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Select Regulatory T Cells in Thymus

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Cutting Edge: Hematopoietic-Derived APCs Select Regulatory T Cells in Thymus

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Recognition of self-peptide–MHC complexes by high-affinity TCRs and CD28 signaling are critical for the development of forkhead-winged helix box transcription factor 3+ regulatory T cells (Tregs) in thymus. However, the type of APCs that are responsible for selecting Tregs has remained unclear. To dissect the role of hematopoietic-derived APCs (HCs) and thymic epithelial cells (TECs) in Treg selection, we constructed bone marrow chimeras with disrupted CD28/B7 signaling in the HC or TEC compartment and analyzed the generation of Tregs in the thymus. We found that both HCs and TECs were independently able to fully reconstitute the Treg population in the thymus of bone marrow chimeras. In addition, Treg selection requires the TCR signal and CD28 costimulation presented in cis on the same APC type in vivo. This study demonstrates a new role, to our knowledge, for HCs in the development of Tregs in thymus. The Journal of Immunology, 2010, 185: 3819–3823.

Forkhead-winged helix box transcription factor 3 (Foxp3)+ regulatory T cells (Tregs) develop in the thymus and are required for the maintenance of self-tolerance (1). Tregs develop from thymocytes with high affinity for self-peptide–MHC complexes (2) and require CD28/B7 signaling for their generation, peripheral homeostasis, and functional activation (3–5). Two types of thymic APCs, thymic epithelial cells (TECs), and dendritic cells (DCs), express B7 molecules and have the capacity to present self-Ag–MHC class II complexes to developing thymocytes. A recent study showed that medullary TECs presenting promiscuously expressed tissue-restricted Ags under the control of the autoimmune regulator Aire, but not DCs, play an important role in Treg selection (6).

Thymic DCs play a critical role in negative selection of self-reactive thymocytes (7). However, two studies suggest that peripheral DCs can migrate into the thymus and contribute to the induction of nondeleitional tolerance (8, 9). We have shown that human thymic DCs conditioned by thymic stromal lymphopoietin expressed by the epithelial cells within Hassall’s corpuscles induce the differentiation of thymocytes into CD4+Foxp3+ Tregs in culture (10). Previous studies have used the bone marrow (BM) chimera class II knockout into wild-type (WT) host to describe the contribution of TECs versus hematopoietic-derived APCs (HCs) in the selection of Tregs and suggested HCs do not play a major role in selection based on the lack of class II on HCs did not decrease the number of Foxp3+ cells generated in the thymus. Another group, using the same model, indicated the contribution of HCs based on a 30% reduction in production of Tregs in the thymus, and, using a thymic transplantation system, they demonstrated that DCs in the periphery can migrate to the thymus, where they induce Treg generation (11, 12).

Our studies differ from previous ones in that we generated BM chimeras in which CD28/B7 signaling was disrupted in either the radiosensitive HCs or radioresistant TECs. Using this model, we were able to discriminate between the function of TECs versus HCs. Our results show that lack of B7 costimulation in both TEC and DC compartments resulted in a significant decrease in the number of Foxp3+ Tregs in the thymus. Restoration of B7 only in HCs induced complete reconstitution of the number of Tregs, indicating that in the absence of functional TECs, HCs have the capacity to induce the development of Tregs. Treg selection by HCs required both class II and B7 expression, indicating that TCR and CD28 signaling cannot be segregated and provided by different APCs in vivo.

Materials and Methods

Mice

C57BL/6, Pep3b (B6.SJL-Pep3b/J, B6), and CD80/86-deficient mice (B6.129S4-Cd80tm1Shr Cd86tm1Shr/J, MHC class II-deficient mice (B6.129-H2dIAb1-Ea/J), on the B6 background, were from The Jackson Laboratory (BAR Harbor, ME). Foxp3GFP reporter mice were provided by F. Xiao-Fen Qin (MD Anderson Cancer Center, Houston, TX). CD11cYFP mice were provided by Michel C. Nussenzweig (The Rockefeller University, New York, NY). All experiments were performed in accordance with federal guidelines approved by the University of Texas, MD Anderson Cancer Center Institutional Care Committee.
BM chimeras
Recipient mice were lethally irradiated (10^3 rad). Hematopoietic progenitors were isolated from BM; cells were sorted on the basis of lineage-negative, c-kit^+ Sca^-CD45^- after T cell and lineage depletion. Recipient mice were injected i.v. with 1–5 × 10^5 progenitor cells and maintained on 0.025% Baytril for 2 wk. Chimeric mice were analyzed 8–10 wk postreconstitution.

Abs and flow cytometry
Abs were purchased from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), and CalTag Laboratories (Burlingame, CA). Intracellular Foxp3 (eBioscience) and CCL22 (BD Biosciences) staining was done as recommended by the manufacturer. Cell events were collected using an LSR II cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR). For thymic DC isolation, thymus was treated with 2 mg/ml collagenase (Life Technologies, Rockville, MD) and 10 μg/ml DNase (Roche, Basel, Switzerland) for 30 min at 37°C. CD4^+ cells were depleted by incubating with microbeads, and cells were purified on MACS LD columns (Miltenyi Biotec, Auburn, CA). TECs were isolated by enzymatic digestion as described elsewhere (6). TECs were identified by CD45^-epithelial cell adhesion molecule^+ MHC class II^-

Treg suppression assay
Tregs were isolated from spleen and thymus of BM chimeric mice or Foxp3-GFP mice as control by incubation with anti-CD8 microbeads followed by MACS LD columns (Miltenyi Biotec) and sorted on the basis of CD4^+ CD8^- CD25^- or CD4^+ CD8^- Foxp3-GFP expression. Sorted CD4^+CD25^- cells (5 × 10^4) were cocultured with irradiated T depleted APCs (10^5) and 1 μg/ml anti-CD3 (2C11; BD Biosciences) in the presence or absence of the indicated ratio of purified CD4^+CD25^- or CD4^+Foxp3+ Tregs. Cultures were incubated for 72 h, with the inclusion of 1 μCi well[^H]thymidine deoxyribosyl for the final 16 h.

Chemokine assay
Freshly isolated CD4^+ thymocytes were resuspended at 1 × 10^6 and loaded into the upper chamber of a transwell chamber (5 μm pore; Costar, Corning, NY). Bottom wells were loaded separately with different concentrations of recombinant mouse chemokine CCL22 (R&D Systems, Minneapolis, MN), sorted CFSE-labeled TECs (5 × 10^4), and HCs (5 × 10^4) in 0.6 ml RPMI 1640. After 4 h at 37°C, cells that had migrated to the lower chamber were collected and counted by the manufacturer. Cell events were collected using an LSR II cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR). For thymic DC isolation, thymus was treated with 2 mg/ml collagenase (Life Technologies, Rockville, MD) and 10 μg/ml DNase (Roche, Basel, Switzerland) for 30 min at 37°C. CD4^+ cells were depleted by incubating with microbeads, and cells were purified on MACS LD columns (Miltenyi Biotec, Auburn, CA). TECs were isolated by enzymatic digestion as described elsewhere (6). TECs were identified by CD45^-epithelial cell adhesion molecule^+ MHC class II^-

Results and Discussion
HC3 and thymic epithelial APCs can independently contribute to the development of Tregs in thymus
To discriminate between the role played by HCs versus TECs as APCs in the development of Foxp3^+ Tregs in the thymus, we generated BM chimeras in which CD28/B7 signaling was disrupted in either the radiosensitive HCs or radioresistant TECs by using mice deficient in CD80 and CD86 costimulatory molecules (B7^-/-) as either donor or host. The BM chimeras with B7-deficient TECs and costimulation-competent (B7-sufficient) HCs were generated by reconstituting lethally irradiated B7^-/- mice with sorted BM stem cells, isolated from WT CD45.1 congenic donors. Alternatively, BM chimeras expressing B7-deficient HCs and B7-sufficient TECs were generated by reconstituting lethally irradiated WT-CD45.1 mice with BM stem cells isolated from B7^-/- mice. For controls, lethally irradiated B7^-/- mice were reconstituted with sorted stem cells from B7^-/- donor mice (HC and TEC B7-deficient) or lethally irradiated WT mice were reconstituted with the stem cells from WT CD45.1 donors (HC and TEC B7-sufficient). Eight weeks later, BM chimeras were analyzed for the development of CD4^+CD25^+ and Foxp3^+ Tregs in the thymus by flow cytometry. As shown in Fig. 1A, B7^-/- mice have a dramatic reduction in the percentage of Tregs (0.08 ± 0.07) within single-positive (SP) CD4^+ T cells in the thymus as compared with WT mice (2.7 ± 0.8). Strikingly, analysis of the frequency of Tregs within the SP CD4^+ T cells in the thymus of BM chimeras with disruption in the CD28/B7 pathway on either HCs (B7^-/- → WT) or TECs (WT → B7^-/-) showed a normal percentage and number of Tregs (2.1 ± 0.7 and 2.5 ± 0.4, respectively), which was approximately equivalent to that seen in the control WT chimeras (WT → WT) (2.8 ± 0.7) (Fig. 1C, 1D). Also, the mean fluorescence intensity (MFI) of Foxp3 expression was comparable (Supplemental Fig. 2B). Importantly, all BM chimeras were completely reconstituted by the donor cells, as demonstrated by the expression of the CD45.1 congenic marker (Fig. 1B). Also, the expression of B7 molecules restricted to either HCs or TECs in the indicated BM chimeras was demonstrated by flow cytometry (Supplemental Fig. 1). In addition, all BM chimeras produced comparable frequencies of CD4^+ SP T cells in thymus (9 ± 0.5) (Fig. 1C). In contrast, disruption of CD28/B7 in both HCs and TECs (B7^-/- → B7^-/-) resulted in a highly significant reduction in the percentage of Tregs in the thymus (0.1 ± 0.1) comparable to the B7-deficient mice (Fig. 1D). In addition, these Tregs had a reduced MFI of Foxp3 (Fig. 2B). Analysis of the total number of CD4^+CD25^+Foxp3^+ Tregs within the SP CD4^+ T cells in the thymus of BM chimeric mice clearly showed that lack of B7 molecules in either HCs or TECs has some impact on the
development of Tregs (Fig. 1E). These data demonstrate that HCs expressing B7 costimulatory molecules are sufficient for the development of Tregs in the thymus in the absence of functional TECs. Also, both TECs and HCs have a comparable capacity to induce the development of Tregs in the thymus.

MHC class II presentation by HCs is required for generation of Tregs

Because B7−/− mice contain a very small population of Tregs in the thymus, the reconstitution of Foxp3+ Tregs in the WT → B7−/− BM chimera (Fig. 1C) could be the result of Tregs selected by self-Ag presentation by TECs and their subsequent expansion induced by B7 costimulation on HCs. To discriminate between expansion or generation of Tregs mediated by HCs, BM chimeras with class II-deficient, B7−-competent HCs and class II-competent but B7−-deficient TECs were generated. Irradiated B7−/− mice were reconstituted with stem cells isolated from BM of MHC class II-deficient mice (class II−/− → B7−/−), and the frequency of Tregs generated was compared with WT → B7−/− and class II−/− → WT BM chimeras. Eight weeks later, thymocytes from these groups of mice were analyzed by flow cytometry. The results (Fig. 2A, 2C) show that absence of MHC class II in the hematopoietic compartment results in an accumulation of SP CD4+ T cells, confirming the role of HCs in the negative selection of self-reactive T cells (7). Concomitant to the lack of negative selection, the percentages of Foxp3+ Tregs in the class II−/− → B7−/− BM chimera were highly reduced (0.2 ± 0.5) as compared with class II−/− → WT BM chimeras (2.5 ± 0.8) (Fig. 2A, 2C). Interestingly, lack of negative selection in the class II−/− → WT BM chimera did not alter the reconstitution of CD4+ Foxp3+ cells in the thymus; those Tregs had similar levels of Foxp3 as WT → WT BM chimeras and were functional in an in vitro suppressive assay (Fig. 2A, Supplemental Fig. 4). These results indicate that competent TECs can compensate as an APC for the development of Tregs, and this process is independent of negative selection mediated by HCs. Similar results were recently reported by Liston et al. (12) postreconstitution of class II−/− into RAG−/−. In contrast, Proietto et al. (11) demonstrated a 30% reduction in the total numbers of Tregs in the class II−/− → B7−/− BM chimera; this difference in Foxp3 T cell numbers may have been attributed to a reduction of thymus size in this BM chimera as mice age (data not shown).

The MHC class II requirement by HCs for the development of Tregs in the B7−/− mice was also reflected in the total numbers of CD4+ Foxp3+ Tregs within the SP CD4 T cells in the thymus (Fig. 2D). Class II−/− → B7−/− BM chimeras had a 70% reduction in Tregs as compared with WT → B7−/− and class II−/− → WT BM chimeras, which had competent HCs or TECs as APCs, respectively (Fig. 2D). Interestingly, class II−/− → B7−/− BM chimeras had a very small thymus and low numbers of Tregs (Fig. 2D) with no functional suppressive capacity (Supplemental Fig. 4B). Those mice developed severe autoimmune disease characterized by exocrine pancreatitis and gastritis, leading to death of the mice at 12 wk postreconstitution (data not shown). This autoimmune phenotype showed a remarkable similarity to spontaneous autoimmune pancreatitis described in NOD.CD28−/− mice (13). Overall, these data demonstrate that HCs directly present self-Ags to developing thymocytes and induce the generation of Foxp3+ Tregs in the thymus. In addition, the requirements for TCR signaling and CD28 costimulation in the generation of Foxp3+ Tregs in the thymus cannot be separated in time or space and need to be provided on the same APC type.

CD4+Foxp3+ Tregs selected by HCs in the thymus are functional

To examine the phenotypic and functional characteristics of Foxp3+ Tregs selected by HCs, BM chimeras were constructed by transferring stem cells from WT-Foxp3GFP mice into lethally irradiated B7−/− mice (Foxp3GFP → B7−/−). Eight weeks later, thymus was collected, and the percentage of CD4+GFP+ cells was analyzed. As shown in Fig. 3A, the percentage of GFP+...
Tregs in thymus from Foxp3^GFP → B7^−/−^ BM chimeras (3 ± 0.2) was comparable to the number of Tregs in the WT-Foxp3^GFP^ mice (2.8 ± 0.4). The two Treg populations (HC selected versus WT) expressed high levels of CD24 and CCR7, characteristic of recently differentiated thymic Tregs, and they also expressed high levels of Treg markers such as FR4 and glucocorticoid-induced TNF-like receptor molecules (Fig. 3B). We also compared the phenotype of HC- and TEC-selected Tregs and found that the expression levels of several Treg molecules between the two freshly isolated naive Treg subsets was very similar (Supplemental Fig. 2). We then analyzed the suppressive capacity of thymic Tregs generated by HCs (Foxp3^GFP → B7^−/−^) and compared them to the Treg generated by both TECs and HCs (Tregs isolated from WT-Foxp3^GFP^ mice) (Fig. 3C, Supplemental Fig. 4A). In addition, we compared the capacity of these two Treg subsets to upregulate the expression of granzyme B, and our results show that freshly isolated HCs and TEC-selected Tregs expressed low levels of granzyme B and that both Treg subsets have the capacity to upregulate this molecule after in vitro activation (Supplemental Fig. 3). Most importantly, the two subsets of Tregs show comparable ability to suppress the proliferation of CD4^+CD25^+ T cells in a coculture assay. Thus, HC-selected Tregs and Tregs selected on both HCs and TECs are similar with respect to their suppressive function and phenotype.

**Hematopoietic-derived donor DCs in thymus express high levels of CCL22**

The development of T cells in the thymus requires a series of migration, proliferation, and differentiation events that take place in specific anatomical regions in the thymus (14). To investigate the characteristics of HCs able to select Treg in the thymus, we used stem cells isolated from CD11c^YFP^ reporter mice to reconstitute lethally irradiated B7^−/−^ mice (CD11c^YFP → B7^−/−^). Eight weeks later, CD11c^+^ DCs were isolated from thymus, and we found that all CD11c^+^ cells coexpressed YFP, indicating full reconstitution from the donor cell origin (Fig. 4A). CD11c^+^YFP^+^ DCs in thymus were CD8α^+^ and expressed high levels of CD80/86 and high levels of chemokine CCL22 (Fig. 4B). The expression of CCL22 on hematopoietic cells may facilitate the interaction with CCR4^+^ Tregs within the thymus. In addition, we found that TECs and HCs isolated from thymus have the same ability to attract thymocytes (Fig. 4C). Thus, hematopoietic-derived APCs, most likely DCs expressing high levels of B7 molecules and CCL22, play a critical role in the selection of thymic Tregs.

The key finding from this study is that, in addition to TECs, HCs can also support the generation of Tregs in the thymus. Although we have not yet identified the specific HCs able to select Tregs, we found that thymic DCs in the BM chimeras were fully reconstituted from donor origin, which express high levels of costimulation molecules CD80/86 and Tregs attracting chemokine CCL22. The self-Ag spectrum presented by DCs could be complementary to that presented by TECs, because not only have DCs been shown to be more efficient at presenting exogenous Ags than TECs, but also they can take up and present medullary TEC-derived tissue-restricted Ags during cell death and renewal (15). Although the requirement for coengagement of TCR plus CD28 for the differentiation of DP thymocytes into Tregs has been previously demonstrated in vitro (5), it was not clear if these two signals could be provided by different APCs to induce Tregs. We found that efficient generation of Tregs in vivo requires a TCR and CD28 signal provided in cis by the same APC type. The ability of...
both hematopoietic and nonhematopoietic APCs for the development of Tregs in the thymus suggests that Tregs selected by these two different APCs may express TCRs that recognize different but complementary repertoires of self-Ags.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplementary figure 1

A

WT→WT
WT→B7−/−
B7−/−→WT
class II−/−→WT

thymic epithelial cells

B

dendritic cells

CD11c

FSC

class II

B7

class II
Supplementary figure 2

A  Phenotype of DC-Treg and TEC-Treg

<table>
<thead>
<tr>
<th>% of Max</th>
<th>CD103</th>
<th>CD62L</th>
<th>CCR7</th>
<th>CD24</th>
<th>CD69</th>
<th>CD101</th>
<th>CD73</th>
<th>CXCR4</th>
<th>CD39</th>
</tr>
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Legend:
- TEC-Treg
- DC-Treg
- Foxp3-GFP Treg
Supplementary figure 3

Activation induced upregulation of Granzyme B on Treg subsets

FoxP3GFP-Treg  DC-Treg  mTEC-Treg

% of Max

Granzyme-B

- - 0h
- - 96h
- Isotype-mlG1
Supplemental figure 4

A
- Foxp3GFP-Thymus
- Foxp3GFP-Spleen
- B7\(^{-/-}\) → WT Thymus (TEC-Treg)
- WT→B7\(^{-/-}\) Thymus(HC-Treg)

3H-TdR uptake (cpm)

B
- Foxp3GFP-Thymus
- Class II\(^{\text{−/−}}\)→WT-Thymus
- Class II\(^{\text{−/−}}\)→B7\(^{-/-}\)-Thymus
- Foxp3GFP→B7\(^{-/-}\)
- Foxp3GFP→B6.WT

3H-TdR uptake (cpm)
Supplemental figures legends:

Supplemental figure 1. Expression of B7 and class II on thymic antigen presenting cells of BM chimeras. Thymi from the indicated groups of BM chimeras were harvested eight weeks after reconstitution and the thymic epithelial cells and dendritic cells were enriched as described in methods and analyzed by flow cytometry. (A) Histograms shows the expression of B7 and class II molecules on TECs of indicated BM chimeras, TECs were gated on CD45−, EpCAM+ and class II+ cells. (B) Histogram shows the expression of B7 and class II molecules on dendritic cells isolated from the thymus of indicated BM chimeras, DC were gated on CD11c+ and class II+ cells. Representative of two independent experiments.

Supplemental figure 2. Phenotype of DC- and TEC-selected Treg
Freshly isolated thymocytes from 8 week BM chimeras WT→B7−/− (DC-selected Treg), B7−/−→WT (TEC-selected Treg) and WT-Foxp3-GFP mice were stained and gated on CD4+ and Foxp3+ and analyzed for expression of indicated molecules. The Treg subsets were compared to their isotype control Ig and to Treg isolated from WT-Foxp3-GFP and analyzed by FlowJo software. Data representative of two independent experiments.

Supplemental figure 3. Activated induced up-regulation of Granzyme-B in Treg subsets. SPCD4+CD25+ or SPCD4+GFP+ thymocytes were purified by sorting thymocytes from indicated BM chimeras. Sorted Treg cells were cultured in vitro with anti-CD3, anti-CD28 and IL-2 for 24h or 96 h. Intracellular granzyme expression on DC-selected Treg, TEC-selected Treg and WT-Foxp3-GFP in freshly isolated Treg or in vitro cultured for 24 or 96h with plate bound anti-CD3, anti-CD28 and 100U of IL-2. Representative of two independent experiments.

Supplemental figure 4. Suppression activity of Treg subsets. SPCD4+CD25+ or SPCD4+GFP+ thymocytes were purified by sorting thymocytes from indicated BM chimeras. Treg subsets were coculture with CD4+CD25− T cells at the indicated ratio and irradiated T depleted APC with anti-CD3 for 72h and pulsed with [3H] thymidine as described in methods. The figures are representative of two independent experiments.