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*J Immunol* 2007; 179:993-1003; doi: 10.4049/jimmunol.179.2.993

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Deletional Self-Tolerance to a Melanocyte/Melanoma Antigen Derived from Tyrosinase Is Mediated by a Radio-Resistant Cell in Peripheral and Mesenteric Lymph Nodes

Lisa A. Nichols,* Yiming Chen,2* Teresa A. Colella,* Clare L. Bennett,† Björn E. Clausen,† and Victor H. Engelhard3*

Self-tolerance to melanocyte differentiation Ags limits the ability to generate therapeutic antimalanoma responses. However, the mechanisms responsible for CD8 T cell tolerance to these Ags are unknown. We have used a newly generated TCR-transgenic mouse to establish the basis of tolerance to one such Ag from tyrosinase. Despite expression of tyrosinase transcripts in the thymus, central deletion does not shape the tyrosinase-specific CD8 T cell repertoire. We demonstrate that this endogenously expressed melanocyte Ag is constitutively presented in both peripheral and mesenteric lymph nodes, leading to abortive activation and deletion of tyrosinase-specific CD8 T cells. Importantly, this Ag is not presented by either radio-sensitive dendritic cells, or by radio-resistant Langerhans cells. Thus, for this endogenous Ag, cross-tolerization does not appear to be an operative mechanism. Instead, we find radioresistant tyrosinase mRNA expression in lymphoid compartments where CD8 T cell deletion occurs. This suggests that direct presentation of tyrosinase by radio-resistant lymph node resident cells is entirely responsible for tolerance to this endogenous melanocyte differentiation Ag. The Journal of Immunology, 2007, 179: 993–1003.

Many of the identified peptide Ags used in therapeutic vaccination against melanomas are derived from normal tissue-specific proteins (1). As such, the desired outcome of tumor immunotherapy—to elicit an effective CD8 T cell response against the tumor—may be compromised by self-tolerance. In contrast, immune responses against tissue-specific Ags derived from melanocyte differentiation proteins (MDPs)3 in melanoma patients are frequently accompanied by skin depigmentation (2–4). This highlights the correlation between autoimmunity and antitumor immunity and suggests that development of tolerance for these Ags may be limited or inefficient. Understanding the mechanisms of self-tolerance to MDP-derived Ags is important to development of therapeutic vaccines against these targets.

Several peripheral mechanisms that control CD8 T cell reactivity or survival based on specific recognition of other tissue-specific self-Ags have been previously described. Depending on Ag levels, location, and context, a CD8 T cell that encounters self-Ag can either be extrinsically controlled by regulatory T cells (5), or alternatively, undergo anergy (6) or deletion (7–9). Dendritic cells (DCs) mediate tolerogenic presentation of exogenous Ag introduced in the absence of an inflammatory stimulus (10, 11). Similarly, for Ags ectopically expressed in pancreas, deletion of specific CD8 T cells was shown to be due to steady-state presentation by DCs (12). This presentation was limited to lymph nodes (LN) draining the pancreas, suggesting that the responsible DC had acquired Ag while present in this organ. Although these results have established a paradigm of DC-mediated deletional CD8 T cell tolerance, it is unknown whether it is generalizable to proteins expressed in other tissues, and particularly those derived from skin and melanocytes.

Only a limited number of studies have examined peripheral tolerance to transgenic Ags expressed in skin. Expression of the intact H-2Kb molecule on skin keratinocytes induced CD8 T cell anergy (13). Interestingly, tolerance in this model is induced during the neonatal period after Ag encounter in the skin, rather than in draining LN as is the case for Ags expressed in pancreas and intestine (8, 9). However, this model is unusual because the use of an alloantigen precludes the possibility of cross-presentation. In contrast, ectopic expression of conventional Ags in skin keratinocytes leads to rapid development of autoimmune disease after adoptive transfer of specific T cells (14, 15), suggesting that peripheral tolerance mechanisms for Ags expressed in this site are less robust and perhaps more dependent on central deletion in establishing tolerance to this tissue. Importantly, no studies have examined mechanisms of peripheral self-tolerance to Ags expressed in melanocytes despite their relevance in tumor immunotherapy and it is not clear whether the limitations of self-tolerance evident in the skin keratinocyte models apply to these molecules as well. Finally, it is unknown what peripheral cell type, if any, mediates tolerogenic presentation of normal skin Ags in the steady state or whether such tolerance is based solely on thymic selection.

Clonal deletion in the thymus is a well-described mechanism for establishing T cell self-tolerance to ubiquitously expressed proteins (16). A role for the thymus in generation of tolerance to

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Received for publication October 3, 2006. Accepted for publication May 1, 2007.

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This work was supported by Grants CA78400 and AI059996 from the National Institutes of Health (NIH; to V.H.E.). L.A.N. was supported by NIH Training Grants AI07486 and GM08136. B.E.C. is a fellow of the Landsteiner Foundation for Blood Transfusion Research (LSBR 0414).

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4 Abbreviations used in this paper: MDP, melanocyte differentiation protein; DC, dendritic cell; LN, lymph node; mTEC, medullary thymic epithelial cell; BM, bone marrow; LC, Langerhans cell; pLN, peripheral LN; mesLN, mesenteric LN; Ct, cycle threshold; SP, single positive; mTyrVac, recombinant vaccinia expressing full-length murine tyrosinase; DT, diphtheria toxin.

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tissue-specific Ags has also been proposed based on the detection of mRNAs from both transgenic and endogenous tissue-specific Ags within medullary thymic epithelial cells (mTECs) (17–20). For transgenic model Ags, the presence of these tissue-specific mRNAs has been associated with intrathymic deletion of transgene-specific thymocytes (19–24). However, these models may not accurately reflect physiological levels of endogenous gene products (25). Studies examining intrathymic deletion mediated by the products of endogenous tissue-specific mRNAs have given equivocal results. Although mTEC expression of the liver Ag, serum amyloid P component (18), and of the myelin sheath Ag, proteolipid protein (26, 27), were sufficient to induce CD4 T cell tolerance within the thymus, expression of the α-subunit of a gastric ATPase was not (28). In addition, whether any endogenous transcript in mTECs can lead to central deletion of CD8 T cells has not been reported. Significantly, mRNAs for the MDPs MART-1 and tyrosinase have been detected in mTECs of both mice and humans (18, 29). However, the impact of these transcripts in shaping the melanoma-specific T cell repertoire through central tolerance is completely unknown. Altogether, while a variety of tolerance mechanisms have been described using transgenic models, the actual factors that influence the behavior and survival of MDP-specific T cells, and ultimately the steady-state fate of these cells that may determine tumor vaccination strategies, have not been identified.

Tyrosinase is an MDP expressed by sessile melanocytes in the epidermis and hair follicles and by the retinal pigment epithelium in the eye (30). Several tyrosinase-derived MHC class I-restricted epitopes recognized by human melanoma-reactive T cells have been defined. A unique opportunity to study self-tolerance to tyrosinase is created by the availability of mice with a mutation (c38R145L) that results in the deletion of the entire tyrosinase-coding sequence (31). In contrast, mutations in other murine MDPs alter protein folding but not expression and are thus not suitable for an evaluation of self-tolerance. Although, no tyrosinase-derived epitopes presented by murine class I MHC molecules have been reported, the human Tyr369 epitope, comprising residues 369–377 of the protein, is presented by HLA-A*0201 (32). Murine tyrosinase contains only a single conservative amino acid substitution in this region relative to the human sequence. This homologous epitope is processed and presented in murine cells that express either HLA-A*0201 or a chimeric MHC class I molecule, AAD, which contains the peptide-binding domains of HLA-A*0201 and the CD8-binding domain from H-2D<sup>α</sup> (33). We have previously shown that endogenous expression of tyrosinase in AAD<sup>+</sup> mice leads to profound CD8<sup>+</sup> T cell tolerance to murine Tyr<sup>369</sup> (33).

In this study, we examine the mechanism of self-tolerance for this melanocyte-associated Ag using a transgenic mouse expressing a TCR specific for Tyr<sup>369</sup>:AAD. We find no evidence for central tolerance of tyrosinase-specific T cells during their development. However, tyrosinase presentation within LN leads to rapid peripheral T cell deletion. Surprisingly, tyrosinase presentation was not mediated to any extent by radio-sensitive bone marrow (BM)-derived cells, which would include most subsets of DC. It is also not mediated by radio-resistant Langerhans cells (LC). Instead, we find that tyrosinase is produced locally within LN by a radio-resistant cell, suggesting that direct presentation is responsible for induction of tolerance to this melanocyte protein.

Materials and Methods

**Mouse**

AAD<sup>+</sup> mice express an MHC class I molecule composed of the α1 and α2 domains of HLA-A*201 and the α3 domain of the H2-D<sup>α</sup> (34). Mice expressing this transgene in conjunction with a homoygous radiation induced deletion at the tyrosinase (ε) locus on mouse chromosome 7 (338R145L/c38R145L) (31) have been previously described (33). AAD<sup>+</sup>Thy1.1<sup>+</sup> mice on either albino (ATA) or tyrosinase<sup>+</sup> backgrounds (ATT) were generated by backcrossing Thy1.1 congenic C57BL/6 mice purchased from The Jackson Laboratory. Langerin DTR<sup>+</sup> mice on the C57BL/6 background (35) were crossed to AAD<sup>+</sup> mice to produce F<sub>1</sub> offspring expressing both genes. FH transgenic mice were generated from TCR genes isolated from a T cell clone specific for amino acids 369–377 of tyrosinase (Tyr<sup>369</sup>), derived from an AAD<sup>+</sup> albino mouse. Full-length TCR α- and β-chains were cloned, sequenced, and subcloned into the hCD2 transgenic expression vector, linearized, and injected into B6 × CBA F<sub>1</sub> male pronucleus of fertilized eggs by the University of Virginia Gene Targeting and Transgenic Facility. Founder mice were screened for PCR for integration of the TCR transgene and then bred in our facility on the AAD<sup>+</sup> albino and tyrosinase<sup>+</sup> C57BL/6 backgrounds. Animals were maintained in pathogen-free facilities at the University of Virginia and procedures were approved by the University of Virginia Institutional Animal Care and Use Committee.

**Analysis of cell surface markers**

Peripheral LN (pLN; pooled axillary, brachial, and inguinal), mesLN, spleen, and thymus were separately homogenized to single-cell suspensions and incubated with anti-CD16/CD32 (eBioscience) before surface staining. Staining reagents used include: anti-CD8α-FITC, anti-CD8α-PE, anti-CD90-PE, anti-Thy1.2-PE (all obtained from eBioscience), anti-CD4-PerCP-Cy5.5, anti-CD8-PerCP, anti-V<sub>α8.3</sub>-biotin, anti-V<sub>β11</sub>-PE (all obtained from BD Biosciences). Ag-specific cells were identified using Tyr<sup>369</sup>-HLA-A2 or Tyr<sup>369</sup>-AAK tetramers. Apoptosis was determined using anti-annexin-V-PE (BD Biosciences). BM chimeras were evaluated using CD11c-allophycocyanin, CD11b-PE, CD8-FITC, CD3ε-PE (eBioscience), purified anti-ε-cadherin (Zymed Laboratories) in combination with anti-HLA-A2-biotin (clone CR11-351). Secondary reagents used were anti-rat IgG-Fc (Jackson ImmunoResearch Laboratories), streptavidin-PerCP (BD Biosciences), and streptavidin-allophycocyanin (eBioscience). Samples were collected on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Production and immunization of thymic chimeras**

Thymic lobes of 2- to 4-day-old neonates were removed by vacuum suction. Three days postthymectomy, neonates were T cell depleted by i.p. injection of purified anti-CD4 (GK1.5), anti-Thy1 (T24-40.7), and anti-CD8 (80H415) (all gifts from Dr. M. McDuffie, University of Virginia, Charlottesville). Depletion was verified 6 wk later by analysis of PBLs by flow cytometry. Donor thymic tissue was harvested from AAD<sup>+</sup> albino or tyrosinase<sup>+</sup> mice, incubated for 5 days in 1.35 mM 2-deoxyxygosonine, and implanted under the kidney capsule of thymectomized recipient mice. Animals were allowed 6 wk to reconstitute the periphery and then immunized with 10<sup>9</sup> PFU of murine tyrosinase-expressing vaccinia virus (mTyrVac). Three weeks postimmunization, spleens were harvested and cultured for 6 days in the presence of 1 μM Tyr<sup>369</sup> peptide or for 5 days with BALB/c spleen cells, followed by restimulation of cytotoxic activity for 3 days (31). At time of sacrifice, all hosts were negative for thymic remnants by histology and T cell reconstitution in the spleen was 30–60% of normal controls (data not shown).

**Adoptive transfer of FH and ATT T cells**

Single suspensions of cells from pooled LN and spleens of either FH albino or ATT (AAD<sup>+</sup>Thy1.1<sup>+</sup>tyrosinase<sup>+</sup>) mice were enriched for CD8<sup>+</sup> T cells by negative selection (StemCell Technologies). Cells were consistently >90% CD8 by flow cytometry. For some experiments, cells were labeled with CFSE (Molecular Probes). CD8<sup>+</sup>-enriched FH cells (3 × 10<sup>6</sup>) were injected alone or with an equal number of CD8<sup>+</sup>-enriched ATT cells into lateral tail vein, for cotransfers of FH/ATT, CD8<sup>+</sup>-enriched ATT cells were mixed with FH at a ratio of 1:1–2. mTyrVac-treated mice were infected with either 1.5 × 10<sup>5</sup> or 10<sup>5</sup> PFU of recombinant vaccinia expressing full-length murine tyrosinase at time of T cell transfer.

**Generation of BM chimeras**

C57BL/6 or AAD<sup>+</sup> (tyrosinase<sup>+</sup> or albino) mice were lethally irradiated (650R×2) then injected with either AAD<sup>−</sup> or B6 T cell-depleted BM (1–2 × 10<sup>6</sup> cells i.v., respectively). For adoptive transfer experiments, animals were sacrificed 3–6 mo after transplants. Before experiments, peripheral blood cells from some animals were stained for expression of AAD and it was determined that <5% were of host origin. At time of experiment, chimeras used were re-evaluated for AAD expression on LN and splenic cells.
**Diphtheria toxin (DT) depletion**

DTR+ cells in Langerin DTR-AAD mice were depleted by biweekly i.p. injection of 16 ng/g DT (Calbiochem) for 3–5 wk, with a final dose of toxin administered 24 h before T cell transfer. Efficacy of depletion was verified by immunofluorescent staining of epidermal sheets, prepared as described (36), with anti-Langerin (Imgenex) or anti-MHC class II (eBioscience) Abs. Depletion was further verified by analyzing single-cell suspensions of epidermal cells for surface expression of CD11b, CD11c, MHC class II, and Langerin (eBioscience) expression by flow cytometry. Epidermal cell suspensions were obtained by using trypsin digestion to release epidermal sheets from dorsal ear halves followed by homogenization.

**Detection of tyrosinase mRNA**

Total RNA was isolated from single-cell suspensions of pLN, mesLN, spleen, eyes, or B16 melanoma using TRIzol (Invitrogen Life Technologies). cDNA was generated using oligo(dT) primers (First Strand rt-PCR kit; Fermentas). PCR primers (Oaperon) were as follows: tyrosinase forward (Fwd)1031–1040 5'-ACCACAGTCCATGCACTAC-3' and reverse (Rev)1160–1179 5'-CCTGT GAGTGGACTGGCAAAT-3', β-actin Fwd120–139 5'-ACGTAGCCATCCAGGC GCTGTTG-3' and Rev1160–125 5'-TGCGTGGAGGGAAGACAT-3'. PCR conditions were 94 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 65 °C for 1 min. Reaction products were run on 1.5% agarose gels containing ethidium bromide. Real-time PCR samples were run in duplicate using SYBR Green Master Mix and analyzed with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Cycle threshold (Ct) was determined in relation to that of β-actin by calculation of the change in tyrosinase Ct relative to the β-actin Ct using the formula ΔCt = (average (Avg) Ctyr - Avg Cβact), Tyrosinase expression in different compartments was normalized to tyrosinase expression in the mesLN as 2^−ΔΔCt, where ΔΔCt = (ΔCt compartment - Avg ΔCt mesLN) (37).

**Results**

The number of peripheral Tyr369-specific T cells is reduced by endogenous tyrosinase expression

Previous work from our laboratory demonstrated that the CD8 T cell response to the tyrosinase-derived AAD-restricted peptide epitope, Tyr369, is significantly reduced in mice expressing endogenous tyrosinase compared with that in albino mice lacking the tyrosinase gene (33). We generated a transgenic mouse expressing a Tyr369-AAD-specific TCR to examine the nature of this CD8 T cell self-tolerance. This TCR (designated as “FH” for FMDGT nase) was isolated from an immunized cell self-tolerance. This TCR (designated as “FH” for FMDGT nase). Similar data were obtained in both albino and tyrosinase (Fig. 1B and/or Langerin (eBioscience) expression by flow cytometry. Epidermal CD8 T cell compartment, reflected in the skewing of the CD8:CD4 T lymphocyte ratio from 0.7 to 2–3 (Fig. 1C). Additionally, up to 93% of the peripheral CD8 T cells costained with Vβ11 and/or Vβ11-specific Abs (Fig. 1A). Similar data were obtained in both albino and tyrosinase mice that did not express AAD (data not shown). Thus, T cells expressing the FH TCR are undergoing positive selection by an endogenous H-2b molecule. However, in FH mice that express AAD and tyrosinase, both the percent of CD8 lymphocytes and the proportion that were Vß8/Vβ11 were significantly reduced in lymphoid compartments (Fig. 1C). Although variable in extent among individual mice, this consistent decline in Tyr369-specific T cell numbers (Fig. 1D) suggests that endogenous tyrosinase expression leads to deletion of Tyr369-specific T cells.

**Tyrosinase-specific T cells are not deleted during thymic selection**

We examined whether central tolerance shaped the size and function of the tyrosinase-specific T cell repertoire of FH mice. Thymic cellularity was similar in 30- to 35-wk-old FH albino and FH tyrosinase mice at 2.9 × 107 ± 1.6 × 107 and 2.0 × 107 ± 1.4 × 107, respectively. Additionally, the percentages of CD4+CD8+ double-positive and CD8 single-positive (SP) thymocytes were comparable in FH albino and tyrosinase mice (Fig. 2, A and B). No differences were observed in either the frequency of FH TCR-expressing CD8SP thymocytes, or their level of FH TCR expression (Fig. 2, C and D). Finally, there was no difference in the CD8SP, HSA+ populations representing mature, naive thymocytes that will be soon entering the periphery (data not shown). Thus, despite previous demonstration of tyrosinase transcripts in murine mTECs (18), endogenous tyrosinase expression does not lead to central deletion of Tyr369-specific FH T cells.

To generalize our results to the broader tyrosinase-specific CD8 T cell repertoire, and to ascertain whether mechanisms other than deletion were induced intrathymically, we assessed Tyr369-specific functional responses in thymic chimeras. Thymi from tyrosinase mice were transplanted into thymectomized and T cell-depleted albino recipients. The peripheral T cell compartment was allowed to reconstitute for 6 wk and animals were then immunized with

![FIGURE 1. Both spleen and pLN lymphocytes are enriched for Tyr369-specific lymphocytes in FH albinos relative to FH tyrosinase+ mice. Splenocytes from FH albino (A and B) and tyrosinase+ (C) mice were stained with Abs against CD4, CD8, Vß8, and Vß11 and analyzed by flow cytometry. A and C, Representative plots gated on live cells (top) or CD8 cells (bottom). Numbers indicate percentage of total lymphocytes. B, Tyr369 tetramer staining on gated CD8 lymphocytes from pLN of FH albino (solid) and non-TCR-transgenic animals (line). D, Combined data for percent of Vß8/Vß11 cells among splenocytes (□□), and pLN (□□) lymphocytes of FH albino (□□) and tyrosinase mice. Each point represents an individual animal. Mean values of FH tyrosinase+ and FH albino spleen and pLN are significantly different (unpaired, two-tailed t test; p = 0.04 and p = 0.03, respectively).](http://www.jimmunol.org/content/jimmunol/199/6/959/F1.large.jpg)
murine tyrosinase-expressing vaccinia virus (mTyrVac). Three weeks later, splenocytes were harvested, restimulated with Tyr369 peptide-pulsed splenocytes in vitro, and Tyr369-specific CTL responses were assessed. When tyrosinase gene expression was limited to thymic tissue in this manner, Tyr369-specific responses in these chimeras were comparable to those in albino mice (compare Fig. 3, A and B) and substantially higher than in tyrosinase+ animals (compare Fig. 3, A and D). Primary allogeneic responses were comparable in all three types of mice. We also produced reciprocal thymic transplants, in which tyrosinase+ mice received thymi from albino donors. Tyr369-specific T cell responses in these mice were comparable to those of nonthymectomized tyrosinase+ controls (compare Fig. 3, C and D), and substantially lower than those of nonthymectomized albino controls (compare Fig. 3, C and B). Collectively, these results establish that the presence or absence of an expressible tyrosinase gene in the thymus does not influence the representation or functional activity of Tyr369-specific T cells and demonstrate that tolerance to this epitope is the result of peripheral, not central, mechanisms.

Peripheral presentation of Tyr369 induces deletion of tyrosinase-specific T cells

The minimal Tyr369-specific responses in immunized tyrosinase+ mice observed in Fig. 3 and in previous work (33) suggested that self-tolerance to tyrosinase was due to anergy or deletion resulting from Ag encounter in the periphery. To examine this, we tracked CFSE-labeled naive FH cells from albino donors after adoptive transfer into AAD+ tyrosinase+ or AAD+ albino recipients. These recipients were also Thy1.1+ to facilitate identification of transferred Thy1.2+ FH cells that may have diluted CFSE or downregulated their TCRs. Additionally, an equal number of CFSE-labeled CD8 T cells from a non-TCR-transgenic AAD+ Thy1.1+ tyrosinase+ (ATT) donor were cotransferred to allow direct determination of the proportion of FH donor cells that divided and/or survived in the tyrosinase+ host. Three days posttransfer, cells from pLNs were stained for CD8, Thy1.2, and tetramer, and assessed for CFSE dilution. No dilution was observed in FH T cells reisolated from naive albino mice, but those isolated from albino mice infected with mTyrVac had undergone extensive cell division (Fig. 4A). Using the cotransferred ATT as an internal control and comparing the FH:ATT ratio in naive and mTyrVac-immunized mice, we observed that FH cells increased by a factor of 5 within 60 h after infection with 10^6 PFU of virus (Fig. 4A), demonstrating an appropriate FH cell response to cognate Ag in an immunogenic context. FH cells reisolated from tyrosinase+ mice also had undergone several rounds of division, indicating that they had encountered Tyr369 presented by endogenous cells. However, there was little or no increase in the numbers with each successive division (Fig. 4A), suggesting that activated FH cells did not survive. In comparison to cells activated by tyrosinase-expressing vaccinia, T cells encountering endogenous tyrosinase-derived Ag 60 h posttransfer were also significantly more positive for annexin V, a marker of apoptosis (Fig. 4B). By 2 wk posttransfer, FH cells in tyrosinase+ animals were essentially undetectable in any lymphoid compartment examined, while those transferred into albino hosts remained quiescent and undiminished in the periphery (Fig. 4C).

Together, these data show that the Tyr369 epitope is constitutively presented to circulating naive FH T cells and that this induces abortive activation leading to apoptotic death and deletion of tyrosinase-specific lymphocytes.

Presentation of Tyr369 occurs in pLN and mesLN, but not in spleen

The above results were obtained from analyses of pLN (pooled axillary, brachial, inguinal) that are well-established to drain the skin. To establish the association between skin drainage and presentation of tyrosinase-derived Ag to T cells, we examined spleen
and mesLN on day 3 posttransfer of FH cells. Neither of these lymphoid compartments is thought to directly drain the skin. Unexpectedly, we noted divided FH T cells in both of these compartments (Fig. 5A). To determine whether FH cells were primed in these sites, we examined CD69 expression, which is transiently up-regulated within hours after both tolerogenic and immunogenic Ag encounter (7). Tyr369-specific lymphocytes harvested from both skin-draining (axillary/brachial/inguinal) and mesLN expressed CD69 18–24 h after transfer into tyrosinase/H11001 hosts, suggesting presentation of the epitope occurs within these compartments (Fig. 5B). Importantly, activation in the spleen was generally nonexistent. These results demonstrate that presentation of tyrosinase is associated with both skin-draining and non-skin-draining LN. The absence of T cell activation in the spleen suggests that tyrosinase presentation occurs in the periphery but is not due to free circulation of Ag.

To definitively rule out a role for presentation within the tissues, rather than within the LN, we pretreated mice with anti-CD62L to block entry into LN of naive FH cells. CFSE-labeled FH cells were adoptively transferred into these mice, blockade was maintained for an additional 48 h, and pLN, mesLN, and spleen were then assessed for the presence of dividing FH cells. This anti-CD62L blockade efficiently prevented T cell entry into LN (Fig. 6, left). However, the displaced cells evident in the spleen remained unactivated (Fig. 6, right). This result establishes that endogenous presentation of the Tyr369 epitope leading to self-tolerance occurs only within LN.

Radio-resistant cells mediate presentation of Tyr369

To gain insight into the nature of the cell responsible for steady-state presentation of endogenous tyrosinase to FH CD8 T cells, B6 (AADneg tyrosinase/H11001) mice were irradiated and reconstituted with AAD/H11001 BM to generate AAD/H11001 BM3AADneg tyrosinase/H11001 chimeras. The reciprocal AADnegBM3AAD/H11001 tyrosinase/H11001 chimeras were also produced. After allowing 3–6 mo for reconstitution, FH cells were adoptively transferred into chimeric mice and skin-draining LN were harvested 24 h or 3 days later. Strikingly, based on the absence of CD69 up-regulation or CFSE dilution among...
transferred cells, reconstitution of the LN compartments of AAD<sup>neg</sup> tyrosinase<sup>+</sup> mice with AAD<sup>+</sup> BM-derived cells did not rescue presentation of tyrosinase within the skin-draining LN (Fig. 7, A and B, top). In contrast, activation of FH cells transferred into the reciprocal AAD<sup>neg</sup>BM→AAD<sup>+</sup> tyrosinase<sup>+</sup> control mice (Fig. 7, A and B, bottom). FH cells in these latter chimeras also showed increased apoptosis with further divisions as compared with tyrosinase-vaccinia immunized controls (Fig. 7C) and were undetectable by 2 wk posttransfer (Fig. 7D). Together, this demonstrates that a radio-resistant cell is responsible for steady-state presentation of Tyr<sub>369</sub> within pLN. Similar results were observed in the mesLN (data not shown). Thus, presentation by this cell is necessary and sufficient to induce tolerogenic deletion of self-specific CD8 T cells. Importantly, absence of presentation in the AAD<sup>+</sup>BM→AAD<sup>neg</sup> chimeras also establishes that the DC subsets generally associated with deletional tolerance of peripheral T cells (12, 38, 39) are not involved in tolerance to tyrosinase.

**LCs are not the radio-resistant population that presents Tyr<sub>369</sub>**

The results in BM chimeras demonstrated that tyrosinase is presented by a radio-resistant cell. LCs are a radio-resistant DC in the epidermis and have been shown to carry melanin granules to draining LN in the steady state (40). To test the hypothesis that LCs are responsible for the presentation of Tyr<sub>369</sub> from endogenous tyrosinase in LN, we used mice expressing the human DT receptor (DTR) under control of the Langerin promoter. In these mice, LCs are rapidly and selectively depleted from the epidermis within 24 h after administration of DT and remain absent for >2 wk (35). Thus, AAD<sup>−</sup>tyrosinase<sup>−</sup>Langerin DTR<sup>+</sup> mice were produced and treated with DT on a biweekly basis to maintain long-term depletion of epidermally derived LCs in skin and pLN (Fig. 8, A–C). Following ablation of LC for 3–4 wk, FH T cells were transferred into host animals and presentation of tyrosinase was determined by assessing up-regulation of CD69 on transferred FH cells in the LN. Ablation of LC had no effect on the extent of CD69 up-regulation among the Tyr<sub>369</sub>-specific cells (Fig. 8, D and E). Presentation in the mesLN was likewise unaffected by LC ablation (data not shown). These results demonstrate that LC are not required for steady-state presentation of tyrosinase in either pLN or mesLN.

**Steady-state presentation of Tyr<sub>369</sub> is linked to radio-resistant expression of tyrosinase in different lymphoid compartments**

Both pLN and mesLN of AAD<sup>neg</sup>→AAD<sup>+</sup> chimeras were examined for presence of radioresistant AAD<sup>+</sup> hemopoietic cells. Although LC were identified in the pLN of the AAD<sup>neg</sup>BM→AAD<sup>−</sup> chimeras, we were unable to detect an alternative/analogous CD11b<sup>+</sup> or CD11c<sup>+</sup> population that remained AAD<sup>+</sup> in the mesLN (data not shown). In the absence of any tyrosinase-presenting radio-resistant DC or macrophage within the LN, we entertained the hypothesis that peripheral deletion was mediated by LN-resident cells that expressed the tyrosinase gene. Using RT-PCR, we reproducibly amplified mRNA specific for tyrosinase from both the pLN and mesLN of tyrosinase<sup>−</sup>, but not albino, animals (Fig. 9A). Tyrosinase mRNA expression was positively correlated with presentation of tyrosinase in these LN compartments and was not detected in the spleen. In addition, we generated...
chimeras in which irradiated tyrosinase+ mice were reconstituted with BM from albino animals. Animals were sacrificed 4 wk postirradiation and analyzed for expression of tyrosinase mRNA in presenting (mesLN) and nonpresenting (spleen) lymphoid compartments. mesLN were evaluated to eliminate possible contamination of pLN with Tyr+ cells from skin perturbed by recent irradiation. Tyrosinase expression was maintained in the mesLN, but remained undetectable in the spleen, of albino

FIGURE 7. Presentation in tyrosinase+ mice is mediated by a radio-resistant APC. CFSE-labeled naive FH CD8-enriched cells were transferred into tyrosinase+ AAD3 bm→AAD3+ chimeras, AAD3+bm→AAD+ chimeras, or AAD+ tyrosinase+ and albino controls. pLN cells were reisolated and analyzed by flow cytometry 24 h (A), 3 days (B) and (C), or 2 wk (D) later. A, Left panels, CD69 expression on gated CD8 Tyr369 tetramer+ lymphocytes 24 h after transfer into tyrosinase+ chimeras (filled histograms), or tyrosinase+ controls (solid line), or albino controls (dotted line). Right panel, Aggregate CD69 expression data for CD8 Tyr369 tetramer+ pLN cells 18–24 h after transfer into AAD+ bm→TyraAD+ (n = 9), AAD+bm→AAD+ (n = 6), or AAD+ BM→AAD+ (n = 4) chimeras. B, CFSE dilution in gated Vo88′ Vβ11′ CD8 lymphocytes 3 days after transfer into tyrosinase+ chimeras (filled histogram) or albino controls (solid line). C, Annexin V staining on gated Vo88′ Vβ11′ CD8 lymphocytes harvested 3 days after transfer into AAD+ albino mice ± mTyrVac (1.5 × 104 PFU), or AAD+bm→tyrosinase+ AAD+ chimeras. D, Unlabeled CD8-enriched FH cells and whole LN/spleen cell suspensions from ATT (AAD+ tyrosinase+Thy1.1) mice were transferred at a ratio of 1:10 into the indicated recipient. Numbers on plots show percent of ATT (upper gate) and FH cells (lower gate) among CD8 lymphocytes 2 wk posttransfer.

FIGURE 8. Depletion of LC does not diminish Tyr369 presentation in the LN. Langerin DTR-AAD+ mice were treated biweekly for 3–5 wk with DT before adoptive transfer of FH cells. A, Immunofluorescent microscopy of epithelial sheets isolated from Langerin DTR-AAD+ animals either untreated (top) or DT-treated biweekly (bottom) and stained with anti-Langerin, bar = 200 μM. B, Epithelial sheets from the same animals were homogenized and single-cell suspensions were analyzed by flow cytometry. Plots are gated on CD11c+ LC. C, pLN from the same samples. Langerin expression on CD11c+ DCs in pLN of the mice analyzed in A and B. Number in plot indicates percent of CD11c+ cells with the upper right quadrant of untreated (left) vs DT-treated (right) cells. D, CD69 expression on CD8 Tyr369 tetramer+ FH cells isolated from pLN 24 h after transfer into untreated (top) or DT-treated (bottom) Langerin DTR-AAD+ hosts. E, CD69 expression data pooled from two independent experiments, error bars = SE.
Tyrosinase mRNA is detected in both pLN and mesLN, but not in spleen. A, Tyrosinase and \( \beta \)-actin expression in tissues from albino and tyrosinase\(^+\) animals was determined using 40-cycle RT-PCR. Representative of five independent experiments. B, Left panel, Representative data from AlbBM→Tyr\(^+\) (\( n = 3 \)), Tyr\(^+\)BM→Alb (\( n = 4 \)), and control chimeras evaluated for tyrosinase expression (top band) with \( \beta \)-actin as internal control. Right panel, Summary of BM chimera mRNA expression data. C, Relative tyrosinase levels in different lymphoid compartments were quantified using real-time PCR as described in Materials and Methods. All samples were run in duplicate. For tyrosinase\(^+\) samples, \( n = 2 \) average shown on graph, albino control (\( n = 1 \)).

BM→tyrosinase\(^+\) chimeras (Fig. 9B). We also produced the reciprocal tyrosinase\(^+\)BM→albino chimeras, and found that the mesLN compartments of these mice remained negative for tyrosinase expression (Fig. 9B). These results establish a link between radio-resistant expression of tyrosinase mRNA in peripheral lymphoid compartments and radio-resistant Ag presentation leading to deletion of Tyr\(_{369}\)–specific T cells.

The above results were somewhat paradoxical, in that we had failed to see a tolerogenic influence of tyrosinase expression in the thymus on these same T cells. To understand this, we compared tyrosinase mRNA levels in both compartments. Under the amplification conditions used to visualize tyrosinase expression in mesLN in Fig. 9B, we found that the signals from several thymi of tyrosinase\(^-\) mice were weak or undetectable (data not shown). Further comparison of the tissue mRNAs using real-time PCR showed that, while tyrosinase mRNA was more abundant in thymus than spleen, the level in the thymus was only 0.5% of that in mesLN (Fig. 9C). This suggests that the lack of central deletion of Tyr\(_{369}\)–specific T cells is because the levels of tyrosinase or the numbers of tyrosinase-expressing cells are much lower in thymus than in LN.

We attempted to directly identify the tyrosinase-expressing cell within the LN. However, we were unable to detect ex vivo presentation of endogenous tyrosinase by coculture of tyrosinase-specific T cells/clones with LN suspensions from tyrosinase\(^+\) animals (data not shown). Additionally, using immunofluorescent analysis, we were unable to detect cells expressing tyrosinase above background levels within the LN, although melanocytes present in the ear epidermis fluoresced brightly using the same Ab combinations (data not shown). This indicates that the cells expressing tyrosinase are either very rare or express very low levels of the protein. Nonetheless, the correlation of tyrosinase mRNA with the sites of Ag presentation suggests that direct presentation within the LN by a tyrosinase-expressing cell is responsible for deletional tolerance to this Ag.

**Discussion**

In this study, we have addressed the mechanisms of self-tolerance to an endogenously expressed melanocyte Ag that is a relevant target in antitumor immunity. Using a newly generated TCR-transgenic mouse in conjunction with both tyrosinase\(^+\) and albino animals, we tracked the fate of CD8 T cells specific for Tyr\(_{369}\), an MHC class I-restricted epitope derived from tyrosinase. We found no evidence for a thymic role in development of tolerance to this melanocyte Ag. Instead, Tyr\(_{369}\) is constitutively presented in the periphery to naive CD8 T cells. For the Tyr\(_{369}\)–specific FH T cells used in this study, which use a high-affinity TCR isolated from albino mice, this encounter leads to activation and rapid deletion. In contrast to pancreatic Ags that are cross-presented in the periphery by radio-sensitive DCs, deletional tolerance to Tyr\(_{369}\) is based on presentation by a radio-resistant cell. Strikingly, despite its occurrence within the LN, this presentation is not mediated by the dominant epidermal DC population, the Langerhans cells. Instead, the radio-resistant expression of tyrosinase mRNA in LN where deletion occurs suggests that tolerance to tyrosinase is dependent on direct presentation by LN-resident cells.

The absence of any effect on the selection or function of Tyr\(_{369}\)–specific T cells in the thymus demonstrates that the previously reported expression of tyrosinase transcripts in mTECs (18, 29) does not lead to self-tolerance to this Ag. This contrasts with the intrathymic deletion of CD8 T cells mediated by OVA expressed in mTEC under the control of an insulin promoter (19, 20, 22). The reasons for this difference are not clear. The AAD molecule is expressed in transgenic mice at similar levels to endogenous murine class I MHC molecules and efficiently mediates both positive and negative selection (34). OT-1 has an extremely high affinity for its Ag, which may enhance its susceptibility to deletion during thymic development. Ectopic expression of OVA may also lead to substantially higher levels of mRNA expression in mTECs than that of genes expressed in their native context. In this regard, it is of interest to note that tyrosinase mRNA expression in bulk thyrocytes was much lower than that of mesLN. In keeping with this, the previous report of tyrosinase mRNA expression in thymus required isolation and enrichment of the rare mTEC cells (18). Thus, it is likely that intrathymic message is at extremely low levels, or in only a very small number of cells. Although it is possible that, as a rule, physiologic expression of proteins within mTECs does not suffice for adequate presentation to CD8 T cells, it is likely that as with CD4\(^+\) T cell central tolerance (18, 28), the relevance of these thymic transcripts is variable. Nonetheless, it has been proposed that the simple presence of thymic transcripts may indicate a given Ag is a poor candidate relative to other tumor-associated Ags not expressed intrathymically (41). Our findings show such generalizations are premature and that detection of mRNA within thymic epithelia is not alone sufficient criteria to eliminate an Ag as a potential immunotherapeutic target.
Our data establish that activation-induced peripheral deletion, is a principal tolerogenic pathway for high-affinity tyrosinase-specific CD8 T cells. We have previously shown that a subset of low-avidity Tyr_{369}^+/specific T cells persist in tyrosinase^+/ mice (33, 42), and that these are sufficient to control the outgrowth of tyrosinase expressing melanoma cells (43). Thus, the effect of peripheral deletion is to skew the overall repertoire of Tyr_{369}^-reactive cells to lower average avidity, rather than eliminating it altogether. This deletional process is superficially similar to one previously described for CD8 T cells specific for transgenic Ags expressed in the pancreas (8), in that it is confined to LN, and distinct from a previously described process of tolerance that depends on presentation of a keratinocyte Ag to naive T cells within the skin (13). The occurrence of activation-induced deletion after adoptive transfer into mice expressing endogenous tyrosinase also distinguishes it from the autoimmune disease that occurs after adoptive transfer of CD8 T cells recognizing transgenic Ag expressed in skin keratinocytes (14, 15). These results demonstrate that the expression of Ag in different skin cell subsets can lead to distinct immunological outcomes.

One aspect of our results differs significantly from the established deletional peripheral tolerance paradigm. Tolerance to Ags expressed in the pancreas is mediated by radio-sensitive CD11c^-CD8α^+ DCs via cross-presentation (12). However, Tyr_{369}^- is not presented to any significant extent by radio-sensitive BM-derived cells, of which the CD8α^- DCs are a subset. Instead, the cells within LN that are necessary and sufficient to induce deletion of Tyr_{369}^-specific T cells are radio resistant. It should be noted that radio-sensitive APC present viral Ags after infection of skin epidermis (44) and we have shown elsewhere that immunogenic cross-presentation of Tyr_{369}^+ from B16 melanoma tumors in other compartments is mediated by a radio-sensitive APC (45). Thus, radio-sensitive APC can present skin-derived Ags and contain at least one subset that is competent for processing and cross-presentation of tyrosinase. Hence, the absence of steady-state presentation of Tyr_{369}^- from endogenously expressed tyrosinase by radio-sensitive cells indicates that this Ag is not available to them. This further points out that tyrosinase is not freely circulating in lymph to the draining nodes where it may be picked up and presented by resident DCs (46). Instead, its availability is likely to be determined by either the localized distribution of tyrosinase-producing cells or the ability of radio-resistant cells to carry Ag into the LN.

LCs, the prototype skin-resident DC, are known to be radio resistant (47, 48). Because LCs are localized in juxtaposition to melanocytes in the epidermis and hair follicles, contain melanin granules, and constitutively drain to peripheral LN (40, 49), it was expected that they may be responsible for steady-state presentation of tyrosinase leading to tolerance. Surprisingly, long-term selective ablation of LC demonstrated that this was not the case. This result highlights the disparate involvement of LC in a variety of immune responses. LC initiate graft-vs-host disease (47) and “cross-present” an OVA peptide expressed under a keratinocyte promoter leading to autoimmune disease (14). However, LC cross-present peptides derived from whole protein at relatively low levels (50) and do not cross-present viral Ags after epidermal infection (44, 51). Their relative inefficiency may render them incapable of cross-presenting proteins that are expressed at lower levels or are restricted to the less frequent melanocytes. It has also been suggested that LC may simply carry Ag to LN for handover to resident APC for presentation (44). However, because long-term LC ablation did not diminish presentation, and introduction of AAD^+ DCs into AAD^- mice did not rescue presentation, such a mechanism cannot account for the presentation of tyrosinase in LN. It remains to be determined whether, outside of the steady-state, LC are involved in presentation of Ags from necrotic melanocytes, or in acutely inflamed epidermis, and whether this might lead to the development of autoimmune vitiligo.

Recently, a population of radio-resistant DC that reside in the dermis has been identified (52) and it is possible that these cells could be responsible for steady-state presentation of Tyr_{369}^- the skin-draining LN. However, analysis of AAD^-/BM → Langerin_{Pyr}^- AAD^+ chimeras has shown that DT deletion eliminates the entire radio-resistant cohort of CD11c^- AAD^- cells in skin-draining LN (data not shown). This demonstrates that the radio-resistant DC population in these LN is not derived from Langerin^+ dermal DC. Furthermore, dermal DCs would not be expected to traffic into the mesLN and would not account for presentation within this compartment. In total, we observed no evidence for steady-state presentation of tyrosinase by either radio-sensitive DC or radio-resistant LC populations.

Our observation of T cell activation in non-skin-draining mesLN was unexpected and suggested a source of tyrosinase for lymph node presentation other than skin melanocytes. Tyrosinase is also expressed in retinal-pigmented epithelial cells (53), whose lymphoid drainage is controversial but may include the mesLN (54, 55). It is possible that steady-state tolerogenic presentation of tyrosinase from both skin and/or retina may be mediated by an as yet undescribed radio-resistant BM-derived APC present in both LN and absent in spleen. However, the presence of tyrosinase mRNA in both the pLN and the mesLN, and its absence in spleen, correlates completely with the sites of presentation. This correlation was strengthened by the observation that tyrosinase mRNA expression in LN was radio resistant. Thus, we suggest that direct presentation of tyrosinase in LN is responsible for deletional tolerance. We are aware of the seeming contradiction in proposing that tyrosinase expression in LN leads to deletion, while expression in thymus does not. However, as already mentioned above, tyrosinase mRNA is expressed at lower levels in thymus than LN. Our analysis is not correlated with protein, but suggests that a higher level of tyrosinase expression in the LN is sufficient for tolerance induction, whereas the lower levels in the thymus are not.

Unfortunately, histological analysis of LN sections was insufficiently sensitive to identify a tyrosinase-expressing cell. One possibility is melanocytes. Although generally considered nonmotile, there are a small number of reports documenting their presence in low numbers in the LN of melanoma-bearing mice (56) and patients (57). However, melanocytes would be expected to stain brightly for tyrosinase based on their appearance in epidermal sheets and perhaps to appear more frequently in LN draining the tissue in which they are abundant. However, PCR consistently shows a brighter tyrosinase band in mesLN samples. Alternatively, tyrosinase may be expressed by LN stromal/endothelial cells that express tissue-specific proteins promiscuously, analogous to mTECs. Aire protein, which is thought to regulate tissue-specific Ag expression in mTECs, is also expressed by some secondary lymphoid hematopoietic cells. In keeping with this, direct expression of insulin in secondary lymphoid tissue has been observed. However, expression occurred in spleen as well as LN and occurred in DCs and macrophages which would be expected to be radio sensitive (58). Further work will be required to definitively identify the cell expressing tyrosinase in the LN, and to establish its direct role in mediating tolerance to this and other tissue specific Ags, particularly those derived from other MDP-based tumor targets.

In total, our work provides insight to the steady-state tolerance mechanisms at work for an endogenous tumor-associated melanocyte Ag and has demonstrated a role for peripheral, but not central...
deletion. The strong correlation of tyrosinase mRNA with sites of Ty3/Gypsy presentation indicates that this peripheral Ag is produced locally by a LN resident radio-resistant cell. After the original submission of this manuscript, Lee et al. (59) demonstrated that transgenic OVA expressed under the control of an intestine-specific promoter was also expressed by radio-resistant stromal cells in a variety of pLN, and this was associated with activation-induced peripheral deletion of CD8 T cells in these compartments. Although the expression of this OVA transgene may not be entirely reflective of endogenous gene expression, our observations with endogenous tyrosinase expression and theirs are in accord. Thus, we suggest that direct presentation of Ags derived from tissue-specific genes by cells residing in LN is likely to be a phenomenon of general importance for the establishment of CD8 T cell tolerance.

One interesting difference between our study and that of Lee et al. (59) is that, while OVA was also cross-presented by radiosensitive DCs in intestine-draining lymphoid compartments, we failed to find a comparable cross-presentation of tyrosinase in skin draining LN. Our model is one of very few models in which the Ag-encoding gene in nontransgenic, therefore, our finding that no professional APC, i.e., LC/DC, is responsible for presentation of this tumor immunotherapy target challenges assumptions for how tolerance is maintained for different peripheral Ags, and particularly tyrosinase and other MDPs. It is of interest to know whether this tolerogenic cell is inefficient, or can undergo a change in properties that allows the development of autoimmune vitiligo and/or autoimmunity, and use for cancer immunotherapy.

Acknowledgments
We thank J. Gorman with her assistance in the animal facility and S. Lewis for generation of tetramer reagents used. We also thank all members of the Engelhard laboratory, and Drs. M. McDuffie and K. Tung for discussion and critical reading of the manuscript.

Disclosures
The authors have no financial conflict of interest.

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