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

Danielle F. Atibalentja, Craig A. Byersdorfer, and Emil R. Unanue

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-Ak. 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.

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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 cloning 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. Efficiency of labeling was determined by measurement, single cell suspensions of thymocytes were made and stained with mAb (40) for 3A9 T cells. To evaluate negative selection and Treg induction, sorting. In some experiments, DCs were isolated using a Nycodenz gradient without HEL as previously described (42, 43).

**In vivo induction of negative selection and Tregs**

Six- to 8-week-old mice were injected i.v. with the indicated amount of HEL. HEL was purchased from Sigma Chemical and further purified to remove endotoxins and other impurities. Sterile, pyrogen-free saline (PSF) was used as a diluent and a control in all experiments. Mice were sacrificed 3 days postinjection and thymic cells were examined by light 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. Statistical significant differences are indicated in the figures.

**Flow cytometry**

Flow cytometry data were acquired using a FACSCalibur or LSR II 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-Vα2 (B20.1; eBioscience) for LB11.3 T cells or IgG1 mAb (40) 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-Vα2 (B20.1), or IgG1 mAb. Intracellular staining with anti-Foxp3 (FJK-16s) was performed according to manufacturer’s protocol (eBioscience).

**Thymic uptake of HEL**

HEL (1 ng) was labeled with 1 μCi 125I (Amersham Biosciences) using the chloramine T method. Efficiency of labeling was determined by measuring radioactivity in the fractions of a Sephadex-G25 separation after tricine-SDS polyacrylamide gel electrophoresis. T cells transferred to recipient mice were analyzed by magnetic bead isolation using anti-CD90.2 (Thy1.2) beads (Miltenyi Biotec). The radioactivity of the enriched cells was determined using a gamma counter (PerkinElmer). For autoradiography, DCs were spun onto slides that were coated with NTB liquid emulsion (Eastman Kodak), dried, and exposed at 4°C for variable periods of time. Development was performed with Kodak D19 developer followed by fixation (Eastman Kodak). Cells were counterstained with the Hema3 stain set (Fisher Scientific) and examined microscopically.

**Results**

**Generation and characterization of the LB11.3 TCR transgenic mouse**

Processing of HEL by I-As2–expressing APC results in the presentation of four peptides with different numbers of pMHC complexes. Among these, the chemically dominant 48–62 peptide is represented at a molar ratio 250-fold greater than the minor 20–35 peptide (36). T cells from the 3A9 TCR transgenic mice directed to HEL48–62 are available (38, 40). A second HEL TCR transgenic mouse (LB11.3) was generated using TCR genes from a T cell hybridoma directed to HEL20–35 peptide. LB11.3 mice had a normal thymic profile with efficient selection of T cells that expressed the correct Vα- and Vβ-chains and proliferated specifically to HEL20–35 (supplemental Fig. S1). A about 30-fold difference in the proliferative response of LB11.3 T cells compared with 3A9 T cells was noted when APCs were cultured with HEL, but both responded identically to peptide (supplemental Fig. S2). Using an in vitro thymocyte assay, LB11.3 thymocytes in contrast to 3A9 thymocytes required more HEL to achieve a level of pMHC density sufficient for deletion of double positive (DP) thymocytes and activation of CD4 single positives (SP) (supplemental Fig. S2). Therefore, the differential response to HEL was likely a function of the several hundred fold difference in the presentation of these two epitopes.

**Uptake of HEL by the thymus**

The response of 3A9 and LB11.3 thymocytes was examined after the i.v. injection of graded amounts of HEL. This approach allowed the precise control of HEL levels in the thymus. To determine the kinetics of HEL entry and uptake in the thymus, protein-bound radioactivity in the thymus, spleen, and blood was measured following injection of 125I-HEL. HEL circulated briefly, most of it leaving the circulation in ~30 min (Fig. 1, A and B). By 24 h there was a negligible amount remaining in the thymus and the blood (data not shown). At 1 h, only 0.25% of the injected HEL had entered the thymus such that with injection of 5 μg, 1.25 ng (0.87 pmol) entered, corresponding to 5.2 × 1011 molecules (Table I). HEL staining of the thymus had a vascular-like pattern at the corticomedullary junction and within the medulla itself (Fig. 1, C–G). No HEL was localized to the cortex. Permeability was highly influenced by the size of the circulating protein with proteins below albumin being more permeable (supplemental Fig. S3).

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4 The online version of this article contains supplemental material.
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 μg HEL for complete negative selection (Fig. 2A and B), but an effect was found with as little as 1 μg. LB11.3 required 30–100 μg HEL for complete negative selection (Fig. 2A 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 μg HEL, although a similar effect was noted in about half the mice at the 5-μg dose (Fig. 2B). 3A9 mice injected with 1 μg HEL had a mild but statistically significant increase in Foxp3⁺ T 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
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 1G12<sub>hi</sub> and 1G12<sub>lo</sub> 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 gating 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 Va\(^{2high}\)CD4SP cells, less so in cells that expressed intermediate Va2 levels and there was no significant effect on the Va2-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 (\(\geq 100,000\) 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\(^{hi}\) 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 cTEC and mTEC (Fig. 4B). By 36 h, presentation by CD45\(^+\) EpCAM\(^+\) cells from mice injected with HEL was 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-Ak 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, CD8αSirpα− cDC that stem from the periphery and CD8αSirpα+ cDC that originate in the thymus (47, 49). Additionally, we have observed a fourth CD11c subset that is CD11cintCD45RAlow, 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: CD11chighCD8αSirpα− (intrathymic cDC), CD11chighCD8αSirpα+ (extra-thymic cDC), CD11cintCD45RAhigh (pDC), and CD11cintCD45RAlow (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, CD8α+ subsets were more efficient than CD8α− 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 Sirpα+ and Sirpα−) 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
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).

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>Percentage entering thymus</th>
<th>0.25%</th>
<th>pmol HEL in thymus</th>
<th>0.17 pmol</th>
<th>Molecules HEL in thymus</th>
<th>10^11 molecules</th>
<th>Assume 10% uptake</th>
<th>10^10 molecules</th>
<th>Efficiency of processing</th>
<th>0.1% of uptake</th>
<th>Number of pMHC</th>
<th>10^7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated number of 48–62 complexes/DC</td>
<td>25–250/DC</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>

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

Our estimate of pMHC/DC is based on FACS data showing that 10^5–10^6 DCs are recovered from a B10.BR thymus and 40% of those DCs are positive for HEL (Table I).

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

<table>
<thead>
<tr>
<th>pMHC/DCa</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>----------</td>
<td>---------</td>
<td>----------</td>
</tr>
<tr>
<td>3A9</td>
<td>1 µg</td>
<td>25–250</td>
</tr>
<tr>
<td>LB11b</td>
<td>5 µg</td>
<td>0.1–1</td>
</tr>
</tbody>
</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.

Our estimate of pMHC/DC is based on FACS data showing that 10^5–10^6 DCs are recovered from a B10.BR thymus and 40% of those DCs are positive for HEL (Table I). Estimates for LB11 assume a 250-fold difference in presentation of HEL48–62 versus HEL20–35 based on experimental data (36).
among $\sim 10^5$–$10^6$ 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 (20 $\mu$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 $\mu$g 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$\to$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 TCR$\alpha$-chain gene rearrangement, although in its absence the induction was inefficient even at low HEL doses. Moreover, the absence of endogenous $\alpha$ rearrangement altered the kinetics of negative selection in both 3A9 and LB11.3, decreasing the threshold of HEL required. Our findings support an avian model of Treg selection whereby TCR-dependent interaction of Treg precursors with Ag at the “right” window of pMHC density induces the final maturation 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 Sirp$^{-}$$\alpha$cDC entering the thymus from the periphery are a specialized APC for the induction of central tolerance to blood-borne Ags. We find the authors’ data showing the unique localization of Sirp$^{-}$$\alpha$cDC inside perivasculare regions of blood vessels in the cortex intriguing and deserving of further examination in other experimental models. We are less convinced about data ascribing unique participation of these cells in tolerance induction to blood-borne Ags. The results examining negative selection in CCR2$^{-}$$\beta$$\alpha$– mice that have decreased numbers of Sirp$^{-}$$\alpha$cDC showed a very modest reduction. This could suggest that other cell types, such as Sirp$^{-}$$\alpha$CD8$\alpha$$\beta$cDC, participate in the induction of tolerance to blood-borne Ags and that Sirp$^{-}$$\alpha$cDC are not required and may in fact have a minor role in this process. This view is supported by our own data showing that both Sirp$^{-}$$\alpha$cDC and Sirp$^{-}$$\alpha$cDC presented HEL to a similar extent (Fig. 5, A and B), suggesting that both these cell types can efficiently acquire blood-borne HEL to mediate negative selection. Similarly to Baba et al., our results show that the fast kinetics of uptake of blood-borne Ag by thymic DC make it unlikely that Ag is being brought in by Sirp$^{-}$$\alpha$cDC 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

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