Absence of MHC-II expression by lymph node stromal cells results in autoimmunity

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How lymph node stromal cells (LNSCs) shape peripheral T-cell responses remains unclear. We have previously demonstrated that murine LNSCs, lymphatic endothelial cells (LECs), blood endothelial cells (BECs), and fibroblastic reticular cells (FRCs) use the IFN-γ-inducible promoter IV (pIV) of the MHC class II (MHCII) transactivator CIITA to express MHCII. Here, we show that aging mice (>1 yr old) in which MHCII is abrogated in LNSCs by the selective deletion of pIV exhibit a significant T-cell dysregulation in LNs, including defective Treg and increased effector CD4+ and CD8+ T-cell frequencies, resulting in enhanced peripheral organ T-cell infiltration and autoantibody production. The proliferation of LN-Tregs interacting with LECs increases following MHCII up-regulation by LECs upon aging or after exposure to IFN-γ, this effect being abolished in mice in which LECs lack MHCII. Overall, our work underpins the importance of LNSCs, particularly LECs, in supporting Tregs and T-cell tolerance.

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Introduction

T-cell precursors undergo thymic negative selection, which ensures the elimination of developing T cells expressing TCR-recognizing self-Ags with excessive affinity. However, some autoreactive T cells escape this process of clonal deletion and exit the thymus to populate secondary lymphoid organs (SLOs). Therefore, additional mechanisms of T-cell tolerance are required in the periphery to avoid the development of autoimmunity. Among them, resting DCs, which constantly sample self-Ags in peripheral tissues and reach the draining LNs through the afferent lymph, present self-Ag-derived peptides to naïve T cells. In the absence of danger, this phenomenon leads to clonal deletion, or anergy of autoreactive T cells (Steinman et al, 2003; Mueller, 2010). Alternatively, Tregs, by exhibiting suppressive immunoregulatory functions, can inhibit autoreactive T cells. Different subsets of Tregs have been described so far. Natural Tregs bear an autoreactive TCR, are induced in the thymus, and express the transcription factor Foxp3. Peripheral-induced Tregs can express Foxp3 or not, and differentiate in SLOs (Chen et al, 2003; Swee et al, 2009; Wirnsberger et al, 2011). Preservation of Treg function and biology is crucial for peripheral tolerance.

Lymph node stromal cells (LNSCs) have recently been promoted to the rank of new modulators of T-cell responses. After being considered for years as simple scaffolding, forming routes, and proper environment for Ag-lymphocyte encountering, we recently learned that they also impact both DC and T-cell functions. Lymphatic endothelial cells (LECs) promote DC entry into and T-cell egress from LNs (Sixt et al, 2005; Pham et al, 2010; Braun et al, 2011), whereas CCL19/CCL21−producing fibroblastic reticular cells (FRCs) control immune cells entry and proper localization into LNs (Link et al, 2007; Tomei et al, 2009). Blood endothelial cells (BECs) control T-cell homing to LNs (Bajenoff et al, 2003). In addition, LECs and FRCs are the major source of IL-7 in LNs, ensuring T-cell homeostasis. In inflammatory situations, however, LECs and FRCs produce nitric oxide to constrict T-cell expansion (Khan et al, 2011; Lukacs-Kornek et al, 2011; Siegert et al, 2011), whereas LECs further impair DC maturation in a contact-dependent fashion (Podgrabinska et al, 2009). In the context of peripheral tolerance, LNSCs, and in particular LECs and FRCs, ectopically express a large range of peripheral tissue Ags (PTAs), and further present PTA-derived peptides through MHC class I (MHC) molecules to induce self-reactive CD8+ T-cell deletion (Cohen et al, 2010; Fletcher et al, 2010, 2011; Tewalt et al, 2012). We have previously demonstrated that, in addition to inducing CD8+ T-cell dysfunction by presenting peptide-MHC class II (MHCII) complexes acquired from DCs, LECs, BECs, and...
FRCs endogenously express MHCII molecules (Dubrot et al., 2014). Central tolerance of self-reactive CD4+ T cells is partially mediated by thymic epithelial cells (TECs), in which MHCII molecules are loaded with peptides derived either from phagocytosis and processing of extracellular Ags (Stern et al., 2006), or from autophagy and endocytosis of intracellular Ags (Adamopoulou et al., 2013; Aichinger et al., 2013). Whether these pathways can be involved in MHCII-restricted Ag presentation by LNSCs, and impact peripheral self-reactive T-cell responses, is currently unknown.

Here, we have used genetically modified mice in which MHCII expression by non-hematopoietic cells is abrogated. Upon aging, and compared with their control counterparts, these mice exhibit an enhancement of spontaneous autoimmune processes, with enhanced T-cell activation in SLOs and effector T-cell infiltration in peripheral tissues, as well as the production of autoantibodies. In contrast, the Treg compartment is significantly impaired in SLOs. Furthermore, Rag2−/− mice transferred with T cell isolated from LN of aging MHCII-deficient LENS mice displayed similar immunological and clinical perturbations compared with recipient injected with age-matched control T cells, suggesting a direct link between MHCII expressed by LNSCs and the appearance of T-cell-mediated signs of autoimmunity. Accordingly, upon aging or IFN-γ treatment, LECs up-regulate MHCII molecules, and interact with Treg to promote T-cell proliferation through MHCII-restricted Ag presentation.

Results

Expression of MHCII by LNSCs

Our previous work has demonstrated that LNSCs impact CD4+ T-cell biology by presenting peptide-MHCII complexes acquired from DCs (Dubrot et al., 2014). Although these findings revealed a novel function of the lymphoid stroma as a local APC, whether endogenous MHCII Ag presentation by LNSCs can also impact T-cell responses remains to be determined. To abrogate cell-intrinsic pIV-mediated MHCII expression by LNSCs, we have used mice deficient for the IFN-γ-inducible pIV of CIITA (pIVKO) (Waldburger et al., 2001). As expected, steady-state MHCII expression by LECs, BECs, and FRCs was only partially abrogated in pIVKO mice compared with control mice (Fig 1A). Indeed, we recently described that MHCII expression by LNSCs partially results from a combination of both DC-acquired and CIITA promoter-driven, endogenously expressed, MHCII molecules (Dubrot et al., 2014). DCs use the pi promoter of CIITA, and therefore, MHCII transfer to LNSCs is not abolished following pIV deletion (Dubrot et al., 2014). However, pIV deletion led to the abrogation of endogenous MHCII expression by LECs, BECs, and FRCs. We confirmed this by analysing a second CTIIA-regulated gene, H2-M, the oligomorph MHCII molecule involved in MHCII Ag loading. Ex vivo LECs, BECs, and FRCs express low H2-M and MHCII levels in the steady state, but substantially up-regulate H2-M and I-AK molecules at both mRNA and protein levels after IFN-γ treatment (s.c. injection), suggesting a potential role for MHCII-restricted Ag presentation by LNSCs during inflammation. Both steady state and IFN-γ-inducible endogenous MHCII expressions were abrogated in pIVKO mice (Fig 1B and C).

Abrogation of endogenous MHCII in LNSCs

MHCII expression in cortical TECs (cTECs) depends on pIV. Because of the absence of MHCII expression by cTECs, pIVKO mice consequently exhibit a defect in CD4+ T-cell thymic positive selection, and lack peripheral CD4+ T cells (Waldburger et al., 2003). Therefore, pIVKO mice were crossed with transgenic mice expressing CIITA under the keratin 14 (K14) promoter, which is active in cTECs (Lauber et al., 1996), but not in LNSCs (Fig 2A and B). MHCII expression by cTECs was efficiently restored in K14 CIITA tg x Ciita pIV−/− (referred as K14tgpiIVK0 mice) compared with pIVKO mice, and to a similar extent compared with cTECs isolated from WT or control (K14 Ciita tg x Ciita pIV−/−, referred to as K14tg mice (Fig 2C). As a consequence and as described before (Irla et al., 2008; Tholemann et al., 2014, 2016), K14tgpiIVKO mice exhibit normal CD4+ T-cell frequencies in SLOs (Fig 2D). We did not observe any significant expression of the K14 transgene in LNSCs purified from these mice (Fig 2B). However, to avoid any off-target effect of the K14 Ciita transgene in LNSCs or other cells, K14tgpiIVKO mice were always compared with K14tg controls in all experiments. Importantly, MHCII expression by mTECs is not altered in pIVKO and K14tgpiIVKO mice (Fig 2C), for the respective, following reasons: first, mTECs express the pII of CIITA in addition to pIV (Irla et al., 2008), and second, the K14 promoter is not active in terminally differentiated mTECs (Sukseere et al., 2012). Therefore, negative CD4+ T-cell selection is unaffected in K14tgpiIVKO mice. Accordingly, we have previously demonstrated that CD4+ thymocytes and peripheral CD4+ T cells that developed in K14tgpiIVKO mice (6–10 wk-old) were indistinguishable from WT with respect to cell numbers and TCR V-β repertoire (Irla et al., 2008).

IFN-γ–induced endogenous and pIV–dependent MHCII up-regulation, which was efficiently abolished in LECs, BECs, and FRCs from K14tgpiIVKO mice compared with control mice (Fig 2E). However, the expression of MHCII by other LN cells, i.e., different DC subtypes, B cells, or monocytes/macrophages, which rely on pI and/or pII of CIITA (Waldburger et al., 2001), was found unaffected in K14tgpiIVKO mice (Fig 2F). Altogether, our data demonstrate that endogenous MHCII expression is efficiently abrogated in LNSCs of K14tgpiIVKO mice.

Mice deficient for MHCII expression in LNSCs develop spontaneous signs of T cell-mediated autoimmunity

LNSCs have been so far described to function as tolerogenic Ag-presenting cells. Therefore, we tested whether the absence of MHCII expression by LNSCs would result in self-reactive T-cell tolerance breakdown and lead to immunopathology. Analysis of peripheral organs showed that the percentage of CD4+ and CD8+ T cells infiltrating the spinal cord (SC) and salivary glands (SGs) were markedly increased in K14tgpiIVKO compared with K14tg control
elderly (18 mo-old) mice (Fig 3A). No difference was observed in 4-mo-old mice (Fig 3A). In addition, intestines of 18-mo-old K14tgpIVKO exhibit increased frequencies of CD4+ T cells producing the proinflammatory cytokine IFN-γ compared with K14tg controls (Fig 3B). Other peripheral organs, such as the liver and the lungs, also exhibit a tendency of enhanced infiltrating CD4+ and CD8+ T cell numbers in 18-mo-old K14tgpIVKO mice, although not significant (not shown). Therefore, peripheral tissue T-cell infiltration was increased in a variety of non-lymphoid organs in elderly K14tgpIVKO mice. In agreement with a possible autoimmune syndrome, serum obtained from 18-mo-old K14tgpIVKO mice contained a broader spectrum of autoantibodies with enhanced reactivity to proteins from several tissues compared with K14tg control mice (Fig 3C), indicating an exacerbated development of systemic autoimmunity upon aging. Altogether, our data provide evidence that MHCII molecule expression by non-hematopoietic cells contribute to peripheral T-cell tolerance by providing a brake in the development of spontaneous signs of T cell-mediated autoimmune inflammation in peripheral tissues of elderly mice.

**MHCII abrogation in LNSCs enhances T-cell activation and impairs the Treg compartment in LNs**

We next sought to determine whether exacerbated signs of T cell-mediated autoimmune inflammation in peripheral tissues of elderly K14tgpIVKO mice result from enhanced T-cell activation in SLOs. Analysis of CD4+ and CD8+ T cells in LN did not show any differences between 4-mo-old K14tgpIVKO and K14tg mice (Fig 4A). However, in aging mice (18 mo), we noticed a significant enhanced T-cell activation, with decreased frequencies of naive (CD62L+CD44lo) and increased frequencies of activated (CD62L−CD44hi) CD4+ and CD8+ T cells in LNs of K14tgpIVKO compared with K14tg mice (Fig 4A).
addition, the frequency of effector CD4+ and CD8+ T cells expressing PD-1, and the frequency of CD4+ and CD8+ T cells producing IFN-γ, were enhanced in skin LNs from K14tgpIVKO compared with K14tg mice (Fig 4B). Because both CD4+ and CD8+ T-cell phenotypes and effector functions were affected, we postulated that an active mechanism of T-cell inhibition might have been lost in LNs of K14tgpIVKO mice upon aging. Accordingly, the frequency of Foxp3+ Tregs was significantly impaired (two-fold reduction) in LNs of aging K14tgpIVKO compared with K14tg control mice (Fig 4C). Foxp3+ staining on LN sections confirmed this phenotype (Fig 4D). In contrast, no difference in Treg frequencies was observed in 4-mo-old mice (Fig 4C). With Treg frequencies being reduced in aging K14tgpIVKO mice compared with K14tg controls, we next wondered whether this cellular population exhibited any phenotypic or functional alteration. We did not observe any alteration in the expression of several typical markers characterizing the Treg compartment, such as PD-1, CD44, CD25, or Foxp3 (not shown). In contrast, compared with control mice, LN Treg isolated from aging K14tgpIVKO mice exhibited an impaired ability to suppress the proliferation of naive CD4+ T cells in vitro at the Treg:T naive ratio of 1:5 (Fig 4E). The fact that no difference was observed when increased Treg numbers were used suggests that impaired Treg functions in knockout mice can be overcome with high Treg numbers.

So far, we have accumulated convincing data that upon aging, K14tgpIVKO exhibits signs of spontaneous autoimmune defects. Organ-infiltrating T cells, and also the production of autoantibodies are increased, suggesting that the lack of MHCII expression by LNSCs in LNs of knockout elderly is likely to be responsible for this phenotype. However, these observations might also result from the lack of pIV of CIITA in the target organs, as other non-hematopoietic cells from peripheral tissues may up-regulate MHCII and contribute to this phenotype. Indeed, peripheral tissue-resident cells can up-regulate MHCII molecules in an IFN-γ–inducible pIV-dependent pathway and, by re-stimulating infiltrating T cells, possibly influence the outcome of T-cell responses (Duraes et al, 2013). Therefore, to determine whether enhanced signs of spontaneous autoimmunity in aging K14tgpIVKO mice were a direct consequence of MHCII deficiency in LNSCs, we adoptively transferred T cells isolated from LN of K14tgpIVKO or control K14tg mice into Rag2−/− mice. 4 mo later, recipients that received T cells from >18-mo-old K14tgpIVKO mice exhibited a significant loss of weight compared with mice injected with T cells isolated from age-matched K14tg controls (Fig 5A). Accordingly, immunofluorescence staining on small intestine sections revealed that Rag2−/− hosts that received LN T cells from K14tgpIVKO mice exhibited increased infiltrating CD45+ cells, and in particular in the CD3+ T-cell compartment (Fig 5B).
Interestingly, the levels of expression of MHCII molecules by LECs were significantly higher in elderly (18 mo) compared with young (4 mo) mice (Fig 6A). A slight, but not significant, increase in MHCII expression by BECs, but not by FRCs, was also observed. These observations can be a consequence of increased levels of IFN-γ in old versus young LNs, (although undetectable by ELISA, not shown) and/or an enhanced sensitivity to IFN-γ. Accordingly, LECs and BECs express higher levels of IFN-γ receptor in LNs from elderly compared with younger mice (Fig 6A). These results suggest a more substantial contribution of MHCII-restricted Ag presentation by LECs in aging mice, and provide a possible explanation of why the phenotype observed in K14tgpIVKO only appeared after several months.

Treg frequencies were found impaired in aging (18 mo), but not in young adult (4 mo) K14tgpIVKO mice lacking MHCII expression in LNSCs. Therefore, we tested whether Treg proliferation may be affected by the loss of MHCII expression in LECs. Immunofluorescence staining on LN sections from K14tgpIVKO and control elderly showed that the frequency of proliferating (red, Ki67+) Tregs (green, Foxp3+) in close interaction with LECs (Lyve-1+, in white) was significantly increased in aging controls compared with younger mice (Fig 6B). Again, to determine whether increased Treg expansion in LNs following IFN-γ injection was dependent on MHCII expression by LNSC, most likely by LECs, we performed similar experiments in K14tgpIVKO and K14tg control mice.

Deletion of MHCII on LNSCs decreases Treg proliferation

Interestingly, the levels of expression of MHCII molecules by LECs were significantly higher in elderly (18 mo) compared with young (4 mo) mice (Fig 6A). A slight, but not significant, increase in MHCII expression by BECs, but not by FRCs, was also observed. These observations can be a consequence of increased levels of IFN-γ in
mice. We observed an increased Treg proliferation in LNs of K14tg controls, and further demonstrated that proliferating (red, Ki67+) Tregs (green, Foxp3+) in close proximity of LECs (white, Lyve-1+) (Fig 6C). In contrast, Treg proliferation in LEC proximity (red arrows) was lower in LNs of K14tgpIVKO mice, demonstrating that the increase of proliferation of Tregs interacting with LECs was dependent on their expression of MHCII (Fig 6C). In contrast, we did not observe any increase in the proliferation of non-Treg (Foxp3neg) CD4+ T cells upon IFN-γ stimulation, nor difference between K14tg controls and K14tgpIVKO mice (not shown), suggesting that the Treg compartment is specifically affected. Finally, to examine a potential direct contribution of LECs as MHCII-restricted APCs in inducing Treg proliferation, we repeated the above experiments in mice in which MHCII expression was selectively abrogated in LECs. For that, we used Prox-1-CreERT2 mice, expressing the Tamoxifen-inducible Cre recombinase under the promoter Prox-1, which is selectively expressed in adult LECs (Bazigou et al, 2011). Prox-1-CreERT2 mice were crossed with MHCIIfl mice, allowing the selective deletion of MHCII molecules in LECs upon Tamoxifen treatment (not shown). Immuno-fluorescence analyses of LN sections demonstrated that the frequency of proliferating Tregs in contact with LECs 6 d after IFN-γ injection was significantly reduced in mice in which LECs do not express MHCII molecules (Prox-1-CreERT2 MHCIIfl) compared with control mice (MHCIIfl) (Fig 6D). Altogether, our results demonstrate that IFN-γ-mediated MHCII up-regulation by LNSCs promotes Treg proliferation, with a major contribution of LECs.

Discussion

Our results provide the first evidence for an in vivo role of LNSCs in impacting peripheral T-cell tolerance as MHCII-restricted APCs. We have recently published that subsets of murine LNSCs present MHCII–peptide complexes acquired from DCs to induce CD4+ T-cell dysfunction (Dubrot et al, 2014). Here, we further show that LNSCs inhibit autoreactive T-cell responses by directly presenting Ags through endogenous MHCII molecules. A recent study suggested that, although LECs express surface MHCII molecules, they cannot present a model Ag to CD4+ T cells presumably because of the lack of expression of H2-M in steady state, preventing the

Figure 4. K14tgPIVKO mice exhibit enhanced T-cell activation and impaired Treg frequencies in LNs upon aging.

K14tgPIVKO (white) and K14tg control mice (black) were analysed at 4 and 18 mo. (A) Frequencies of naive (CD62LhiCD44lo) and activated/memory (CD62LloCD44hi) CD4+ and CD8+ T cells. (B) Frequencies of PD-1+, IFN-γ, and IL-17 producing cells among CD4+ and/or CD8+ T cells. (C, D) Foxp3+ Treg identification by flow cytometry (CD4+ CD25+ Foxp3+) (C) and IHC staining (Foxp3+) (D) in lymph nodes of indicated mice. (A–D) Data are representative of three experiments with 3–7 mice/group. *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant. Error bars depict mean ± SEM. (E) CD4+ CD25hi T cells (Treg) from total skin LNs of 18-mo-old K14tgPIVKO and K14tg mice were cultured (ratio 1:5, 1:3, and 1:1) with proliferation dye-labeled naive CD4+ CD25neg T cells. Labeled T-cell proliferation was assessed by flow cytometry after 5 d of co-culture. Data are representative of two experiments. Two-way Anova; **P < 0.01.
loading of endogenous antigenic peptides onto MHCII molecules (Rouhani et al, 2015). However, we show that LECs, BECs, and FRCs that express the IFN-γ-inducible-CIITA pIV, require IFN-γ to up-regulate H-2M molecules, as they do for MHCII expression, these two genes being co-regulated by CIITA (Reith & Mach, 2001). In addition, LNSCs were described to express considerable amounts of invariant chain (ii) and cathepsin L (Rouhani et al, 2015), and therefore seem well equipped for the presentation of antigenic peptides through MHCII in situations involving the presence of IFN-γ.

Genetic abrogation of MHCII in LNSCs in vivo leads to impaired regulatory T-cell frequencies and in vitro functions, enhanced effector T cell differentiation LNs and peripheral tissue infiltration, and subsequent development of T cell–mediated autoimmunity in elderly mice. Indeed, mice lacking MHCII in LNSCs exhibit spontaneous signs of autoimmunity in elderly mice. LNSCs express a broad range of self-Ags. Therefore, our hypothesis is that abrogation of MHCII in these cells will lead to an inflation of polyclonal memory/effector T cells. In agreement, no difference was observed in the frequency of several TCR Vβ chains expressed by CD4+ and CD8+ T cells isolated from 6- to 10-wk-old K14tgpIVKO mice and those from young adult mice (Fig 2E), confirming that the K14 ciita transgene does not promote any MHCII expression in LNSCs. However, because peripheral non-hematopoietic tissue-resident cells can up-regulate MHCII molecules in an IFN-γ–inducible pIV-dependent manner (Duraes et al, 2013), this mouse model does not represent per se a valid mouse model to study the specific role of endogenous MHCII expression by LNSCs in shaping peripheral CD4+ T-cell responses. For instance, intestinal epithelial cells (IECs), hepatocytes and liver sinusoidal endothelial cells, pancreatic β cells, or astrocytes were postulated to be capable of MHCII-mediated Ag presentation, although very little in vivo data are available on the potential impact on peripheral CD4+ T-cell responses (reviewed in Duraes et al [2013]). Recently, using the mouse model we are presently exploiting, two studies have highlighted opposite roles for MHCII expression in distinct target tissues. First, IFN-γ–mediated MHCII up-regulation by IECs exerts anti-inflammatory effects and protect against colitis (Thelemann et al, 2014). In contrast, in the context of experimental autoimmune myocarditis, elevated IFN-γ–inducible cardiac endothelial MHCII expression exacerbates the disease (Thelemann et al, 2016). Together, these findings provide evidence of a critical role of pIV-mediated non-hematopoietic MHCII expression in peripheral tissues.
upon inflammation, the impact on T-cell responses being largely dependent on the target organ. Here, we add a distinct role of LN-resident stromal populations in shaping CD4+ T-cell responses, providing support to Treg homeostasis and maintaining peripheral tolerance. We cannot totally exclude a role for a lack of MHCII expression by cells in peripheral tissue on local T-cell reactivation in K14tgIVKO mice. Indeed, it is possible that T cells in infiltrating peripheral organs are maintained in a tolerogenic state by local MHCII+ cells, and that this phenomenon is abrogated in our mouse model. However, it is unlikely, for several reasons. First, a similar phenotype was obtained after adoptive transfer of T cells from LN of K14tgIVKO mice into Rag2−/− mice with competent MHCII peripheral tissues. Importantly, T-cell compartments in the recipient Rag2−/− mice recapitulated the effects observed in the donor transgenic mice indicating a contribution of LNSCs to peripheral T-cell alterations. Second, although a contribution of MHCII expression by tissue-resident cells has been described before, it was always in inflammatory settings, in which these cells indeed up-regulate MHCII in contrast to our study which was performed in the steady-state (Duraes et al, 2013), minimizing their possible contribution as MHCII-restricted Ag presenting cells in the absence of model-induced inflammation. Finally, LN-resident Tregs exhibit impaired proliferation in LNs not only in K14tgIVKO, but also in Prox-1-CreERT2 MHCIIfl mice, supporting a pro-tolerogenic role for MHCII-restricted Ag presentation by LNSCs, in particular LECs, in dampening T-cell autoimmune reactions.

We have previously shown that endogenous MHCII expression by LECs, BECs, and FRCs was significantly increased in WT mice compared to IFN-γ receptor-deficient mice (Dubrot et al, 2014), demonstrating that the presence of low levels of IFN-γ in naive mice drives pIV-mediated MHCII expression in steady-state LNSCs. This was confirmed by reduced MHCII expression by LNSCs in steady-state
LNs from K14tgPIV KO compared with control mice. Therefore, basal (and presumably variable) levels of circulating steady-state IFN-γ promote endogenous MHCII expression by LNSCs, and confer them the ability to function as competent MHCII-mediated APCs. Increased inflammatory status over time might be due to the accumulation of punctual and mild infectious processes or altered gut microbiotic composition upon aging (Claesson et al., 2012).

Last, we provide evidence for a direct effect of MHCII-restricted Ag presentation by LECs in promoting Treg proliferation. Genetic selective abrogation of MHCII expression by LECs in mice results, similarly to what was observed in K14tgPIV KO mice, in impaired proliferation of Treg in contact with LECs. Therefore, among the LNSC subsets, LECs seems to be the main contributors in inducing Treg proliferation. Distinct sets of PTA expression, together with differential levels of expression of co-inhibitory molecules, such as PDL-1 (Tewalt et al., 2012), by LNSC populations might give rise to distinct impacts on T-cell responses, such as T-cell apoptosis, T-cell anergy, or Treg induction. Elevated PDL-1 expression by LECs compared with BECs and FRCs might explain a role for those cells in impacting Tregs, which express high levels of PD-1. Although we did not elucidate the molecular mechanisms, a selective effect on Tregs, which mostly express a self-reactive TCR, could result from the ability of LECs to present endogenously expressed PTAs. One explanation could be the use of the autophagy pathway by LECs to present endogenous PTAs through MHCII molecules. This hypothesis would be in accordance with previous results obtained in the thymic epithelium (Nedjic et al., 2008; Aichinger et al., 2013). Future experiments in the laboratory will use mice genetically deficient for autophagy to firmly demonstrate the implication of this pathway in the ability of the different LNSC subsets to present PTAs through MHCII molecules and to alter the Treg compartment. Our experiments do not rule out another possible mechanism of action by which MHCII expression in LNSC may support T-cell inactivation in an Ag-independent manner. MHCII is a natural ligand for the molecule lymphocyte activation gene 3 (LAG-3), expressed on activated T cells and can be induced by IFN-γ stimulation. This immune checkpoint has an inhibitory role for LAG-3 in controlling both CD4 and CD8 T-cell proliferation in vitro and in vivo (Workman et al., 2004; Grosso et al., 2007). In addition, LAG-3 expression on Tregs has been shown to be important for their suppressive function (Grosso et al., 2007; Okamura et al., 2009). Moreover, the co-expression of LAG-3 with the inhibitory receptor PD-1 on exhausted T cells or Tregs correlates with a greater state of effector T-cell exhaustion and the suppressive function of Tregs. The relative contribution of both Ag-dependent and/or Ag-independent pathways remains to be addressed.

In conclusion, our work identifies novel in vivo functions of MHCII expression by LNSCs in the maintenance of peripheral T-cell tolerance by inhibiting autoreactive T cells. We also define the LECs as important MHCII expressing cells supporting Treg homeostasis.

Materials and Methods

Mice and treatments

C57BL/6 WT mice were purchased from Charles River. pIV−/− (Waldburger et al., 2003), Ciita−/− and K14tgPIV KO (Laufer et al., 1996), and Rag2−/− (Jackson Laboratories) Prox-1-CreERT2 MHCII mice have been previously described (Bazigou et al., 2011). To selectively abrogate MHCII expression in LECs, Prox-1-CreERT2 MHCIIfl/fl (and MHCIIfl/fl control) mice were injected with tamoxifen (i.p.) twice a day for four consecutive days (2 mg/mice/d). 2 wk after the last injection, mice were treated with IFN-γ and FTY720 as described below. These mouse strains are on a C57BL/6 background and were housed and maintained under SPF conditions at the Geneva Medical School animal facility and under EOPs conditions at Charles River, France. All animal husbandry and experiments were approved by and performed in accordance with guidelines from the Animal Research Committee of the University of Geneva. Genotyping was carried out by using PCR on ear biopsies with the Phire Tissue Direct PCR Master Mix (F170L; Thermo Fisher Scientific). When indicated, IFN-γ (Peprotech) was injected subcutaneously (both flanks and neck, 1 μg/50 μl), and FTY720 (Sigma-Aldrich) (20 μg) was injected every day for 6 d before the experiment. Mice from each group were randomized before treatments.

LNSC and cell tissue isolation

LNSCs were obtained as previously described (Dubrot et al., 2014). In brief, total skin or skin-draining LNs from individual or 10–15 pooled mice were cut into small pieces and digested in RPMI containing 1 mg/ml collagenase IV (Worthington Biochemical Corporation), 40 μg/ml DNase I (Roche), and 2% FBS. Undigested cells were further digested with 1 mg/ml collagenase d, and 40 μg/ml DNase I (Roche). The reaction was stopped by addition of 5 mM EDTA and 10% BSA. Samples were further disaggregated through a 70-μm cell strainer and blocked with anti-CD16/32 antibody. Single-cell suspensions were negatively selected using CD45 microbeads and a magnetic bead column separation (Miltenyi Biotec).

Cells from LN, the spleen, the SC, and SGs were isolated by digesting organ fragments with an enzymatic mix containing collagenase D (1 μg/ml) and DNase I (10 μg/ml) (Roche) in HBSS [14]. For SC and SG, single-cell suspensions were further centrifuged through a discontinuous 30:70% percoll (Invitrogen) gradient. T cells were further analysed by flow cytometry.

Small intestines were excised and transferred into PBS 2% FCS. Peyer’s patches and adipose tissue were carefully removed. The small intestines were cut longitudinally and cleaned of faecal content. The small intestines were next incubated in RPMI containing 10% FCS, 2 mM EDTA, and 1 mM DTT for 30 min at 37°C in a shaking incubator to remove epithelial cells, the supernatant being discarded. The remaining tissue was digested twice in RPMI containing 1 mg/ml of collagenase D (Roche) for 30 min at 37°C in a shaking incubator. Cells were recovered from the supernatant and filtered through a 70-μm cell strainer.

Antibodies, flow cytometry, and cell sorting

Anti-gp38 (8.1.1), anti-CD31 (390), anti-CD11c (N418), anti-CD44 (IM7), and anti-IAb (AF6.120.1) mAbs were from BioLegend. Anti-CD45 (30F11), anti-CD16/32 FcγRIII (2.4G2), anti-I-A^b/I-Ed (2G9), anti-IFNγR (GR20), and H2-M (Z5A) were from BD. Anti-CD9 (1D3), anti-CD8 (53–6.7), anti-CD4 (GK1.5), anti-CD11b (M1/70), anti-CD62L (MEL-14), anti-PDCA-1 (eBio927), anti-PD-1 (J43), anti-IFN-γ (XMG1.2),
anti-IL-17 (eBio17B7), and anti-Foxp3 (FJK-16s) were from eBio-science. Anti-EpCAM (caa7-9G8) and anti-Ly5.1 (6C3) were from Miltenyi Biotec.

For LNSC flow cytometry sorting, enriched CD45<sup>neg</sup> cells were stained with mAbs against CD45, gp38, and CD31. In some experiments, cells were also stained with mAbs against MHCII or isotype control as indicated. For cTEC and mTEC sorting, enriched CD45<sup>neg</sup> cells were stained with mAbs against CD45, EpCAM, and Ly5.1.

Intracellular cytokine stainings were carried out with the Cytofix/Cytoperm kit (BD) for IFN-γ and IL-17 staining. Foxp3 staining was performed with the eBioscience kit, according to manufacturer’s instructions. For IFN-γ and IL-17 staining, LN cells were cultured in RPMI containing 10% heat-inactivated fetal bovine serum, 50 mM 2-mercaptoethanol, 100 mM sodium pyruvate, and 100 μM penicillin/streptomycin at 37°C and 5% CO2. Cells were stimulated for 18 h with PMA/ionomycin and Golgi stop solution (BD) was added during the last 4 h of culture before the staining.

Cells were either acquired on a Gallios or sorted using a MoFlowAstrios (Beckman Coulter), and analysed using FlowJo (Tree Star) or Kaluza softwares.

**Immunofluorescence microscopy**

Mice were transcardiacaely perfused with PBS, and intestines were fixed in paraformaldehyde before inclusion in paraffin. Preserved organs were cut into 7-μm-thick sections and deparaffinised in xylene–ethanol. Ag retrieval was performed in citrate buffer. Sections were then stained with labelled antibodies against CD45 (30-F11) and CD3 (17A2) and DAPI (Sigma-Aldrich) counter-staining. Sections were mounted with Mowiol fluorescent mounting medium (EMD). Images were acquired with a confocal microscope (LSM 700; Carl Zeiss Inc. and SP5; Leica).

Skin LNs from untreated mice or mice treated with IFN-γ and FTY720 were frozen in OCT medium. 10-μm-thick sections were cut and fixed with paraformaldehyde 4% for 20 min. After washing and permeabilization, the sections were stained overnight at 4°C using a rabbit anti-Lyve-1 antibody (Reliatech GmbH). Secondary staining was performed using a Alexafluor488-labelled donkey anti-rabbit antibody, Alexafluor488-labelled anti-Foxp3 (150D) antibodies and ef660-Ki67 (SolA15), for 2 h at room temperature. After DAPI (Sigma-Aldrich) staining, sections were mounted with Mowiol fluorescent mounting medium (EMD). Images were acquired with a confocal microscope (LSM 700; Carl Zeiss Inc. and SP5; Leica). Tregs in “close proximity” (<5 μm) of LECs were quantified as proliferating (Ki67<sup>+</sup>Foxp3<sup>+</sup>) or non-proliferating (Foxp3<sup>-</sup>) cells, and the ratio of proliferating Tregs was calculated. At least 20 images/condition were quantified.

**Treg suppression assay**

In vitro Treg suppressive assays were performed as follows: CD4<sup>+</sup> CD25<sup>hi</sup> T cells (Treg) were purified by flow cytometry from total skin LNs of 18-mo-old K14tgP<sup>IVKO</sup> and K14tg control mice and cultured at the indicated ratio with 2 × 10<sup>3</sup> proliferation dye-labeled naive CD4<sup>+</sup> CD25<sup>neg</sup> T cells in the presence of bone marrow–derived DC and anti-CD3 antibodies. Labeled T-cell proliferation was assessed by flow cytometry after 5 d of co-culture.

**T-cell adoptive transfer into Rag2<sup>-/-</sup> recipients**

LN and spleen of donor mice were harvested, and T cells were negatively selected from the cell suspension using a PAN T isolation kit (Miltenyi biotec) according to the manufacturer’s instructions. Purity generally exceeded 95%. 2–5 × 10<sup>6</sup> T cells were injected intravenously into Rag2<sup>-/-</sup> mice. Recipient mice were randomized before transfer and co-housed during the experiment.

**RNA isolation and quantitative RT-PCR**

Total RNA was isolated using Tri-Reagent (Ambien) from sorted cells. cDNA was synthesized using random hexamers and M-MLV reverse transcriptase (Promega). PCRs were performed with the CFX Connect real-time PCR detection system and iQ SYBR green super mix (Bio-Rad Laboratories). The results were normalized with GAPDH or 60S ribosomal protein L32 mRNA expression and quantified with a standard curve generated with serial dilutions of a reference cDNA preparation. Primer sequences: I-Ab forward, 5′-TCCGAGAGCCCTATGTTGGG-3′ and reverse, 5′-CGTGTAAGATGCATGCAGT-3′. H2-Ma forward, 5′-CTCGAACATCTACACACGTG-3′ and reverse, 5′-TCCGAGAGCCCTATGTTGGG-3′. Ki4 forward, 5′-AGG GAG AGG AGC CCC ACC TT-3′ and reverse, 5′- CCTTGGT GGC GGA TCT GCC CCI-3′

**Lysate preparation and immunoblotting**

Tissues were homogenized in T-PER tissue protein extraction reagent (Thermo Fisher Scientific) supplemented with a cocktail of protease inhibitors (Complete, Roche). Protein extracts were incubated at 95°C for 5 min with of 4× SDS–PAGE–loading buffer (250 mmol Tris–HCl at pH = 6.8, 40% glycerol, 8% SDS, 0.57 mol β-mercaptoethanol, and 0.12% bromophenol blue). Equal amounts of protein were run on 12% SDS–PAGE gels and transferred onto a polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences). Sera from K14tgP<sup>IVKO</sup> or control mice were visualized with HRP-conjugated goat anti-mouse IgG (Bio-Rad Laboratories) and the ECL WesternBright Sirius (advansta).

**Statistical analysis**

Statistical significance was assessed by the two-tailed unpaired t test or two-way Anova, and Log-rank test using Prism software (GraphPad).

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**Author Contributions**

J Dubrot: conceptualization, formal analysis, investigation, methodology, and writing—original draft.
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MHC-II in LNSCs prevent autoimmunity

Dubrot et al.

https://doi.org/10.26508/lsa.201800164
vol 1 | no 6 | e201800164
11 of 12

MHC-II in LNSCs prevent autoimmunity

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https://doi.org/10.26508/lsa.201800164
vol 1 | no 6 | e201800164
11 of 12

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11 of 12

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11 of 12

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vol 1 | no 6 | e201800164
11 of 12

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vol 1 | no 6 | e201800164
11 of 12

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vol 1 | no 6 | e201800164
11 of 12

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vol 1 | no 6 | e201800164
11 of 12

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vol 1 | no 6 | e201800164
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