Thymus-Blood Protein Interactions Are Highly Effective in Negative Selection and Regulatory T Cell Induction

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Using hen egg-white lysozyme, the effect of blood proteins on CD4 thymic cells was examined. A small fraction of i.v. injected hen egg-white lysozyme rapidly entered the thymus into the medulla. There it was captured and presented by dendritic cells (DCs) to thymocytes from two TCR transgenic mice, one directed to a dominant peptide and a second to a poorly displayed peptide, both presented by MHC class II molecules I-A<sup>d</sup>. Presentation by DC led to negative selection and induction of regulatory T cells, independent of epithelial cells. Presentation took place at very low levels, less than 100 peptide-MHC complexes per DC. Such low levels could induce negative selection, but even lower levels could induce regulatory T cells. The anatomy of the thymus-blood barrier, the highly efficient presentation by DC, together with the high sensitivity of thymic T cells to peptide-MHC complexes, results in blood protein Ags having a profound effect on thymic T cells. The Journal of Immunology, 2009, 183: 7909–7918.

PRESENTATION OF SELF-AGS BY THYMIC APCs RESULTS IN NEGATIVE SELECTION AND/OR TREG DIFFERENTIATION

The role of thymic presenting cells in mediating tolerance has been extensively studied, showing both specialized and overlapping functions (8–10). The conditions that dictate whether Ag presentation by specific APC results in negative selection and/or Treg differentiation are not entirely understood. Deciphering the role of APC type in such decisions needs to be examined in the context of blood proteins in which thymic architecture modulates Ag accessibility to various cellular components. Accessibility and the different endocytic and Ag retention capacities of thymic APC subsets (11) could affect the density of peptide-MHC (pMHC) complexes presented, thereby influencing negative selection and Treg development. Both processes require the same costimulatory molecules (12, 13), as well as high avidity interaction between the TCR and pMHC complex (14, 15). A long-standing hypothesis is that Tregs must interact with self-pMHC with an avidity intermediate between positive and negative selection (re-viewed in Ref. 16). It has also been suggested that the APC type dictates whether TCR-pMHC interaction results in negative selection or Treg differentiation. This view is supported by studies in which selective expression of Ag by mTECs (15, 17) or restricted expression of MHC class II molecules or ligands to the cortical epithelium was sufficient for Treg differentiation (18–20). Moreover, in addition to their well-described role in negative selection (21), thymic dendritic cells (DC) have been implicated in Treg development (22–24).

The thymus is permeable to circulating proteins (25–27). Early permeability studies suggested the existence of a blood-thymus barrier, allowing access to low m.w. tracers while mostly excluding high m.w. particles (27–29). The anatomical studies of Raviola and Karnovsky (27), using peroxidase as a tracer, indicated that the venules at the corticomedullary junction were the site of leakage for blood Ags, whereas the capillaries draining the cortex were largely impermeable. A perivascular system in the medulla that effectively traps small blood-borne molecules has been described (30–32). In studies examining the presentation efficiency of different thymic APC, Kyewski et al. showed that circulating protein Ags injected i.v. rapidly entered the thymus and were efficiently presented by thymic rosettes enriched for DCs (28, 29). Entry and presentation were observed over a wide range of molecular weights, albeit with different efficiencies, and was time and dose dependent. Additionally, injection of peptides induced clonal deletion of thymocytes (33, 34) and naturally circulating Ags were shown to induce effective negative selection of TCR transgenic T cells in several experimental models upon presentation by thymic APC (6, 7, 10, 35).

In this study, we examined the response of thymic CD4 T cells from two different TCR transgenic mice to the protein hen egg-white lysozyme (HEL) injected i.v. One TCR transgenic T cell (3A9) recognized a dominant long-lived peptide, whereas a second (LB11.3) recognized a weak and poorly displayed peptide (36, 37). HEL circulated briefly, entered the thymus, and were efficiently presented by thymic rosettes enriched for DCs (28, 29). Entry and presentation were observed over a wide range of molecular weights, albeit with different efficiencies, and was time and dose dependent. Additionally, injection of peptides induced clonal deletion of thymocytes (33, 34) and naturally circulating Ags were shown to induce effective negative selection of TCR transgenic T cells in several experimental models upon presentation by thymic APC (6, 7, 10, 35).

MATERIALS AND METHODS

Mice

Mice were maintained under specific pathogen-free conditions in accordance with institutional animal care guidelines. B10.BR mice were...
purchased from Jackson Laboratory and maintained in our facility. 3A9 TCR transgenic mice against the major HEL48–62 peptide were a gift from Dr. Mark Davis (Stanford University, Stanford, CA) (38).

**Generation of TCR transgenic mice against HEL20–35**

The LB11.3 T cell clone reactive to the 20–35 segment of HEL was isolated from B10.BR mice immunized with HEL. After subcloning the cells, genomic DNA was amplified by PCR and cloned into a shuttle vector (38). Shuttle vectors containing the LB11.3 hybridoma α- and β-chains were linearized and microinjected into oocytes from C57BL/6 mice. Thymi were also purified side by side. Briefly, thymic fragments were subcloned into Sigma Chemical and further purified to remove endotoxins and other impurities. Stirling, pyrogen-free saline (PFS) was used as a diluent and a control in all experiments. Mice were sacrificed 3 days postinjection and thymic cells were examined by flow cytometry to evaluate the effects of HEL on the various T cells. All data were analyzed with GraphPad Prism software (GraphPad Software). Foxp3 induction and negative selection data were analyzed with the nonparametric Mann-Whitney U test. Statistically significant differences are indicated in the figures.

**Flow cytometry**

Flow cytometry data were acquired using a FACSCalibur or LSRII flow-cytometers (BD Biosciences) and analyzed using the FlowJo software (Tree Star). Thymocytes were stained with anti-CD4 (L3T4) and anti-CD8 (Ly-2) mAbs (eBioscience or BD Pharmingen). Transgenic T cells were identified using anti-Vβ2 (B20.1; eBioscience) for LB11.3 T cells or anti-CD25 (B20.1) for 3A9 T cells. To evaluate negative selection and Treg induction, single cell-suspensions of thymocytes were made and stained with anti-CD4 (L3T4), anti-CD8 (Ly-2), anti-Vβ2 (B20.1), or anti-CD12 mAb. Intracellular staining with anti-Foxp3 (FKJ-16s) was performed according to manufacturer’s protocol (eBioscience).

**Thymic uptake of HEL**

HEL (1 mg) was labeled with 1 mCi 125I (Amersham Biosciences) using the chloramine T method. Efficiency of labeling was determined by measuring radioactive fractions in the fractions of a Sephadex G25 separation after tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a γ-counter (PerkinElmer). Six-week-old B10.BR mice were injected i.v. with 45.4 μg 125I-HEL (37.6 × 10^6 cpm). Mice were killed at different time points (1, 8, or 12 h) to harvest thymus and collect blood serum. Radioactivity was determined in thymus and in the trichloracetic acid precipitate from sera.

To localize HEL in the thymus, B10.BR mice were injected with 1 mg biotin-HEL. At 15 min, thymus were fixed on ice for 1 h in 3% formaldehyde. Organs were immersed in 30% sucrose at 4°C overnight, flash frozen on dry ice in Tissue-Tek OCT compound (Sakura Finetek) and sectioned. HEL was detected using a TSA biotin system kit according to the manufacturer’s instructions (PerkinElmer). Alternatively, B10.BR mice were injected with 10 mg purified HEL. Thymus were harvested at 20 min and flash frozen on dry ice. Sections were permeabilized with Triton X-100 and HEL was detected using an anti-HEL mAb (P10.6.66) conjugated to mouse IgG1 Zenon Alexa Fluor 488 (Invitrogen). Images were captured at 20× magnification using an Olympus BX51 microscope (Olympus).

**Isolation and testing of thymic APC**

All sorts were performed using a Dako MoFlo Cell sorter (Dako) or a BD FACSAria cell sorter (BD Biosciences). All Abs used for segregation and phenotyping of thymic APCs are listed in supplemental Table S1.3 DCs were purified from the thymi of 5–8-week-old B10.BR mice injected with HEL and killed 30 min or 36 h post injection. DCs from un.injected mice were also purified side by side. Briefly, thymic fragments were subjected to two rounds of 30-min digestion with 10 μg/ml DNase I (Sigma Aldrich) and 0.14 U/ml Liberase Blendzyme 3 (Roche). T cells were removed by magnetic bead isolation using anti-CD90.2 (Thy1.2) beads (Miltenyi Biotec). Further purification of APCs was performed by cell sorting. In some experiments, DCs were isolated using a Nycodenz gradi-
Density of pMHC modulates negative selection of thymocytes and induction of Tregs

Thymi were harvested from 3A9 and LB11.3 mice 3 days after HEL injection and the percentage and absolute number of CD4SP and Foxp3+/H11001 CD4SP was assessed by flow cytometry. CD4SP and Foxp3+/H11001 CD4SP in 3A9 and LB11.3 thymocytes underwent negative selection, the threshold varying depending on the dose (Fig. 2). 3A9 CD4SP required 5 g HEL for complete negative selection (Fig. 2, A and B), but an effect was found with as little as 1 µg. LB11.3 required 30–100 g HEL for complete negative selection (Fig. 2, A and C); the 10 µg dose was ineffective, but the 30 µg dose had an abrupt and pronounced effect (Fig. 2C). Thus, the threshold for negative selection for LB11.3 CD4SP thymocytes was higher, requiring more Ag to reach a pMHC density sufficient for deletion.

Tregs cells were less sensitive to negative selection compared with conventional CD4SP (Fig. 2, D and E), confirming previous reports (44, 45). Although the frequency of Foxp3+ compared with Foxp3− CD4SP increased with HEL dose (Fig. 2A and supplemental Fig. S4), the greatest increase in their absolute number occurred at doses below that required for complete negative selection (compare Fig. 2B with D and Fig. 2C with E). For 3A9, Foxp3+ CD4SP thymocytes underwent significant depletion with 50 g HEL, although a similar effect was noted in about half the mice at the 5-g dose (Fig. 2B). 3A9 mice injected with 1 g HEL had a mild but statistically significant increase in Foxp3+ CD4SP cells (p = 0.0148; Fig. 2D): four of 11 mice had a 2-fold or more increase compared with the PFS control, three had a 50% increase, and four showed no change. The increase in the number of Foxp3+ T cells in 3A9 mice was largely restricted to CD4SP subset that expressed low levels of the 1G12 clonotype (Fig. 2F). Interestingly, in mice that did not receive HEL, all Foxp3+ T cells were in the clonotype low CD4SP

Table I. Autoradiography of CD11c+ DC isolated from B10.BR thymi

<table>
<thead>
<tr>
<th>Sample</th>
<th>cpm Injected</th>
<th>Amount of Protein Injected (µg)</th>
<th>cpm DC Enriched Fraction</th>
<th>Total No. DC Counted</th>
<th>Total No. Grain Plus DC</th>
<th>Grain Plus DC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>125I-HEL</td>
<td>1.3 × 10⁹</td>
<td>220</td>
<td>1155</td>
<td>1996</td>
<td>726</td>
<td>36.4</td>
</tr>
<tr>
<td>No HEL</td>
<td>0</td>
<td>0</td>
<td>79</td>
<td>895</td>
<td>2</td>
<td>0.2</td>
</tr>
</tbody>
</table>
FIGURE 2. Negative selection and Foxp3 expression in LB11.3 and 3A9 mice injected with HEL. A, Top, Representative CD4 and CD8 flow cytometry profiles of thymocytes from 3A9 and LB11.3 TCR transgenic mice injected with PFS or 5 or 50 μg HEL. Bottom, Corresponding Foxp3 expression gated on CD4SP. B, Total number of thymocytes within CD4SP/DP from 3A9 mice injected with the indicated amounts of HEL. IG12 indicates thymocytes positive for a mAb to the TCR of 3A9. Bars correspond to pooled data from 15 (PFS), 7 (10 ng), 13 (500 ng), 11 (1 μg), 6 (5 μg), 4 (50 μg) and 3 mice (1000 μg). C, Total number of thymocytes within CD4SP/DP subpopulation from LB11.3 mice injected with titrated amounts of HEL. Bars represent pooled data from 17 (PFS), 13 (5 μg), 15 (10 μg), 4 (30 μg), 20 (50 μg) and 4 mice (100 μg). D and E, Each symbol represents an individual mouse. D, Total number of Foxp3⁺CD4SP thymocytes in 3A9 mice shown in B. *, p = 0.0148. E, Total number of Foxp3⁺CD4SP thymocytes in LB11.3 mice shown in C. F, Foxp3 expression within 1G12high and 1G12low CD4SP in 3A9 mice shown in D. G, Foxp3 expression within different Vα2-expressing CD4SP subset in LB11.3 mice shown in E. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. All graphs are based on flow cytometry data gaging on CD4SP cells.
population (Fig. 2F, see PFS). This data suggest that cells that expressed high levels of the 3A9 TCR did not develop into Tregs. Modest deletion of Foxp3\(^{+}\) CD4SP subsets also occurred with 1 \(\mu\)g HEL (\(p = 0.0007\)), indicating that this dose could mark the threshold for negative selection in 3A9 mice.

In comparison, LB11.3 Foxp3\(^{+}\) CD4SP cells were not negatively selected at the 100-\(\mu\)g dose that deleted almost all Foxp3\(^{+}\) CD4SP (compare Fig. 2E with C). LB11.3 mice had to receive as much as 10-fold that amount (1000 \(\mu\)g) for effective deletion of Foxp3\(^{+}\) CD4SP (data not shown). Foxp3\(^{+}\) CD4SP induction occurred over a wide range of doses, in marked contrast to 3A9 (Fig. 2E). The increase in Foxp3\(^{+}\) CD4SP cells happened in the absence of overt negative selection (at the 5–10 \(\mu\)g dose) or with significant deletion of Foxp3\(^{+}\) CD4SP (at the 30–50 \(\mu\)g dose; compare Fig. 2C with E). The response peaked in mice that received 30 and 50 \(\mu\)g HEL, with the increase being more than 2-fold over the PFS control (Fig 2E). The change in the number of Foxp3\(^{+}\) T cells was more robust in V\(\alpha\)2\(^{\text{hi}}\)CD4SP cells, less so in cells that expressed intermediate V\(\alpha\)2 levels and there was no significant effect on the V\(\alpha\)2-negative population (Fig. 2G). Notably, at the doses where Foxp3\(^{+}\) CD4SP cells were induced in LB11.3, both Foxp3\(^{+}\) and Foxp3\(^{+}\) CD4SP in 3A9 mice were negatively selected (compare Fig. 2E with B and D). In LB11.3 and 3A9 mice, Foxp3\(^{+}\) T cells were enriched specifically within the CD4SP set, suggesting that these cells arose during the later stages of T cell development (supplemental Fig. S4). The enrichment in Foxp3\(^{+}\) CD4SP cells was HEL specific. Wild-type B10.BR mice injected with the same amounts of HEL had no changes in total thymic cellularity or in the numbers of Foxp3\(^{+}\) CD4SP (supplemental Fig. S5).

In sum, pMHC density determined the outcome of TCR/pMHC interaction whether that outcome was negative selection or development of Treg precursors. The induction of Foxp3\(^{+}\) CD4SP T cells required and was initiated by TCR interaction with a specific pMHC complex presented at a particular density, irrespective of the final Ag-specificity of the Foxp3\(^{+}\) CD4SP T cells. Thus, although TCR/pMHC interaction may influence the decision of a T cell to become negatively selected or continue to develop as a Treg, it does not necessarily determine whether the T cells that emerge retain the same Ag specificity. Other mechanisms such as receptor editing may influence the final specificity.

The efficiency of Foxp3\(^{+}\) CD4SP T cell induction in LB11.3 and 3A9 mice is dependent on \(\alpha\)-rearrangement

To assess the effects of TCR\(\alpha\)-chain gene rearrangement in Foxp3\(^{+}\) CD4SP induction and/or negative selection, mice crossed to TCR\(\alpha\)-deficient strain were injected with HEL and analyzed. 3A9\(\alpha^{-/-}\) mice were more sensitive to negative selection with a little more than half the CD4SP deleted at the 0.5 \(\mu\)g HEL, a dose that did not delete 3A9 T cells (Fig. 3A). LB11.3\(\alpha^{-/-}\) CD4SP were also more sensitive, with approximately half of the cells deleted at the 10 \(\mu\)g dose, which had no effect on its LB11.3 counterpart (Fig. 3B). These results suggest that at the population level, the loss of the \(\alpha\)-chain rearrangement likely increased the avidity of the TCR/pMHC interaction, decreasing the threshold of pMHC sufficiently for negative selection.

Perturbation of endogenous TCR\(\alpha\) rearrangement did not preclude Foxp3\(^{+}\) T cell development in 3A9\(\alpha^{-/-}\) and LB11.3\(\alpha^{-/-}\), as there were still Foxp3\(^{+}\) CD4SP cells that developed (see PFS controls), though in lesser numbers than in the wild-type mice (Fig. 3, C and D). In 3A9 mice, there was a modest increase in the number of Foxp3\(^{+}\) CD4SP (Fig. 3C), but with much variation among the mice: four of six mice increased Foxp3\(^{+}\) CD4SP at the 0.5 \(\mu\)g dose (\(p = 0.0434\)) and six of 12 mice at the 1 \(\mu\)g amount (\(p < 0.02\)). At a higher dose of HEL (5 \(\mu\)g), there was negative selection of Foxp3\(^{+}\) CD4SP instead of induction. The LB11.3\(\alpha^{-/-}\) mice showed no increase in the number of Foxp3\(^{+}\) CD4SP cells with the 10-\(\mu\)g and 50-\(\mu\)g amounts that previously induced them in wild-type LB11.3 (Fig. 3D). Decreasing the dose of HEL below 10 \(\mu\)g (0.5–5 \(\mu\)g) showed a small level of induction, albeit with great mouse-to-mouse variability; a little less than half of the mice had Foxp3\(^{+}\) CD4SP T cell numbers slightly above the control. This effect was most striking at the 5-\(\mu\)g dose where half the mice (eight of 16) showed induction (\(>100,000\) Foxp3\(^{+}\) CD4SP) and half had Foxp3\(^{+}\) CD4SP T cell numbers similar to the control. This data indicates that TCR\(\alpha\) gene rearrangement is not absolutely required for the generation of HEL-dependent Tregs, although there is a noticeable effect on the efficiency of the process.

The enrichment of Foxp3\(^{+}\) CD4SP cells could be due to proliferation rather than differentiation. Coinjection of HEL and BrdU into LB11.3 showed an increase in the number of Foxp3\(^{+}\) CD4SP primarily within the BrdU-negative population (supplemental Fig. S4E). Thus, the induction of the Treg population observed was likely not a result of expansion, but rather, could be due to other mechanisms such as differentiation of Treg precursors.

**Injected HEL is presented by thymic DC and not TECs**

APC subsets were isolated from the thymi of B10.BR mice at 30 min and 36 h postinjection of 50 \(\mu\)g HEL, a dose effective at inducing negative selection in 3A9 and LB11.3 mice and Tregs in LB11.3. Robust presentation to 3A9 T cell hybridoma occurred when they were cultured with different numbers of CD45\(^{+}\) CD11c\(^{\text{hi}}\) DCs isolated from mice injected with HEL but not from uninjected mice (Fig. 4A). DCs presented HEL when isolated as early as 30 min postinjection, but presentation was significantly lower by 36 h (Fig. 4A). In contrast, there was minimal presentation to 3A9 T-hybridoma at the 30 min time point by CD45\(^{+}\) EpCAM\(^{+}\) cells (where EpCAM is epithelial cell adhesion molecule), which comprise cTECs and mTECs (Fig. 4B). By 36 h, presentation by CD45\(^{+}\) EpCAM\(^{+}\) cells from mice injected with HEL was no different from the control, untreated mice (Fig. 4B). The same results were obtained with primary CD4 T cells isolated from 3A9\(\alpha^{-/-}\) and LB11.3 spleens (Fig. 4, C and D). Only the fractions enriched for CD11c\(^{+}\) DCs induced proliferation of 3A9\(\alpha^{-/-}\) T cells (Fig. 4C) and LB11.3 T cells (Fig. 4D). Further, increasing the Ag dose did not change the weak presentation of HEL by TECs (Fig. 4, E and F); sorted CD45\(^{+}\) EpCAM\(^{+}\) BP-1\(^{+}\) cells from mice injected with 1 mg HEL still presented poorly compared with CD11c\(^{+}\) DCs from the same mice (Fig. 4, E and F). Although there was a small level of 3A9 T cell proliferation induced with sorted mTECs (Fig. 4F), no proliferation was observed when 3A9 T cells were similarly cultured with CD45\(^{+}\) EpCAM\(^{+}\) BP-1\(^{+}\) cTECs (data not shown). CD45\(^{+}\) cells were not intrinsically deficient at presentation as they could still present HEL to 3A9 and LB11.3 T cells when pulsed with soluble HEL ex vivo (data not shown). Given the low level presentation of soluble HEL by mTECs early after injection, it is possible that in vivo these cells are able to capture and present more circulating HEL than suggested by our ex vivo assays. However, our results suggest that presentation by TECs will occur with a significantly much lower efficiency when compared with DCs.

Thus, the CD11c\(^{+}\) cells were the primary early capture cell for HEL, bearing most of the available pMHC complexes, and thymic epithelial cells did not contribute much, if at all, to the presentation of blood-borne HEL. This observation is compatible with the tissue immunofluorescent staining, showing a close apposition of DCs to medullary vessel areas that stained for HEL (Fig. 1, C–G). The fast kinetics of presentation agree with a previous report showing that DCs
in thymic rosettes present injected myoglobin as early as 15 min postinjection (28). The finding that presentation of the 48 – 62 epitope, which has a half-time of dissociation from I-A\(^{k}\) of more than 96 h, wanes over time, indicates turnover of thymic DCs. HEL was cleared from the blood minutes after injection and supplies the thymic DC system briefly (Fig. 1). Additionally, the fast kinetics of presentation rules out any contribution from a circulating APC internalizing HEL in the periphery and trafficking into the thymus (24, 46, 47).

Thymic DC population include both conventional (cDC) and plasmacytoid (pDC) DCs (24, 48 –50). Although thymic pDCs have been shown to migrate in from the periphery, thymic cDC are comprised of two populations, CD8\(^{+/−}\)Sirp\(^{+/−}\) cDC that stem from the periphery and CD8\(^{+/−}\)Sirp\(^{+/−}\) cDC that originate in the thymus (47, 49). Additionally, we have observed a fourth CD11c subset that is CD11c\(^{int}\)CD45RA\(^{low}\), which exhibits intermediate levels of MHC class II and costimulatory molecules compared with cDC and pDC populations, suggesting an immature DC phenotype (data not shown).

To identify further the Ag capturing DC, we sorted thymic DC from mice 30 min after injection of HEL into four sets: CD11c\(^{int}\)CD8\(^+/−\)Sirp\(^+/−\) (intrathymic cDC), CD11c\(^{int}\)CD8\(^−/−\)Sirp\(^+/−\) (extra-thymic cDC), CD11c\(^{int}\)CD45RA\(^{high}\) (pDC), and CD11c\(^{int}\)CD45RA\(^{low}\) (immature DC). Presentation to 3A9 T cell hybridoma or primary 3A9 T cells was assessed. Both cDC subsets captured and presented blood-borne HEL very efficiently, as evidenced by high IL-2 production by 3A9 hybridoma and proliferation of primary 3A9 T cells (Fig. 5, A and B). pDC were significantly less efficient at capturing and presenting to the T cells, but were still superior when compared with the immature DC subset and TEC populations (Figs. 4, C and D, and 5, A and B). Among the intrathymic cDC, CD8\(^+/−\) subsets were more efficient than CD8\(^−/−\) subsets at capturing and presenting HEL, both at 30 min and 36 h after injection of HEL (Figs. 5, C and D). We conclude from these experiments that cDC in the thymus (both Sirp\(^+/−\) and Sirp\(^−/−\)) are the main APC that take up and primarily present blood-borne HEL.

To assay how many DCs took up blood-borne HEL, thymic DC were isolated 1 h after injection of 125I-HEL into B10.BR mice. After the final step of enrichment, only a fraction of the initial radioactivity was recovered (1155 cpm), indicating that only a small amount of the input HEL was cell associated (Table I). Radioactive grains contained in each DC were enumerated by microscopy. Of the DCs counted, 36.4% were positive for 125I HEL grains compared with 0.2% in slides from uninjected mice (Table I). These results point to the efficacy of DC at taking up blood-borne Ags and indicate that HEL becomes concentrated in a few DCs that

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**FIGURE 3.** Role of TCR\(^{α}\)-chain gene rearrangement in Foxp3 \(^{−}\)CD4SP Treg induction in mice injected with HEL. A, Total number of CD4SP and DP in 3A9\(^{−}\) injected with HEL. Data are pooled from two independent experiments with individual bars corresponding to 8 (PFS), 10 (0.5 \(μ\)g), 12 (1 \(μ\)g), and 4 mice (5 \(μ\)g). B, Total number of CD4SP and DP from LB11.3\(^{−}\) mice injected with HEL. Data are pooled from five independent experiments with individual bars corresponding to 17 (PFS), 9 (0.5 \(μ\)g), 9 (1 \(μ\)g), 10 (2.5 \(μ\)g), 17 (5 \(μ\)g), 15 (10 \(μ\)g), and 11 mice (50 \(μ\)g). C and D, Each symbol represents an individual mouse. C, Total number of Foxp3 \(^{−}\)CD4SP cells from 3A9\(^{−}\) mice shown in A. D, Total number of Foxp3 \(^{−}\)CD4SP cells from LB11.3\(^{−}\) shown in B. All graphs are derived from flow cytometry data gating on CD4SP. *, \(p < 0.05\); **, \(p < 0.01\).
are able to effectively mediate negative selection and Treg induction.

Discussion
This study reports on three findings. First, it confirms that the thymus is highly effective at taking up and presenting blood proteins as a result of its DC network surrounding a restricted anatomical area. Second, it presents data that the thymus-blood interaction influences negative selection and Treg development as a function of the level of protein in the blood, resulting in variable pMHC density. Third, it indicates that the pMHC density required for negative selection and Treg development is limiting and different for each function, albeit overlapping.

In this study, we confirm that entry into the thymus by circulating proteins and their presentation by thymic APCs can result in tolerance. Several features were evident. First, the permeability of blood proteins was largely dependent on molecular size and on the anatomical region, the thymic medulla. The blood-thymic barrier restricted access of large m.w. proteins, as others have examined, favoring entry of proteins below the m.w. of albumin (supplemental Fig. S3 and Refs. 27, 29). In our experimental system, thymic DCs, and not TECs, were particularly effective at capturing and presenting blood-derived HEL, agreeing with the early findings of Kyewski et al. (28, 29). HEL was largely presented by both Sirpα-H9251- positive and negative cDC subsets found in the medulla. Together, the results demonstrate the effectiveness of presentation of blood proteins. The presentation of blood constituents may complement the function of the AIRE transcriptional regulator in presentation of tissue-specific Ags. Indeed, some tissue-derived proteins and peptides are found in the circulation, although the extent of this representation still needs examination. This speculation is supported by the findings that AIRE deficiency shows restricted rather
than broad patterns of autoimmunity, as would be expected if it was the sole process for control of autoimmunity. Recent studies have also speculated that mechanisms outside the thymus may maintain tolerance to tissue Ags (51, 52, discussed in Ref. 53).

In examining the dose responses of the 3A9 and LB11.3 T cells (Fig. 2), a consistent quantitative difference was observed between them in the HEL levels required for negative selection and Treg generation (Tables II and III). There are notable differences between the two HEL pMHC complexes. The 48–62-Ak complex is distinguished by high binding to I-Ak molecules and a long persistence. In contrast, the 20–35-Ak complex shows a low degree of binding and a fast dissociation rate. Estimates on the presentation efficacy of these two peptides can be made by combining data from the injection of titrating amounts of HEL, previous studies on uptake and handling of HEL (54), and data showing that the amount of HEL that entered the thymus was 0.25% of input (Fig. 1 and Table II). Although the efficiency of uptake and processing of HEL by thymic APCs is unknown, previous data suggest this will be relatively low (54). Based on the results of that study, it was found that 48–62-Ak complexes represented 10% of the total class II molecules on a given APC, reflecting that only 1 per 1000 molecules of the internalized HEL ends as a pMHC complex for HEL48–62. Similar examination of presentation efficiency using FLT3 ligand-generated splenic DCs showed that HEL 48–62-Ak complexes represent a little less than 3% of the total I-Ak complexes (our unpublished data). Thus, assuming 10% uptake of HEL, we estimate a total of $10^7$ pMHC complexes distributed

Table II. Identifying the pMHC threshold for negative selection and regulatory T-cell generation

<table>
<thead>
<tr>
<th>Amount injected</th>
<th>1 μg</th>
<th>1 μg</th>
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<tbody>
<tr>
<td>Percentage entering thymus</td>
<td>0.25%</td>
<td>0.25%</td>
</tr>
<tr>
<td>pmol HEL in thymus</td>
<td>0.17 pmol</td>
<td>0.17 pmol</td>
</tr>
<tr>
<td>Molecules HEL in thymus</td>
<td>$10^{11}$ molecules</td>
<td>$10^{11}$ molecules</td>
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<tr>
<td>Assume 10% uptake</td>
<td>$10^{10}$ molecules</td>
<td>$10^{10}$ molecules</td>
</tr>
<tr>
<td>Efficiency of processing</td>
<td>0.1% of uptake</td>
<td>0.1% of uptake</td>
</tr>
<tr>
<td>Number of pMHC</td>
<td>$10^7$</td>
<td>$10^7$</td>
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</tbody>
</table>

Estimates for the number of pMHC complexes that mediate negative selection vs Treg generation in 3A9 and LB11.3 mice.

A comparison is shown of the HEL doses that marked the thresholds for negative selection and Treg induction in 3A9 and LB11.3 mice.

Table III. Estimates of pMHC/DC for responses of 3A9 and LB11

<table>
<thead>
<tr>
<th>pMHC/DC</th>
<th>Treg induction</th>
<th>Negative selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount</td>
<td>pMHC/DC</td>
<td>Amount</td>
</tr>
<tr>
<td>3A9</td>
<td>1 μg</td>
<td>25–250</td>
</tr>
<tr>
<td>LB11</td>
<td>5 μg</td>
<td>0.1–1</td>
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</table>

A comparison is shown of the HEL doses that marked the thresholds for negative selection and Treg induction in 3A9 and LB11.3 mice.
among \(\sim 10^2 - 10^6\) thymic DCs (Table II). The number of DCs containing the pMHC has been difficult to obtain, but it is likely that as much as \(\sim 40\%\) bear the complexes (Table I) leading to \(25 - 250\) pMHC of \(48 - 62\) per APC when 1 \(\mu\)g HEL is injected (Tables II and III). These calculations suppose that all thymic APCs have equal access to soluble HEL and present a uniform number of pMHC complexes, which may not be the case, although microscopically there were no discernible differences in the number of grains among the positive cells. A point to note is that our estimate of the fraction of DCs bearing HEL is based on autoradiography analysis of mice injected with high dose (200 \(\mu\)g) HEL. It is more likely that at the small doses used in this study (0.01–100 \(\mu\)g), this value could be significantly lower, suggesting that the pMHC that mediate negative selection and Treg induction are likely in the single digits. The predicted values for pMHC agree with in vitro studies showing that as few as 2–3 pMHC complexes on an APC were sufficient to induce apoptosis of immature DP thymocytes (55, 56). Comparing the two epitopes, for LB11.3 T cells to undergo complete negative selection to the same extent as 3A9, more than a 20-fold higher amount of HEL (1 mg HEL) had to be injected. Making calculations like those in Tables II and III, the range of pMHC complexes that supported negative selection and Treg induction for both 3A9 and LB11.3 T cells must be about the same. Regardless of the degree of error of these estimates, the amounts of pMHC per DC must be very low.

A second feature is that for each T cell there was a very narrow margin between the doses that resulted in the induction of negative selection (of both conventional CD4SP and Tregs) and those that induced Tregs. Although the lowest doses induced Tregs without a change in negative selection, there was also notable overlap in both LB11.3 and 3A9 mice. This overlap may explain the wide range of responses found at critical doses of the injected HEL. Based on the numbers calculated for HEL, it is likely that the expression of most self-peptides falls close to the level required for negative selection or somewhere below it. For some tissue Ags (e.g., those influenced by AIRE), the pMHC density levels on TECs compared with DCs may be more compatible with Treg selection than negative selection. This view is supported by data showing that AIRE induces stochastic and heterogeneous expression of tissue-restricted ectopically expressed Ags (57), such that at the single cell level, the extent to which any one pMHC will be represented on an individual mTEC will vary considerably. Therefore, the sensitivity of both negative selection and Treg induction to pMHC levels ensures that central tolerance will be effective, regardless of the constraints imposed by the levels of Ag expression.

The induction of Tregs had several features. Their generation was not solely a result of selective survival (45), because both a relative and absolute enrichment in the number Foxp3+ CD4SP cells was found even at doses that resulted in increased negative selection. Our results are consistent with a late stage DP→CD4SP differentiation, as Foxp3+ cells accumulated within the CD4SP and not DP when mice were treated with an inductive dose of HEL. Additionally, generation of Tregs in response to HEL occurred independently of TCRα-chain gene rearrangement, although in its absence the induction was inefficient even at low HEL doses. Moreover, the absence of endogenous α rearrangement altered the kinetics of negative selection in both 3A9 and LB11.3, decreasing the threshold of HEL required. Our findings support an avidity model of Treg selection whereby TCR-dependent interaction of Treg precursors with Ag at the “right” window of pMHC density induces the final matuational steps of Treg differentiation, as denoted by induction of Foxp3 expression.

The exquisite efficiency, sensitivity, and stringency of central tolerance took place not only because of the presentation abilities of the DC system, but also because of the low threshold of activation of the T cells during their thymic sojourn. The ability of thymocytes to respond to a few pMHC ensures that autoreactive T cells specific for rare Ags can be effectively removed from the T cell repertoire. Importantly, the required number of pMHC predicted for negative selection and Treg induction was about 10-fold lower than those estimated for mature CD4 response (58–60). As others have discussed, the threshold for responses of T cell changes during their differentiation (61–65). The pMHC threshold for CD4SP negative selection being much lower compared with that required to initiate an effector response in the periphery is the biochemical margin of safety, a process that ensures that T cells that escape negative selection remain dormant in the periphery (55, 66).

Note: While this manuscript was under review, a report by Baba et al. (67) was published. This study reported that thymic Sirpα+ cDC entering the thymus from the periphery are a specialized APC for the induction of central tolerance to blood-borne Ags. We find the authors’ data showing the unique localization of Sirpα+ DC inside perivascular regions of blood vessels in the cortex intriguing and deserving of further examination in other experimental models. We are less convinced about data ascribing unique participation of these cells in tolerance induction to blood-borne Ags. The results examining negative selection in CCR2−/− mice that have decreased numbers of Sirpα+ DC showed a very modest reduction. This could suggest that other cell types, such as Sirpα−/− DC8α+ cDC, participate in the induction of tolerance to blood-borne Ags and that Sirpα+ DC are not required and may in fact have a minor role in this process. This view is supported by our own data showing that both Sirpα+ cDC and Sirpα− cDC presented HEL to a similar extent (Fig. 5, A and B), suggesting that both these cell types can efficiently acquire blood-borne HEL to mediate negative selection. Similarly to Baba et al., our results show that the fast kinetics of uptake of blood-borne Ag by thymic DC make it unlikely that Ag is being brought in by Sirpα+ DC that have captured Ag in the periphery. Therefore, we concur that Ag uptake from the blood-stream is most likely a local event whereby DCs localized in close proximity to the blood-vessels have access to Ag leaking from the blood. It remains to be examined exactly what the contribution of peripheral DC trafficking to the thymus is in mediating tolerance induction to blood-borne Ags at steady state.

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Disclosures

The authors have no financial conflict of interest.

References


Figure S1. Generation and characterization of the LB11.3 TCR transgenic mice

(A) Mice were screened by PCR for the presence of the Vβ4 transgene (upper band). Mouse #586 is a positive founder. #587 is a negative littermate. (B) Representative southern blot of three F2 mice derived from the 586 founder. (+) indicates the integration pattern of mice positive for the LB11.3 transgene. (-) indicates a transgene negative littermate (C) Top panel shows a representative thymic profile of LB11.3 mice. Bottom panel shows Vα2 and Vβ4 expression gated on CD4SP. (D) Proliferative response of LB11.3 lymph node T-cells to different HEL peptides as determined by ³H-thymidine incorporation.
Figure S2. *Ex vivo* response of LB11.3 T-cells

(A) Sensitivity of LB11.3 (▲) and 3A9 (■) lymph node T-cells to full length HEL and (B) cognate peptide as measured by $^3$H-Thymidine incorporation. Graphs are plotted as antigen concentration versus % of maximum as determined by proliferation of cultures that received the highest amount of antigen (10μM). (C) Deletion of DP LB11.3 (□) and 3A9 (■) thymocytes in response to C3.F6 pulsed with titrated amounts of HEL. (D) CD69 activation of CD4SP LB11.3 (□) and 3A9 (■) thymocytes in response to titrated amounts of HEL. Results are plotted as function of % maximum of deletion or activation that was obtained with the highest amount of HEL (30μM).
Proteins of different molecular weights were labeled with $^{125}\text{I}$ and injected intravenously into 6 wk B10.BR mice. As noted in the graphs, the proteins injected were Insulin (Ins, 5.8 kDa), Hen egg white lysozyme (HEL, 14.4 kDa), Ovalbumin (OVA, 45 kDa), Bovine serum albumin (BSA, 67 kDa), Phosphorylase B (PHOS b, 97.4 kDa), IgG1 (150 kDa), Alpha 2 macroglobulin (a2M, 720kDa). Mice were sacrificed at the time-points indicated and protein-bound radioactivity was measured in the thymus and serum. Data is expressed as (A) % input cpm in the thymus for each protein and the ratio of protein found in the thymus compared to the blood at (B) 1h (C) 24 h and (D) 48 h post-injection.
Figure S4. For HEL doses that result in $T_R$ induction, Foxp3$^+$ cells are enriched at the CD4SP stage and do not primarily arise from selective survival or proliferation of pre-existing Foxp3$^+$ cells.

(A) Flow cytometry analysis showing total number of Foxp3$^+$ cells at different doses of HEL gated on total lymphocytes. (**) indicates $p=0.0023$. These data are pooled from two independent experiments with 7 (PFS), 4 (5μg), 6 (10μg) and 7 (50μg) mice in the indicated groups. (B) Total number of CD4SP and DP thymocytes within Foxp3$^+$ subsets in (A) at different doses of HEL. (**) indicates $p=0.0012$. (C) The ratio of Foxp3$^+$CD4SP to Foxp3$^+$CD4SP cells is shown on a log scale at different doses of HEL for 3A9 and (D) LB11.3 mice. (****) indicates $p<0.0001$, (***) indicates $p<0.001$ and (**) $p<0.01$. (E) Total number of cells within Foxp3$^+$BrdU$^-$ subsets (black bars) and Foxp3$^+$BrdU$^+$ subsets (white bars). Each bar contains 2 or 3 mice per group (50μg). For all figures, graphs are made based on flow cytometry analysis gating on either CD4SP cells or total lymphocytes by forward and side scatter (A, E).
Figure S5. Negative selection and $T_R$ induction in Wild type B10.BR mice

(A) Top, CD4 and CD8 flow cytometry profiles of B10.BR thymocytes in response to titrated amounts of HEL, gated on total lymphocytes. Bottom, shows corresponding Foxp3 expression gated CD4SP (B) Total thymic cellularity and (C) total number of Foxp3$^+$CD4SP thymocytes in wild type B10.BR mice. All graphs are based on flow cytometry data gating on CD4SP cells or for in (B) lymphocytes.
Table S1. List of antibodies used for segregating antigen presenting cells from the thymus

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Clone</th>
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<tr>
<td>CD11c</td>
<td>N418</td>
</tr>
<tr>
<td>CD8α</td>
<td>53-6.7</td>
</tr>
<tr>
<td>CD45RA</td>
<td>14.8</td>
</tr>
<tr>
<td>SIRPα</td>
<td>P84</td>
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<td>CD45</td>
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<td>BP-1</td>
<td>6C3</td>
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