Lymph node–resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation

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Peripheral immune tolerance is generally thought to result from cross-presentation of tissue-derived proteins by quiescent tissue-resident dendritic cells to self-reactive T cells that have escaped thymic negative selection, leading to anergy or deletion. Recently, we and others have implicated the lymph node (LN) stroma in mediating CD8 T cell peripheral tolerance. We demonstrate that LN-resident lymphatic endothelial cells express multiple peripheral tissue antigens (PTAs) independent of the autoimmune regulator (Aire). They directly present an epitope derived from one of these, the melanocyte-specific protein tyrosinase, to tyrosinase-specific CD8 T cells, leading to their deletion. We also show that other LN stromal subpopulations express distinct PTAs by mechanisms that vary in their Aire dependence. These results establish lymphatic endothelial cells, and potentially other LN-resident cells, as systemic mediators of peripheral immune tolerance.

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Abbreviations used: Aire, autoimmune regulator; FRC, fibroblastic reticular cell; LEC, lymphatic endothelial cell; mRNA, messenger RNA; mTEC, medullary thymic epithelial cell; PTA, peripheral tissue antigen.

Peripheral tolerance prevents self-reactive T cells that escape thymic negative selection from causing autoimmunity. Intrinsic mechanisms of peripheral tolerance lead to anergy (Redmond et al., 2005) or deletion (Kurts et al., 1997; Hernandez et al., 2001; Liu and Lefrançois, 2004) when CD8 T cells encounter their cognate tissue-restricted self-antigens. In the prevailing model of this process, these antigens are acquired by quiescent tissue-resident DCs, which then migrate to regional LN and cross-present them to naive T cells (Hawiger et al., 2001; Belz et al., 2002; Waithman et al., 2007). This presentation is limited to LN draining the tissues in which the self-antigen is expressed. Recently, we and others have described an alternative mechanism in which CD8 T cell peripheral tolerance is induced by LN-resident cells that directly express otherwise tissue-restricted proteins (Lee et al., 2007; Nichols et al., 2007; Gardner et al., 2008). Thus far, two nonoverlapping LN stromal cell populations have been associated with this mechanism. Using an antigen expressed under the control of the autoimmune regulator (Aire) promoter, Gardner et al. (2008) implicated a subset of $EpCAM^+$ $gp38^{neg}$ $Aire^+$ LN stromal cells. Two other studies using antigens expressed under the control of intestinal epithelial and enteric glial cell promoters implicated $UEA\text{-}1^+$ LN stromal cells (Lee et al., 2007; Magnusson et al., 2008). However, these studies relied on transgenic antigens expressed under the control of tissue-specific promoters, creating uncertainty about whether the ectopic expression observed in LN cells is physiologically relevant.

We have evaluated self-tolerance to a model antigen expressed in its native genetic context: the mouse homologue of a human HLA-A*0201-restricted epitope from the endogenously encoded protein tyrosinase (Tyr_{369}; Colella et al., 2000). This epitope is presented in the context of a transgenic HLA-A*0201-based chimeric MHC I molecule (AAD). The level of expression of AAD is comparable to that of endogenous

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mouse MHC I molecules (Newberg et al., 1996). Interest in this epitope is based on the fact that it and other epitopes derived from pigmentation proteins are broadly recognized by T cells from melanoma and vitiligo patients, despite being unmodified self-proteins expressed in melanocytes (Slingluff et al., 2006). To study CD8 T cell tolerance induction to Tyr_{369}, we generated a transgenic mouse expressing a TCR specific for Tyr_{369}:AAD, designated "FH." We previously demonstrated that Tyr_{369} is constitutively presented in both peripheral and mesenteric LNs but not spleen, leading to abortive proliferation and deletion of FH cells. Importantly, Tyr_{369} is not cross-presented by either radiosensitive DCs or radioresistant Langerhans cells under noninflammatory conditions, excluding cross-tolerance as an operative mechanism. Although tyrosinase expression is normally confined to melanocytes and retinal pigment epithelial cells, where it is involved in melanin biosynthesis, we found tyrosinase messenger RNA (mRNA) in the lymphoid compartments where CD8 T cell deletion occurred. This suggested that direct presentation of tyrosinase by a radioresistant LN-resident cell is entirely responsible for tolerance to this endogenous melanocyte differentiation Ag.

In this paper, we have identified the cell that directly expresses the Tyr_{369} epitope as an LN-resident lymphatic endothelial cell. We have established that these cells express both tyrosinase and another tissue-restricted mRNA independent of Aire. Finally, we show that other subpopulations of LN stromal cells express distinct peripheral tissue transcripts, which differ in their dependence on Aire. Our results suggest that ectopic expression of tissue-specific transcripts by multiple subsets of LN stromal cells is of general importance in establishing peripheral tolerance.

RESULTS AND DISCUSSION

We previously showed that FH T cells undergo deletional tolerance in all LN but not in either thymus or spleen (Nichols et al., 2007; Fig. S1). Tolerance is not mediated by conventional DC or Langerhans cells in the periphery but instead depends on radioresistant LN-resident cells that express tyrosinase directly (Nichols et al., 2007). To identify the radioresistant cells expressing tyrosinase, pooled LN of tyrosinase-expressing B6 mice were collagenase digested to yield single cell suspensions and $CD45^+$ and $CD45^{neg}$ subsets were enriched

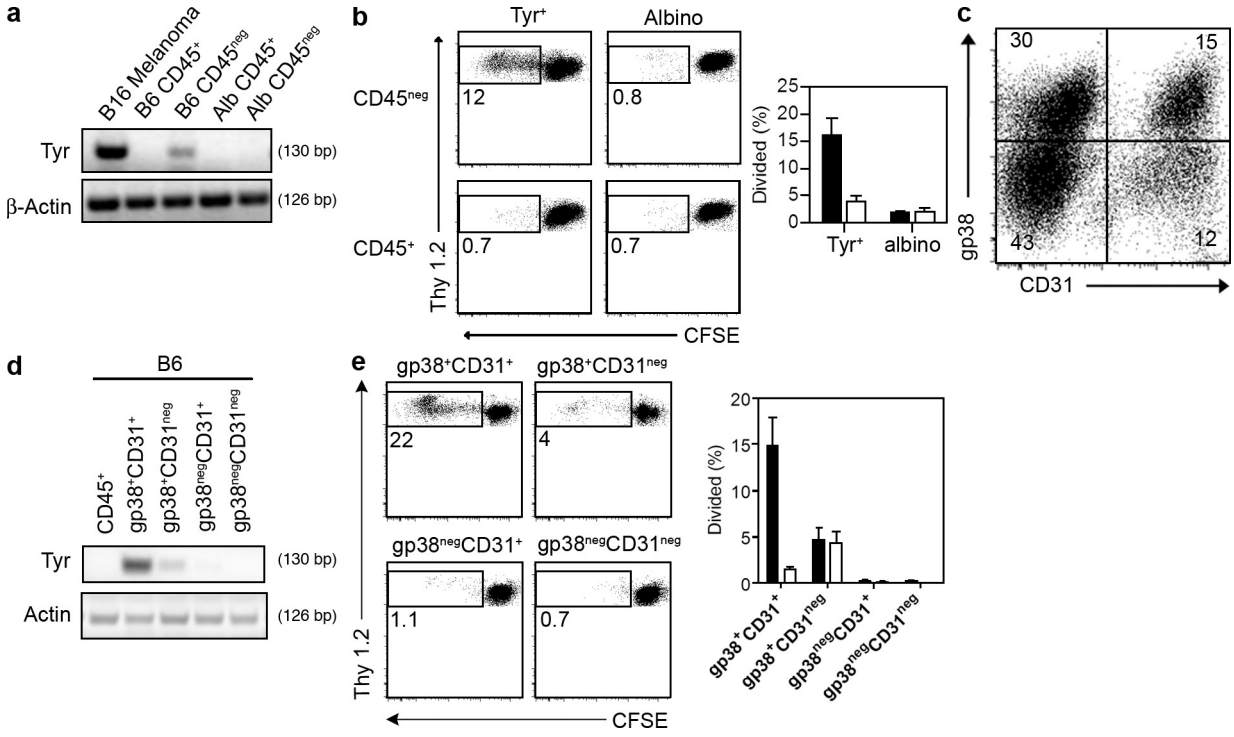

Figure 1. gp38$^+$CD31$^+$ LN stromal cells express and present Tyr_{369} in an Aire-independent manner. (a) Tyrosinase (Tyr) and β-actin expression in $CD45^{neg}$ and $CD45^+$ LN populations from B6 and albino animals was determined using 40-cycle RT-PCR. Data shown are representative of four independent experiments. (b) Proliferation of CFSE-labeled naive Thy1.2$^+$ FH T cells co-cultured with Thy1.1$^+$ $CD45^{neg}$ (black bars) or $CD45^+$ (white bars) cells purified from the LNs of tyrosinase$^+$ and albino animals was assessed after 86 h. Left, representative experiment; right, summary data for two independent experiments. (c) LN stromal cell populations defined based on expression of gp38 and CD31. The representative plot is gated on $CD45^{neg}$ cells. Numbers indicate percentage of total $CD45^{neg}$ cells. (d) Tyrosinase and β-actin expression in $CD45^+$ cells and four LN stromal cell subpopulations identified by differential expression of gp38 and CD31. Purified populations were obtained by electronic cell sorting and 40-cycle RT-PCR was performed. Data shown are representative of six independent experiments. (e) Proliferation of CFSE-labeled naive Thy1.2$^+$ FH T cells co-cultured with Thy1.1$^+$ cells of the indicated purified LN stromal cell subpopulations from tyrosinase$^+$ (black bars) and albino (white bars) animals was assessed after 86 h. Left, representative experiment; right, summary data for three independent experiments. Error bars indicate SEM.

using magnetic beads. RT-PCR of mRNA extracted from these cells reproducibly demonstrated that tyrosinase expression was confined to $CD45^{neg}$ LN stromal cells (Fig. 1 a). As expected, tyrosinase mRNA was not detected in the LN stromal cells of albino mice carrying a complete deletion of the tyrosinase gene. To assess the ability of LN stromal cells to present Tyr_{369}, naive CFSE-labeled FH T cells were co-cultured in vitro with AAD^+ $tyrosinase^+$ $CD45^{neg}$ or $CD45^+$ LN cells. FH T cells proliferated when co-cultured with $CD45^{neg}$ LN cells but not with $CD45^+$ LN cells (Fig. 1 b). This proliferation was antigen specific, as it was not induced by $CD45^{neg}$ LN cells from AAD^+ albino mice. Thus, $CD45^{neg}$ LN cells express tyrosinase mRNA and, based on their ability to induce FH T cell proliferation in vitro, translate and process tyrosinase protein for presentation in the context of MHC I molecules.

Consistent with an earlier study (Link et al., 2007), staining of $CD45^{neg}$ LN stromal cells for the cell surface markers CD31 and gp38 identified four different subpopulations (Fig. 1 c). Single cell suspensions of each of these subpopulations were obtained by electronic cell sorting. RT-PCR demonstrated that tyrosinase was strongly and consistently expressed (six out of six experiments) in $gp38^+CD31^+$ LN stromal cells (Fig. 1 d). We inconsistently detected weak tyrosinase signals in either the gp38 or CD31 single-positive subsets (two and three out of six experiments, respectively), which we attribute to a small and variable number of contaminating $gp38^+CD31^+$ cells in different cell sorts. To establish the relevance of this tyrosinase expression, we co-cultured naive CFSE-labeled FH T cells with each LN stromal cell subpopulation from either $AAD^+tyrosinase^+$ or albino mice. The $gp38^+CD31^+$ subpopulation stimulated robust antigen-specific proliferation, whereas the others did not (Fig. 1 e). This proliferation was physiologically relevant, as the expression level of AAD was comparable to that of endogenous MHC class I molecules (Fig. S2). Thus, $gp38^+CD31^+$ LN stromal cells are the only subpopulation that both expresses tyrosinase and presents Tyr_{369} to FH T cells.

A previous study demonstrated expression of several melanocyte differentiation proteins by LN-resident cells and concluded that they were of the melanocyte lineage (Schuler et al., 2008). However, neither gp38 nor CD31 was detectible on melanocytes in the avascular epidermis (Fig. 2). In addition, although epidermal melanocytes expressed tyrosinase strongly, only a small number of cells in LN stained with anti-tyrosinase antibody, and a comparable number of these cells was also observed in albino mice with a full deletion of the tyrosinase gene (not depicted; Nichols et al., 2007). These data strongly suggest that the $gp38^+CD31^+$ LN stromal cells, the only subset which expresses tyrosinase, are not of melanocytic origin and that the level of tyrosinase protein expression in LN stromal cells is low. LN stromal cells include a subpopulation identified

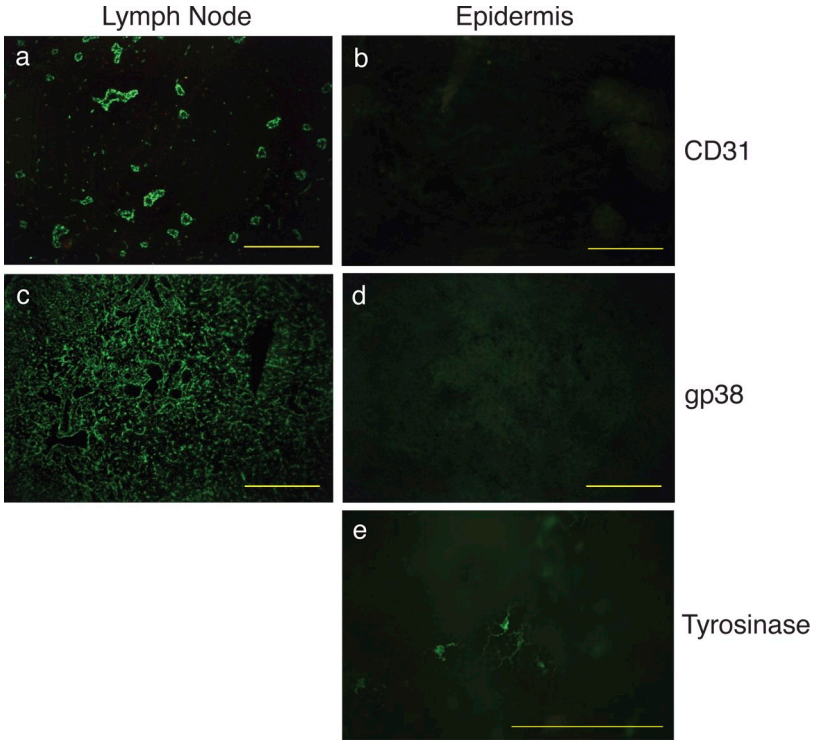

Figure 2. The $gp38^+CD31^+$ LN stromal cells responsible for mediating tolerance to Tyr_{369} are not related to melanocytes. Immunofluorescence of a normal LN section (a and c) and epidermal ear sheets (b, d, and e) stained for the expression of LN stromal cell markers CD31 (100×; a and b) and gp38 (100×; c and d). Melanocytes in epidermal ear sheets were visualized by their tyrosinase staining (200×; e). Staining is representative of multiple magnifications and fields from two independent experiments consisting of two separate LNs and ears from three mice. Bars, 200 µm.

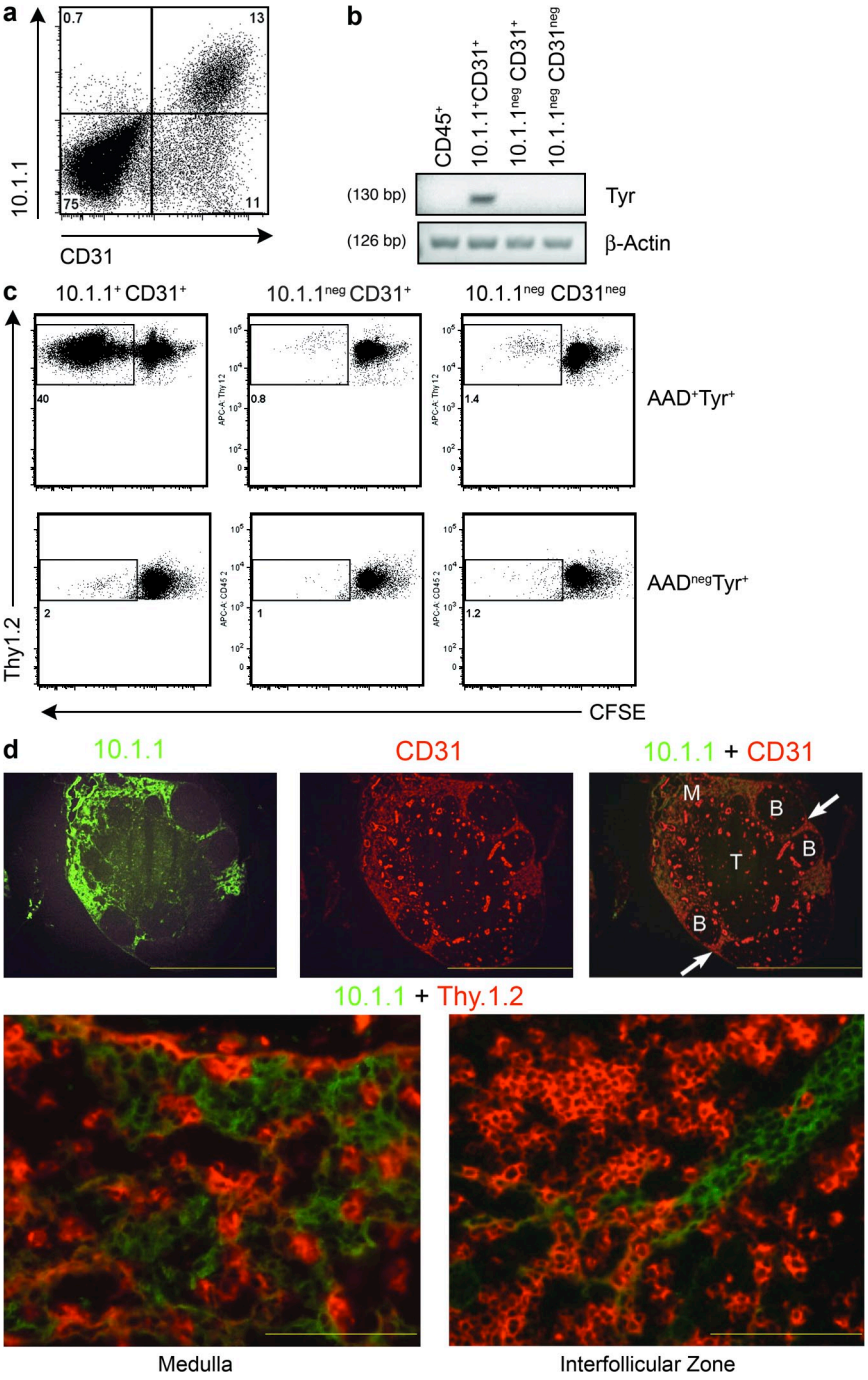

Figure 3. The LN stromal cells responsible for mediating tolerance to Tyr_{369} have characteristics of lymphatic endothelial cells. (a) Staining of $CD45^{neg}$ LN stromal cells with antibodies against 10.1.1 and CD31. (b) RT-PCR for tyrosinase (Tyr) and β-actin mRNA in $CD45^+$ cells and the indicated LN stromal cell subpopulations was evaluated as described in Fig. 1. Results shown are representative of five independent experiments. (c) Proliferation of CFSE-labeled naive $Thy1.2^+$ FH T cells co-cultured with cells of the indicated LN stromal cell subpopulations from $Thy1.1^+$ $tyrosinase^+$ and albino animals was assessed as in Fig. 1. Results are from two independent experiments performed with stromal cells isolated from six pooled mice each. (d, Top) Cells with dual staining of 10.1.1 and CD31 are located in the medulla (M) and in the interfollicular areas (arrows) but not in the T cell zone (T) or B cell follicles (B; 40×). Staining is representative of multiple magnifications and fields from two independent experiments consisting of two separate LNs from two mice. Bars, 1 mm. (d, Bottom) Higher magnification (400×) of the medulla (left) and the interfollicular region (right) showing intermingling of $10.1.1^+$ LEC and $Thy1.1^+$ T cells. Staining is representative of multiple magnifications and fields from two independent experiments consisting of two separate LNs from one mouse. Bars, 400 µm.

as lymphatic endothelial cells (LECs) based on staining with the antibody 10.1.1 (Ruddell et al., 2003; Link et al., 2007), and these are included within the gp38^{+}CD31^{+} subset (Link et al., 2007). To determine if the tyrosinase-expressing cells in LN were LEC, we sorted LN stromal cells into 10.1.1^{+}CD31^{+}, 10.1.1^{neg}CD31^{+}, and 10.1.1^{neg}CD31^{neg} subpopulations (Fig. 3 a). Because of the species of the antibodies, we were unable to determine if all of the gp38^{+}CD31^{+} also stained positive for 10.1.1. However, virtually all 10.1.1^{+} cells were positive for CD31. Moreover, their percentage of representation among LN stromal cells was comparable to that of the gp38^{+}CD31^{+} population. In keeping with this, RT-PCR demonstrated that tyrosinase mRNA was expressed in 10.1.1^{+}CD31^{+} cells but not in either 10.1.1^{neg}CD31^{+} or 10.1.1^{neg}CD31^{neg} cells (Fig. 3 b), although tyrosinase protein was not detected above background by flow cytometry or immunofluorescence (not depicted). In addition, naive CFSE-labeled FH T cells proliferated robustly when co-cultured with 10.1.1^{+}CD31^{+} LEC but negligibly with the other two subpopulations (Fig. 3 c). These results establish that the LN stromal cell that expresses tyrosinase and presents Tyr_{369} has lymphatic endothelial cell characteristics.

To confirm that 10.1.1^{+}CD31^{+} cells were LECs and to determine how they were positioned with respect to T cells within the LN, we examined normal LN sections by immunohistochemistry. All 10.1.1^{+} cells costained strongly with CD31 (Fig. 3 d) and the LEC-specific markers Lyve-1 (lymphatic vessel endothelial hyaluronan receptor 1) and Prox-1 (prospero homeobox protein 1; Fig. S3). These 10.1.1^{+}CD31^{+} LEC were localized predominantly to the medulla and the interfollicular zones and were largely absent from the T and B cell zones (Fig. 3 d). In both medulla and interfollicular zones, a relatively small number of individual T cells was found to interdigitate with the LEC (Fig. 3 d). These results demonstrate that LECs are advantageously positioned to tolerize potentially autoreactive naive T cells as they exit the LN but likely not in the T cell zone.

To determine if LECs have the potential to play a more general role in deletional tolerance, we examined the expression of another melanocyte differentiation antigen, Mart1, and four other peripheral tissue antigens (PTAs) previously shown to be expressed in LN stromal cells (Lee et al., 2007; Schuler et al., 2008). We found that A33, which is considered to be a definitive marker of intestinal epithelial cells (Johnstone et al., 2000), and the pancreatic polypeptide Ppy were also predominately expressed in LEC that had been purified using CD31 and either gp38 or 10.1.1 (Fig. 4 and Fig. S4). In contrast to tyrosinase, a second melanocyte differentiation protein, Mart1, was not expressed in LEC. Instead, it was predominantly expressed in the gp38^{+}CD31^{neg} fibroblastic reticular cells (FRCs) and the 10.1.1^{neg}CD31^{neg} population, which also contains FRC (Fig. 4). The expression of tyrosinase and Mart1 in mutually exclusive LN cell subpopulations further argues against a lineage of LN-resident melanoblasts (Schuler et al., 2008). Like Mart1, preproinsulin 2 (Ins2) was also predominantly expressed in the FRC (Fig. S4). Conversely, Gad67, which is normally expressed in pancreas and neuronal tissue (Yanagawa et al., 1997), was not expressed in LEC but was also found in the gp38^{+}CD31^{neg} FRC and the 10.1.1^{neg}CD31^{neg} population containing FRC, as well as gp38^{neg}CD31^{neg} LN stromal cells and CD45^{+} LN cells (Fig. 4, a and b). Thus, FRC and one or more subsets of both gp38^{neg}CD31^{neg} LN stromal cells and CD45^{+} LN express PTA distinct from those expressed by LEC. These data show that LEC and at least three other LN cell subpopulations have the potential to mediate tolerance to PTA from different tissues.

Aire has been shown to regulate the expression of many PTAs in medullary thymic epithelial cells (mTECs), which can result in the deletion of single-positive thymocytes (DeVoss et al., 2006). Aire is also expressed in LN (Mathis and Benoist, 2009), and it was recently shown that EpCAM^{+}gp38^{neg} LN stromal cells expressing a tissue-specific transgene under the control of the Aire promoter induced CD8 T cell deletional tolerance (Gardner et al., 2008). However, Aire was not expressed in gp38^{+}CD31^{+} or 10.1.1^{+}CD31^{+} LEC, although it was expressed in CD45^{+} LN cells and gp38^{neg}CD31^{neg} LN stromal cells (Fig. 4). In addition, both tyrosinase and A33 continued to be expressed by LEC of Aire$^{-/-}$ mice (Fig. 4). Aire$^{-/-}$ AAD^{+} tyrosinase^{+} LEC also continued to induce the proliferation of FH cells in vitro (Fig. S5 a), and FH cells still underwent deletion after adoptive transfer into Aire$^{-/-}$ AAD^{+} tyrosinase^{+} recipients (Fig. S5 b). Similarly, Aire was not expressed in gp38^{+}CD31^{neg} FRC, and expression of both Mart1 and Gad67 was maintained in Aire$^{-/-}$ mice. However, the expression of Gad67 in both gp38^{neg}CD31^{neg} and CD45^{+} populations was Aire dependent (Fig. 4). This parallels

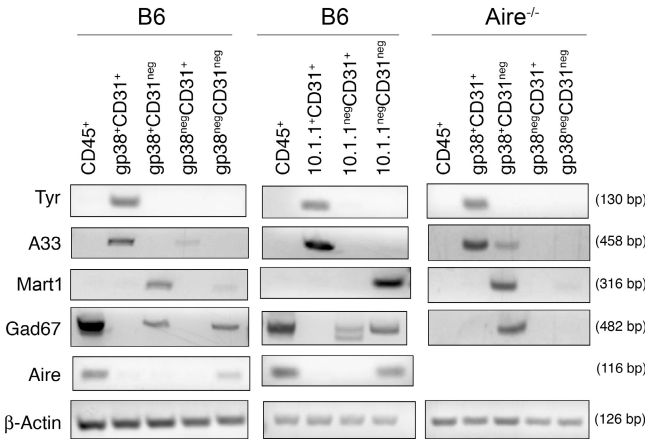

Figure 4. LN stromal cell subsets express distinct groups of PTA that vary in their dependence on Aire. Tyrosinase (Tyr), A33, Mart1, Gad67, Aire, and β-actin mRNAs were amplified by 40-cycle RT-PCR from the indicated subpopulations of LN stromal cells isolated from either C57BL/6 or Aire$^{-/-}$ mice. Data from C57BL/6 mice sorted with gp38 and CD31 are representative of six independent experiments. Data from C57BL/6 mice sorted with 10.1.1 and CD31 are representative of three independent experiments. Data from Aire$^{-/-}$ mice sorted with gp38 and CD31 are representative of two independent experiments.

the expression of Aire in these populations and demonstrates that expression of PTA in both LEC and FRC is controlled by an Aire-independent mechanism.

In this paper, we demonstrate that LN-resident LECs directly present an endogenous antigen to CD8 T cells. We previously demonstrated that FH T cell deletional tolerance is mediated by a radioresistant LN-resident cell and not through cross-presentation by DC (Nichols et al., 2007). Because LECs are the only cells in the LN that express tyrosinase and present Tyr_{369} to induce FH T cell proliferation, we conclude that they are responsible for FH T cell deletion in vivo. It was initially surprising to find that LECs, rather than FRCs, express tyrosinase because FRCs form the stromal network of the T cell zone and interact extensively with naive T cells. However, our results also suggest that FRC and at least two other LN-resident populations ($gp38^{neg}CD31^{neg}$ LN stromal cells and $CD45^+$ LN cells) may also be involved in peripheral tolerance induction to different PTA. It will be of significant interest to determine the full breadth of PTA expression by these distinct subpopulations in comparison with one another to obtain a better understanding of their potential relevance in peripheral tolerance. It will also be of interest to determine how PTA expression patterns by LN populations compare with those of thymic mTECs to understand the extent to which redundancy is built into central and peripheral tolerance pathways. It is significant that in the case of the Tyr_{369} epitope, tolerance is based on peripheral rather than central presentation, despite expression of tyrosinase mRNA in both LN and the thymus (Nichols et al., 2007). Thus, a full evaluation of this issue must be based on the availability of TCR transgenic models that enable an assessment of tolerance to endogenous antigens.

Several recent studies have demonstrated direct expression of PTA in LN. Schuler et al. (2008) suggested that expression of several melanocyte differentiation proteins in LN was the result of a resident population of melanocytic lineage cells. However, we find that tyrosinase and Mart1 are predominantly expressed by LEC and FRC, respectively. Based on their surface markers and expression of gut and pancreatic PTA, neither of these cell types is of melanocytic origin. Gardner et al. (2008) demonstrated that peripheral tolerance to a transgenic antigen expressed under the control of the Aire promoter was mediated by a subset of $EpCAM^+$ $gp38^{neg}$ LN stromal cells. Consistent with their work, we showed that both $gp38^{neg}CD31^{neg}$ LN stromal cells and $CD45^+$ LN cells expressed Aire and that expression of the PTA Gad67 in both populations was strongly Aire dependent. It has also been reported that two tissue-specific transgenes, Gad67 and A33, were expressed in LN stromal cells that bind to the lectin UEA-1 (Lee et al., 2007; Magnusson et al., 2008). However, this lectin stains 40–50% of LN stromal cells and is expressed on all of the subpopulations evaluated in this paper (unpublished data). Importantly, our results show that Gad67 and A33 are actually expressed in distinct LN stromal subpopulations. This emphasizes the importance of using more discriminating markers to clarify which LN stromal cell subsets are capable of mediating self-tolerance. Nonetheless, these results collectively suggest that peripheral tolerance is mediated by the coordinated efforts of $CD45^+$ and different LN stromal populations expressing distinct PTA that vary in their dependence on Aire.

Direct expression of PTA leading to tolerance has been most frequently associated with mTEC. In most cases, expression of PTA transcripts in mTEC has been shown to be Aire dependent (Derbinski et al., 2001, 2005). The expression of different subsets of PTA has been associated with the progressive differentiation of mTEC to express CD80 and Aire (Farr et al., 2002; Derbinski et al., 2005; Gillard and Farr, 2005; Kyewski and Klein, 2006; Yano et al., 2008). In contrast, our results show that different subsets of PTA are expressed by cell subpopulations in LN, at least some of which are not developmentally related. It is conceivable that subsets of PTA-expressing cells in these compartments induce tolerance by distinct mechanisms or act on different subsets of lymphocytes. Our results also emphasize the importance of Aire-independent expression of other PTA in that it can lead to tolerance in LN. The Aire-independent expression of tyrosinase and A33 by LEC, and of Gad67 and Mart1 by FRC, suggests that an alternative transcriptional regulator controls the expression of these PTA. Recent work has established that Deaf1 (deformed epidermal autoregulatory factor 1), a member of the SAND transcription factor family which includes Aire, regulates expression of PTA in pancreatic LN (Yip et al., 2009). Although we have found Deaf1 in all of the $CD45^{neg}$ subsets identified in this paper (unpublished data), it is not yet clear to what extent it regulates their expression of various PTA.

DC-mediated cross-presentation of self-antigen has been the established paradigm for peripheral tolerance induction. However, our results suggest that direct presentation of PTA in secondary lymphoid organs is a critically important process in subduing autoreactive T cells. Indeed, in situations where peripheral tolerance to a particular PTA is not mediated by conventional DC, such as Tyr_{369} (Nichols et al., 2007), LN stromal cell expression of PTA becomes vital to the deletion of self-reactive T cells. Because DCs that have acquired antigen from tissue traffic only to local LN (Kurts et al., 1997; Hawiger et al., 2001; Hernandez et al., 2001; Vermaelen et al., 2001; Belz et al., 2002; Scheinecker et al., 2002; Waithman et al., 2007), DC-mediated tolerance is limited. Conversely, PTA-expressing LN stromal cells are found in all LN, enabling systemic peripheral tolerance induction. This increases the likelihood that rare circulating autoreactive T cells will encounter a tolerizing APC. It will be fascinating to determine the relative contribution of DC versus LN stromal cell–mediated peripheral tolerance.

MATERIALS AND METHODS

Mice. C57BL/6 mice carrying the AAD transgene or a fully deleted tyrosinase gene ($c^{38R145L}$) have been previously described (Newberg et al., 1996; Colella et al., 2000). FH mice were generated using TCR genes from a Tyr_{369}-specific T cell clone derived from an AAD^+ albino mouse (Nichols et al., 2007). $Aire^{-/-}$ mice were purchased from The Jackson Laboratory and bred

to C57BL/6 AAD$^+$ mice to produce Aire$^{+/-}$AAD$^+$ mice, which were then intercrossed to generate Aire$^{-/-}$AAD$^+$ offspring. Animals were maintained in pathogen-free facilities and procedures were approved by the University of Virginia Animal Care and Use Committee.

Isolation of LN stromal cells. LN capsules were opened and digested with collagenase and DNase to obtain single cell suspensions (Link et al., 2007). LN stromal cells were negatively selected using CD45 beads and the MACS system (Miltenyi Biotec) and were >90% pure by flow cytometry. CD45^{neg} cells were stained with mAbs against gp38 (8.1.1; Developmental Studies Hybridoma Bank) or 10.1.1 and CD31 (eBioscience) and electronically sorted into subpopulations (FACSVantage; BD).

In vitro antigen presentation assay. CD45$^+$ and CD45^{neg} LN cells or LN stromal cell subsets were isolated from pooled peripheral and mesenteric LN of AAD$^+$tyrosinase$^+$ or AAD$^+$albino mice as described in the previous section. FH T cells were purified from spleen and peripheral LN of albino mice using CD8 beads and the MACS system. Naive CFSE-labeled FH T cells (5×10^4) were incubated with 10^5 APC in the presence of 10 U/ml IL-2 in for 86 h and analyzed by flow cytometry.

RT-PCR. RNA isolated from tissue or sorted cell populations (RNeasy Mini kit; QIAGEN) and random hexamers were used to generate complementary DNA (First Strand cDNA Synthesis kit; Fermentas). Amplification was done using Sahara polymerase mix (Bioline) with the following program: 95°C for 10 min; 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. Primers used were the following: tyrosinase forward, 5′-CCAGGCTCCCATCTTCAGC-3′, and reverse, 5′-CCTGTGAGTGGACTGGCAAAT-3′; A33 forward, 5′-CCGAAGTCAGACGGAAAGAG-3′, and reverse, 5′-TGCTGGAGGTGCAGATGTAG-3′; Gad67 forward, 5′-TGCAACCTCCTCGAACGCGG-3′, and reverse, 5′-CCAGGATCTGCTCCAGAGAC-3′; MART1 forward, 5′-CTTTGGTTATCCCAGGAAGG-3′, and reverse, 5′-TGAATAAGGTGGCGGTGAAG-3′; AIRE forward, 5′-CCAGCAGGTGTTTGAGTCAG-3′, and reverse, 5′-CACTCCGGGCCTTGTTCTTC-3′; salivary protein 1 forward, 5′-GGCTCTGAAACTCAGGCAGA-3′, and reverse, 5′-TGCAAACTCATCCACGTTGT-3′; and β-actin forward, 5′-ACGTAGCCATCCAGGCTGTG-3′, and reverse, 5′-TGGCGTGAGGGAGAGCAT-3′.

Immunofluorescence microscopy. LNs were cut into 6-μm sections on Superfrost/Plus slides (Thermo Fisher Scientific). Epidermal ear sheets were prepared as previously described (Ratzinger et al., 2002). Tissue sections were fixed, blocked, and permeabilized and stained with FITC-labeled or biotinylated anti-CD31 (eBioscience) or unlabeled anti–Lyve-1 (Millipore), anti-Prox-1 (Abcam), or anti-Tyrosinase$_{cTerminus}$ (AnaSpec), followed by FITC anti–rabbit IgG (SouthernBiotech), biotinylated anti–rabbit IgG (Vector Laboratories), or Texas red streptavidin (SouthernBiotech). For signal amplification, sections were blocked with PBS containing 3% H_2O_2 and 0.1% NaN_3, or using a Biotin-Avidin Blocking kit (Vector Laboratories), and stained with biotinylated anti-Thy1.2 (BD) and either anti-gp38 or 10.1.1, followed by FITC anti–syrian hamster IgG (Jackson ImmunoResearch Laboratories). Fluorescein and biotin tyramide signal amplification (Perkin-Elmer) was performed as per the manufacturer's instructions.

Online supplemental material. Fig. S1 shows that FH T cells undergo abortive proliferation and deletion when adoptively transferred into AAD$^+$ tyrosinase$^+$ animals but not in AAD$^+$ albino animals. Fig. S2 shows endogenous MHC class I and AAD transgene cell surface staining of LEC. Fig. S3 shows immunofluorescence staining of LN sections with 10.1.1 and the LEC-specific markers Lyve-1 or Prox-1. Fig. S4 shows expression of pancreas-specific PTA by different LN stromal cell subsets. Fig. S5 shows in vitro Tyr$_{369}$ presentation by Aire$^{-/-}$ AAD$^+$ tyrosinase$^+$ LEC and in vivo abortive proliferation of FH T cells in Aire$^{-/-}$ AAD$^+$ tyrosinase$^+$ animals. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092465/DC1.

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