test whether MHC II molecules on LEC contain an elevated level of the CLIP peptide using the 15G4 antibody, but the high level of background staining seen on LEC hindered our ability to detect a positive signal. Another possible approach is to stain LEC with the BP107 antibody[251] (ATCC), which recognizes I-$A^b$ or I-$A^d$ molecules containing non-CLIP peptides[252–254]. The specificity of BP107 was demonstrated through experiments showing that in H2-$M^{-/-}$ mice where MHC II molecules are occupied with CLIP, BP107 staining dropped to background levels

**Figure 43: Roles of LEC in T cell tolerance.**

LEC directly present PTA to CD8 T cells, and induce deletional tolerance through PD-1/PD-L1 and LAG-3/MHC II. LEC do not load PTA onto MHC II, but instead provide these antigens to DC, which induce CD4 T cell anergy. Other groups have also demonstrated that LEC can acquire peptide:MHC II complexes from DC, presentation of which induces CD4 T cell anergy in vitro. LEC can endocytosis and weakly cross-present soluble and tumor-derived antigens to CD8 T cells, leading to decreased effector function and increased apoptosis in vitro. The ability of LEC to endocytose and present soluble antigens to CD4 T cells has not been evaluated.


CD8 T cell tolerance
CD4 T cell tolerance
CD4
CD4
CD8
PD-1
PD-L1
LAG-3
PTAs
Anergy
Abortive proliferation
and deletion
DC
LEC
CD4
Apoptosis
in vitro
PTAs
pMHC II from DCs
Soluble antigens
CD8
?
CD4
PD-1, CD80,
CTLA-4
Apoptosis in vitro
IL-2
IFNy
Soluble &
tumor antigens
Legend:
MHC I
MHC II
PTA

despite normal or increased levels of MHC II molecules[252]. Additionally, H2-M$^{-/-}$Ii$^{-/-}$ double knockout mice that do not express CLIP regain staining with BP107[254]. This has lead to the suggestion that CLIP binding induces a subtle conformational change in MHC II molecules that prevents recognition by the BP107 antibody[253]. To test whether LEC express abnormally high levels of I-A$^{b}$:CLIP complexes, the ratio of BP107 staining seen on unpulsed LEC versus peptide pulsed LEC could be compared to the ratio seen with unpulsed and peptide pulsed macrophages, B cells and DC. Peptide pulsed APC will define the maximal signal detectable by BP107 based on the level of MHC II molecules on the cell surface. If an equivalent fraction of the MHC II complexes on LEC contain peptides compared to those on macrophages, B cells, or DC, then the ratio of BP107 staining in unpulsed versus peptide pulsed cells should be comparable. On the other hand, if the majority of MHC II complexes on the surface of LEC are occupied with CLIP, unpulsed LEC should have diminished BP107 staining, leading to a lower ratio. This would suggest that LEC are less efficient at loading antigenic peptides onto MHC II compared to professional APC.

If LEC do not stain with BP107, one alternative possibility is the MHC II molecules on the cell surface are empty, and do not contain CLIP or antigenic peptides. Empty MHC II complexes have been detected on the surface of immature DC[255] using the KL-304 antibody[256] that recognizes the α-helix on the border of the peptide binding site, and thus detects empty I-A$^{S}$ complexes[255,256]. LEC from I-A$^{S+}$ SJL mice could be stained with the KL-304 antibody (ATCC) to determine whether some of the extracellular MHC II molecules found on LEC are empty, using peptide-pulsed LEC as a negative control. The ratio of KL-304 staining to total MHC II staining on LEC versus macrophages, B cells, or DC could be used to determine whether LEC have an

abnormally high percentage of empty MHC II molecules. Davoust and Banchereau[257] postulated that empty MHC II molecules on the surface of DC may enhance presentation of extracellular peptides or proteins which could bind to the empty MHC II molecules on the cell surface or in recycling endosomes. If empty MHC II molecules are found on LEC, this would be a particularly interesting hypothesis since LEC are advantageously positioned in the lymphatic sinuses to sample peptides or proteins entering the LN through the lymph. Recent proteomic studies have shown that the lymph is a rich source of self-peptides, which can have a half life in biological fluids of over 24 hours[258–260]. Loading peptides onto empty MHC II molecules could be a mechanism to increase the diversity of the peptide repertoire presented, as these peptides may be low-affinity and therefore not presented in the presence of H2-M[46,261–263].

If empty MHC II complexes are found in LEC, one interesting question is how they might form. The mechanism of empty complex formation in DC has not been established. It is possible that these either represent complexes that had initially bound low-affinity peptides that subsequently disassociated, or Ii-independent export of empty MHC II molecules to the cell surface. If empty MHC II complexes are seen on LEC, disassociation of low-affinity peptide:MHC II complexes is an attractive explanation since LEC do not express H2-M, which normally selects for high-affinity peptides[262,263]. However, the low numbers of LEC isolated per mouse makes it difficult to elute peptides from MHC II and directly test their affinity for MHC II. Recent advances have made it possible to elute and analyze peptides from as few as 30 x $10^6$ thymic DC[264], suggesting continued advances may soon make it possible to analyze the endogenous MHC II peptide reservoir from primary LEC. An alternative possibility is that MHC II molecules can be exported from the ER without Ii bound. The ability of MHC II complexes to be

exported from the ER independently of Ii varies depending on the haplotype and cell type studied. Ii is not required for the export of H-$2^K$ MHC II molecules in DC, but is required for export of I-$A^b$ in DC and for both H-$2^K$ and I-$A^b$ in B cells[265,266]. It is not entirely clear why DC do not require Ii to export H-$2^K$ but B cells do, although based on the broad range of molecular weights of H-$2^K$ complexes seen in $Ii^{-/-}$ DC it has been suggested that alternative polypeptides can bind in a pre-Golgi compartment to H-$2^K$ and stabilize it in DC but not B cells[265]. To test whether MHC II expression in LEC is Ii-dependent, the level of MHC II on LEC can be compared in H-$2^{K+}$ B10.BR $Ii^{-/-267}$, I-$A^{b+}$ B6 $Ii^{-/-268}$, and B10.BR or B6 wild-type mice. If Ii-independent expression of MHC II is seen, we can use immunofluorescence microscopy to test whether Ii is correctly localized and therefore able to bind MHC II in wild-type LEC. If Ii and MHC II do not colocalize in wild-type cells, it would support a model where Ii-independent export could lead to empty MHC II molecules on the surface of LEC. Combined, these experiments help determine whether the MHC II molecules on LEC are largely empty, contain CLIP, or contain antigenic peptides presumably loaded in a non-H2-M dependent manner.

While H2-M assists in loading high affinity peptides, some peptides can be loaded onto MHC II molecules in the absence of H2-M[41,42,44–46]. The Eisenlohr lab has developed T cell hybridomas specific for two different epitopes from HA, which differ in their dependence on H2-M for presentation. The S1 hybridoma recognizes $HA_{107-119}$, similar to the Clone 6.5 transgenic mouse. The S3 hybridoma recognizes $HA_{302-313}$. The S1 epitope is loaded onto MHC II in the MIIC compartment in a H2-M dependent manner[42]. In contrast, the S3 epitope is loaded onto MHC II in recycling endosomes, and does not require H2-M for loading. Since the S3 epitope is loaded in recycling endosomes, empty or low-affinity peptide:MHC II complexes on the cell surface should

be loaded with the S3 epitope when the MHC II complexes are internalized. We can use these hybridomas to test whether LEC from Prox1 x HA mice or LEC infected with influenza can load and present the S3 epitope. If LEC can present the S3 epitope but not the S1 epitope, it suggests that the lack of H2-M is the major deficiency preventing PTA presentation. If LEC do not present either epitope, there may be an additional deficiency in the MHC II presentation pathway. These studies will test whether the H2-M deficiency is the only factor preventing LEC from presenting antigens on MHC II molecules.

While LEC do not present PTA on MHC II molecules, MHC II appears to be involved in PTA tolerance as a ligand for the LAG-3 pathway. Deletion of Bg1 CD8 T cells involves both the LAG-3/MHC II and PD-1/PD-L1 pathways, while deletion of tyrosinase-specific FH T cells appears to be entirely dependent on the PD-1/PD-L1 pathway[21]. To confirm that the ligand for LAG-3 is MHC II in our model, antibody blockades could be repeated using PD-L1 blocking antibodies in conjunction with MHC II blocking antibodies, instead of the anti-LAG-3 antibody. It is unknown what determines which pathway(s) are involved in deletion. Proliferating FH and Bg1 cells both upregulate PD-1 and LAG-3, but it is possible that the level of upregulation varies between the T cells, perhaps depending on TCR affinity or the density of antigen displayed by the LEC. To test whether PD-1 and LAG-3 are equivalent expressed on activated FH and Bg1 cells, the T cells could be adoptively transferred into tyrosinase$^{+}$ or Prox1 x β-gal$^{+}$ recipients, and the gMFI of PD-1 and LAG-3 upregulation could be directly compared as well as the percentage of cells co-expressing PD-1 and LAG-3. If differences are seen, we could test whether this correlates with differences in TCR affinity, PTA expression level, or both. TCR affinity can be determined by measuring the

T cell response to different concentrations of peptide in vitro, and qPCR can be used compare the relative levels of expression of tyrosinase versus β-gal in LEC. Alternatively, if LAG-3 and PD-1 are upregulated to a similar extent on FH and Bg1 cells, the different pathways used for CD8 T cell deletion could reflect differences in the level of the ligands expressed on the $PTA^+$ LEC. We have demonstrated that $tyrosinase^+$ LEC are concentrated in the LN medulla[120], while Prox1-$creER^{T2}$ and Lyve-1-cre will induce β-gal expression in cortical and subcapsular sinus LEC in addition to medullary LEC. LEC express varying levels of MHC II, ranging from negligible to intermediate levels. It is possible that the medullary LEC lack MHC II, and therefore do not use the LAG-3/MHC II pathway to induce FH T cell tolerance. Immunofluorescence microscopy could be used to test whether medullary LEC lack or express a lower level of MHC II. The MFI of MHC II staining on medullary, subcapsular and cortical LEC could be quantitated using ImageJ software (NIH) and compared. If medullary LEC express low or negligible levels of MHC II, it suggests that although FH cells upregulate LAG-3, the ligand is not present on cells expressing tyrosinase and therefore this pathway is not relevant for tolerance to tyrosinase. In contrast, since β-gal is expressed in all LEC, Bg1 cells that have upregulated LAG-3 can delete after encountering β-gal-expressing MHC $II^+$ cells. These studies will help determine whether the TCR affinity, level of antigen expression, or co-expression pattern of PTA and inhibitory ligands influence which pathways are used for CD8 T cell deletion.

Although LEC do not directly present MHC II antigens, they transfer PTA to DC and the DC present the antigens and induce anergy. Potential mechanisms of antigen transfer include engulfment of apoptotic LEC, exosomes, or gap junctions. Although transfer of preformed peptide:MHC II complexes has been demonstrated from DC to

LEC[179], this does not account for antigen transfer from LEC to DC, since LEC do not load PTA peptides onto MHC II. Additionally, if peptide:MHC II complexes were being transferred from LEC to DC, antigen presentation should occur in MHC $II^{-/-}$ → Prox1 x β-gal or MHC $II^{-/-}$ → Prox1 x HA bone marrow chimeras. However, adoptively transferred β-gal specific or HA-specific CD4 T cells are not activated in these chimeras, demonstrating that transfer of preformed peptide:MHC II complexes is not a relevant mechanism in this system. Our preliminary results have also suggested that the mechanism of antigen transfer is not solely dependent on phosphatidylserine recognition or DC phagocytosis. A recent paper demonstrated that peptides can be transferred from macrophages to DC through gap junctions; specifically through junctions formed from connexin 43 subunits[198]. Since DC and LEC are in close contact in the LN, it is feasible that gap junctions could form and lead to antigen transfer. According to microarray data from the Immunological Genome Consortium Project[199], connexin 43 is the mostly highly expressed gap junction protein in LEC. As DC have been demonstrated to use connexin 43 to acquire antigens from macrophages[198], this is a likely candidate for antigen transfer from LEC. To test whether connexin 43 is required for antigen transfer, Connexin $43^{flox/flox}$ mice (Jax) could be crossed with Prox1-$creER^{T2}$ x β-$gal^{stop\text{-}flox}$ mice, to generate Prox1-$creER^{T2}$ x β-$gal^{stop\text{-}flox}$ x Connexin $43^{flox/flox}$ mice where β-gal is induced and Connexin 43 is knocked out upon administration of tamoxifen. Bg2 T cells could be adoptively transferred into these mice, and Bg2 proliferation would be used to determine if antigen is transferred to hematopoietically derived cells. It is also possible that LEC use multiple overlapping mechanisms of antigen transfer. In this case, it may be necessary to block multiple pathways in order to see an inhibition of antigen transfer.

Prox1-creER$^{T2}$ x β-gal$^{stop\text{-}flox}$ x Connexin 43$^{flox/flox}$ mice could be treated with Annexin V to block both phosphatidylserine recognition and gap junctions.

The ability to test whether β-gal or HA is transferred through exosomes in vivo is currently limited by a lack of tools to completely prevent exosome release or capture. Therefore, in vitro culture systems could initially be employed to test whether LEC secrete exosomes containing β-gal or HA. To test whether antigen transfer can occur through soluble mediators such as exosomes or apoptotic cell blebs, LEC and DC can be separated with a transwell, and the ability of DC to acquire antigen and present it to Bg2 T cells can be tested. If DC can acquire antigen through the transwell, we can expand LEC from Prox1 x β-gal mice ex vivo using recently published protocols[269] and purify exosomes from the culture. If adding the purified exosomes to DC:Bg2 co-cultures induces Bg2 proliferation, it would indicate that LEC can secrete exosomes containing PTA. Additionally, we could add inhibitors of exosome secretion such as GW4869, spiroepoxide, or manumycin A[270] to LEC:DC co-culture to test whether antigen transfer is reduced without exosome release. To demonstrate the physiological relevance of this mechanism of antigen transfer in vivo, several strategies could be employed. Neutral sphingomyelinase-2 is important for exosomes to bud off of a cell. Manumycin A is a neutral sphingomyelinase-2 inhibitor that has been used in vitro to inhibit exosome release[271], and it has been used in vivo for cancer treatment with low toxicity[272]. Prox1 x β-gal mice could be treated with manumycin A to inhibit exosome release, and the proliferation of adoptively transferred Bg2 T cells could be measured as a proxy of antigen transfer. Alternatively, we could knockdown neutral sphingomyelinase-2 in Prox1 x β-gal mice. Hydrodynamic injection of an expression vector encoding mouse neutral sphingomyelinase-2 shRNA into the tail vein has been

shown to inhibit exosome secretion in the liver[270]. We could test the ability of hydrodynamic injection of neutral sphingomyelinase-2 shRNA to knockdown neutral sphingomyelinase-2 in LEC. Alternatively, in collaboration with Kim Kelly's lab, we could design an adenovirus targeted to LEC to more specifically deliver the shRNA to LEC and knockdown exosome secretion. This would allow us to test whether antigen transfer requires exosome production by LEC. Alternatively, kits to isolate exosomes from plasma and other tissues are available (Life Technologies). LN from MHC $II^{-/-}$ → Prox1 x β-gal mice could be homogenized and exosomes purified from the homogenate. The exosomes could be added to B6 DCs co-cultured with Bg2 cells, and if the Bg2 cells proliferated it would indicate that the exosomes contained β-gal. However, the quantity of exosomes obtained from a LN homogenate may limit the utility of this approach. Regardless, these experiments will further our understanding of whether exosomes play a role in the transfer of antigen from LEC to DC.

LEC have also been shown to cooperate with DC to present archived viral antigens. Recent work from the Kedl laboratory has demonstrated that LEC store viral antigens encountered during a primary immune response[273]. LEC do not directly present these archived antigens on MHC I. Instead, the antigens archived in LEC are transferred to and presented by DC and other hematopoietically derived cells for several weeks after clearance of the viral infection, as measured by proliferation of newly transferred naïve antigen-specific CD8 T cells. Continued antigen presentation enhances the maintenance and/or subsequent re-expansion of antigen-specific memory CD8 T cells. The mechanism by which LEC archive antigen is not entirely clear, but it is interesting to consider how LEC can acquire viral antigens to be archived and keep them separate from PTA, which will be used to induce tolerance. The Kedl laboratory

demonstrated that antigen is not captured through antibody/antigen complexes, as archiving occurs in μMT$^{-/-}$ mice that lack B cells and in complement R2$^{-/-}$ mice[273]. Antigen archiving requires a concurrent T cell response, although the T cell antigen specificity is irrelevant, and correlates with LEC proliferation, as measured by the number of LEC isolated from the LN. This supports a hypothesis in which cytokines produced by activated T cells induce LEC proliferation and antigen archiving, and perhaps newly divided LEC are specialized for antigen archiving while more mature LEC express PTA for tolerance induction. LEC proliferation is predominantly controlled by VEGF-C binding to VEGFR-3[274]. To test whether LEC proliferation (measured by LEC number, BrdU incorporation or Ki67 staining) is required for antigen archiving, VEGF-C and VEGF-D signaling could be blocked prior to viral infection using a soluble VEGFR-3-Ig fusion protein[275] or adenovirus encoding soluble VEGFR-3[143] to act as a decoy receptor. Alternatively, to test whether LEC proliferation is sufficient for antigen archiving, RAG$^{-/-}$ mice (Jax) could be infected with adenovirus expressing VEGF-C[276] to induce LEC proliferation in the absence of a T cell response. If LEC proliferation is required and sufficient for antigen archiving, experiments to determine how activated T cells are inducing LEC proliferation could be performed. TNFα secretion is a likely candidate, as TNFα has been shown to induce VEGF-C secretion by HUVEC cells[277]. To test if TNFα is sufficient, RAG$^{-/-}$ mice can be treated with TNFα during viral infection, and LEC proliferation and antigen archiving could be measured. Alternatively, to determine if TNFα is required for antigen archiving, LEC proliferation and antigen archiving could be tested in TNFα$^{-/-}$ mice (Taconic). If LEC proliferation is found to correlate with but not cause antigen archiving, it suggests that cytokines produced by activated T cells are acting on LEC independently of their effects on proliferation. LEC

express receptors for TGF-β, TNFα, IFNα, or IFNγ[199], suggesting any of these cytokines can act directly on LEC. This could be tested using antibody blockades or knockout mice. These studies could be used to determine what factors are needed to induce antigen archiving.

It is also unclear how or where archived antigens are stored within LEC, and how they are separated from tolerogenic PTA. DQ-OVA is a self-quenched fluorescently tagged version of OVA that fluoresces as it is proteolytically degraded. DQ-OVA does not fluoresce in LEC when administered as an antigen to be archived[273], suggesting that archived antigens are not endocytosed and subsequently degraded in LEC. This suggests that archived antigens may remain closely attached to the surface of LEC, or may cycle through non-proteolytic recycling endosomes, as in follicular DC[278]. To test where archived antigen is localized, LEC with fluorescently labeled archived proteins could be co-stained with EEA1, RAB7, and RAB11 as markers for early, late, and recycling endosomes, respectively, and examined with a high power confocal microscope.

Presentation of both archived antigens and PTA acquired from LEC leads to different T cell fates, suggesting that these types of antigens must be handled separately within LEC. One possibility is that these antigens are found in different cells, either based on the maturation state or location of the LEC. If proliferation is required for antigen archiving, it might suggest that recently divided cells are specialized for antigen archiving, while PTA expression occurs in more mature, quiescent LEC. To test this, LEC with or without archived fluorescent antigen could be flow sorted, and qPCR for tyrosinase or other PTA could be performed. If tyrosinase mRNA is detected in the same

cells with archived antigen, it would suggest that LEC are able to maintain the antigens in separate subcellular compartments or otherwise distinguish between these types of antigens. If tyrosinase mRNA is not found in the same cells as the archived antigens, immunofluorescence microscopy could be used to determine if medullary LEC archive fluorescent antigens. If medullary LEC do not archive antigens, it suggests a functional specialization of the different LEC subpopulations based on their location in the LN.

In this work, we have also shown that if LEC-induced tolerance to tyrosinase is abrogated or overwhelmed, immunogenic activation of FH T cells can occur, causing autoimmunity or an anti-melanoma immune response. We have used TFH mice as a model for autoimmune vitiligo, and have shown that cross-presentation by $CD8\alpha^+$ or $CD103^+$ DC is required for the induction of adult but not juvenile vitiligo. It is unclear what APC are activating neonatal FH cells since $CD8\alpha^+$ or $CD103^+$ DCs are not required. We have shown that other $CD45^+$ APC can activate naïve FH cells in the $Batf3^{-/-}$ x TFH mice. Additionally, others have demonstrated that naïve neonatal T cells can be directly tolerized in the skin[129]. Since activation by melanocytes in the skin would not require $CD8\alpha^+$ or $CD103^+$ DC, this is an attractive explanation as to why TFH and $Batf3^{-/-}$ x TFH mice develop juvenile vitiligo with similar kinetics and severity. To test whether neonatal T cells are being immunogenically activated in the skin, we can treat pregnant mice with LTβR and TNF receptor fusion proteins, which prevents LN development in the pups. If treated litters continue to develop vitiligo with unchanged kinetics and severity from untreated TFH litters, this indicates that vitiligo is induced after direct activation by APC in the skin. Additionally, further work using flow sorting to subset different types of DC and macrophages could elucidate which subsets are able to

present tyrosinase in Batf3$^{-/-}$ x TFH mice. Representation and localization of the responsible subset in the LN of neonatal and adult mice could be compared to determine if these APC have increased access to naïve T cells in neonatal mice. Additionally, the ability of the FH T cells from TFH and Batf3$^{-/-}$ x TFH mice to make effector cytokines such as IFNγ, TNFα, and granzyme B could be compared to determine if antigen presentation by non-CD8α$^{+}$ or CD103$^{+}$ DC leads to more tolerogenic activation.

The work in this thesis has demonstrated the dynamic nature of LEC in tolerance and immunity. The variety of PTA expressed in LEC can be directly presented on MHC I molecules, and can be transferred to DC for presentation on MHC II molecules. In this way, LEC are involved in the tolerance of both CD8 and CD4 T cells. Tolerance induction of CD8 T cells is remarkably robust, as it is not altered by danger signals provided through TLR3 or TLR4 ligation. Future work will continue to further our understanding of how LEC shape the immune repertoire and response.

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