Rare Development of Foxp3+ Thymocytes in the CD4+CD8+ Subset

Hyang Mi Lee and Chyi-Song Hsieh

J Immunol 2009; 183:2261-2266; Prepublished online 20 July 2009;
doi: 10.4049/jimmunol.0901304
http://www.jimmunol.org/content/183/4/2261

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/07/21/jimmunol.0901304.DC1

References
This article cites 25 articles, 11 of which you can access for free at:
http://www.jimmunol.org/content/183/4/2261.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Rare Development of Foxp3+ Thymocytes in the CD4+CD8+ Subset

Hyang Mi Lee and Chyi-Song Hsieh

The CD4+CD8+ (double positive, DP) stage of thymic development is thought to be the earliest period that generates natural Foxp3+ regulatory T (Treg) cells important for the prevention of autoimmunity. However, we found that most Foxp3+ DP cells identified by routine flow cytometry represent doublets comprised of Foxp3+ DP and Foxp3+ CD4+CD8- (CD4SP) cells. This was determined using analysis of flow cytometric height and width parameters, postsort contaminants, and thymocyte mixing studies. Temporal analysis of Treg cell development arising from bone marrow precursors in neonatal bone marrow chimeras suggested that Foxp3+ DP cells are not a major percentage of Foxp3+ thymocytes, and it supported the notion that most Treg cell development occurred at the immature HSAhigh CD4SP stage. Thus, these data demonstrate that the frequency of Foxp3+ cells generated at the DP stage is much smaller than previously recognized, suggesting that additional thymocyte maturation may be required to facilitate efficient induction of Foxp3.


Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00

Mice and reagents

Foxp3GFP reporter mice were provided by Dr. A. Rudensky (19); MHC class II-deficient mice (22) were purchased from The Jackson Laboratory; and B6.SJL CD45.1 congenic mice were purchased from The Jackson Laboratory; class II-deficient mice (22) were purchased from The Jackson Laboratory. Mice were analyzed at 6–9 wk of age, housed in specific pathogen-free facility at Washington University, and used under protocols approved by the Animals Studies Committee.

Flow cytometry and FACS cell sorting

mAbs were purchased from eBioscience and BioLegend. Flow cytometric analysis and sorting were performed using a FACSARia (BD Biosciences).
and FlowJo software (Tree Star). For routine flow cytometry, thymocytes were stained at 40 × 10^6/ml in Ca^2+ /Mg^2+-free Dulbecco’s PBS supplemented with 2.5% FCS and 0.02% sodium azide for 20 min on ice. Free Ab was removed by washing after centrifugation. To reduce cell-cell interaction in certain experiments, we stained in the presence of 1 mM EDTA at 4°C with constant rotation, and instead of washing after centrifugation, the cells were simply diluted 50-fold in PBS immediately before flow cytometry. To assess DNA content, thymocytes were stained with Abs under the condition for routine flow cytometry and fixed by 0.1% paraformaldehyde in Dulbecco’s PBS for at least 2 h. Fixed thymocytes were permeabilized using 70% ethanol for propidium iodide staining.

**Neonatal bone marrow chimera**

Bone marrow cells from Foxp3^{gfp} mice were T cell depleted using autoMACS magnetic bead removal of CD4- and CD8-labeled cells (Miltenyi Biotec). Ten million bone marrow cells were i.p. injected into 2-day-old mice.

**Results**

**Flow cytometric analysis of Foxp3^{+} DP thymocytes**

Previous studies have demonstrated Foxp3^{+} DP cells in both TCR transgenic and polyclonal settings (16 –19), with up to ~30% of Foxp3^{+} thymocytes reported to be present in the DP subset (20). We reasoned that Foxp3^{+} DP and CD4SP cells may represent Treg cells selected on different arrays of peptide-MHC class II complexes presented on cortical vs medullary APCs, which could be revealed by a comparison of the TCR repertoires between DP and CD4SP Foxp3^{+} cells. However, initial attempts to purify Foxp3^{+} DP cells by our usual flow cytometric sorting parameters were hampered by low purity in the 40 – 60% range (Fig. 1). Even double FACS purification did not result in a high purity sort (data not shown). This did not appear to be a mechanical limitation of the sorter, as Foxp3^{+} CD4^{+} CD8^{+} single-positive (CD8SP) cells could be sorted with reasonable purity, even though they are at comparable frequency to Foxp3^{+} DP cells.

Postsort analysis of Foxp3^{+} DP cells showed that a large portion of the contaminants were Foxp3^{+} DP cells (Fig. 1). This was expected since Foxp3^{+} DP cells are by far the most frequent cell type in the thymus. Surprisingly, many of the contaminants were Foxp3^{+} CD4^{+} CD8^{+} thymocytes. Since these cells represent only ~0.3% of total thymocytes, this suggested that contamination was occurring in a nonrandom fashion. Because these contaminants were present...
at a 1:1 ratio (Fig. 1, bottom), this hinted at the possibility that many of the Foxp3+ DP cells recognized by flow cytometry may represent doublets of Foxp3+ DP and Foxp3- CD4SP cells.

To test this hypothesis, we performed additional flow cytometric analysis of Foxp3+ DP cells. An appreciable frequency of Foxp3+ in the DP subset was only observed only when generous forward light scatter-area (FSC-A) and side light scatter-area (SSC-A) gating parameters were used (Fig. 2A), large gate). Area parameters are derived from the integration of height and width parameters (e.g., FSC-H and FSC-W) as the cell passes through the detector. However, we found that most Foxp3+ DP cells exhibited an exagerrated FSC-W compared with FSC-H. The same was true for SSC (data not shown). This traditionally implies that two cells serially passed the detector so close together that they could not be discriminated by the cytometer as individual cells. Rather, this event appears as a single large cell with increased FSC-A/SSC-A due to integration. Consistent with this interpretation, a smaller FSC/SSC gating scheme (small gate) decreased the frequency of Foxp3+ cells in the DP subset by approximately 20-fold, and it eliminated most of the doublets by FSC-H/FSC-W criteria (Fig. 2A). Another interesting observation is that the mean fluorescence 2 A

2B

Mean F 4

3

2

A

B

C

FIGURE 3. Doublets in the Foxp3+ DP thymocyte subset. A, Foxp3+ DP doublets are formed during staining. CD45.1 Foxp3+ thymocytes were mixed with CD45.2 Foxp3+ thymocytes at 1:1 ratio, stained using the normal protocol, and analyzed by flow cytometry using a large FSC-A/SSC-A gate (see Fig. 2). Data shown are gated on Foxp3+ cells and as indicated above the plots, and are representative of two independent experiments. The numbers represent the frequency of the cells in the quadrant. B, Doublet discrimination gating is imperfect for eliminating doublets. Thymocytes in the large FSC-A/SSC-A gate as in A were analyzed using gates with small FSC-W and different FSC-H for the frequency of CD45.1+CD45.2+ doublets. Representative plots and quantification of doublets are shown according to FSC-H gate. In these plots, Foxp3+ was not used for gating. C, Frequency of Foxp3+ DP cells is proportional to doublet formation. The mean frequencies of DP and CD4SP in Foxp3+ thymocytes from each FSC-H gate are plotted.

Summary of CD4/8 distribution of Foxp3+ thymocytes

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Large Gate</th>
<th>Small Gate</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8SP</td>
<td>3.1 ± 0.5</td>
<td>3.2 ± 0.6</td>
<td>4.0 ± 0.1</td>
<td>3.4 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>CD4SP</td>
<td>68.8 ± 10</td>
<td>86.8 ± 0.9</td>
<td>87.8 ± 1.5</td>
<td>91.4 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>DP</td>
<td>21.5 ± 11</td>
<td>41.0 ± 0.2</td>
<td>12.7 ± 0.4</td>
<td>1.4 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

*Data shown are the mean frequencies of Foxp3+ thymocytes that fall into the DP, CD4SP, and CD8SP gates as per Fig. 2. EDTA indicates whether 1 mM EDTA and avoidance of centrifugation was used to diminish cell-cell interactions. Large and small gates represent those shown in Fig. 2.*

4 The online version of this article contains supplemental material.
Characterization of Foxp3+ DP thymocytes

As it is unclear whether previous studies excluded doublets from the analysis of Foxp3+ DP events, we performed a flow cytometric characterization of Foxp3+ DP cells using a small gate. One observation was that the levels of CD4 and CD8 are lower than those found on typical Foxp3+ DP cells (Fig. 2, small gate), suggesting that Foxp3+ DP cells were transitioning out of the DP stage after undergoing positive selection. This is consistent with the observation that Foxp3+ DP cells are TCRγδ+ and express lower levels of heat-stable Ag (HSA) than Foxp3− DP cells (supplemental Fig. 5). However, the HSA levels on Foxp3+ DP cells were higher than those on Foxp3− CD4SP cells. Additionally, Foxp3+ DP cells show a higher level of CD69 expression than do Foxp3− DP cells, indicating that Foxp3+ DP cells may have recently received a positively selecting TCR signal. In other respects, DP and CD4SP cells were similar, expressing Treg cell surface markers such as CD25, GITR, OX-40, and CD103 (supplemental Fig. 5). Thus, these data suggest that Foxp3+ DP cells have undergone positive selection and express traditional Treg cell markers.

Since most Foxp3+ thymocytes are presumably MHC class II restricted, as most reside in the CD4SP subset, we asked whether Foxp3+ DP cells require MHC class II for their development. Consistent with a previous report (19), we observed a marked decrease in Foxp3+ CD4SP cells and an increase in Foxp3+ DP cells in Foxp3−/− MHC class II−/− mice (Fig. 4A). This was also reflected in an overall decrease in frequency of Foxp3+ thymocytes (Fig. 4B). Curiously, many Foxp3+ DP cells in MHC class II−/− mice in the large gate by FSC-A/SSC-A parameters show enhanced levels of CD8 compared with their counterparts in wild-type (WT) mice. Since these cells are not present when the small gate is used, they may represent doublets of Foxp3+ CD4+CD8bright/CD8low DP or CD8SP cells with Foxp3− DP cells in MHC class II−/− mice. Nonetheless, the presence of Foxp3+ CD4+CD8bright/CD8low DP cells in MHC class II−/− mice suggests that Treg cell selection can occur on MHC class I.

Developmental kinetics of Foxp3+ thymocytes

A previous study of neonatal thymic development showed that Foxp3+ DP and CD4SP cells appeared coincidently (21). Since this study examined development in a neonatal and potentially lymphopenic thymic setting, it was possible that the thymic medulla was not fully mature (23–25). We therefore analyzed T cell development in lymphoreplete thymuses using neonatal bone marrow chimeras. It took ~ 2.5 wk after transfer of congenically marked bone marrow cells to see appreciable numbers of developing donor thymocytes (Fig. 5A). We did not observe a period in which DP cells contributed a substantial fraction to the total thymic Foxp3+ population (Fig. 5B). Curiously, the frequency of Foxp3+ cells within the CD4SP subset increased over a period of approximately 1.5 wk until it reached steady-state, consistent with a delay in Foxp3+ cell development at the CD4SP stage (Fig. 5C) observed in neonatal mice (21). These data therefore illustrate that the temporal delay in the generation of Foxp3+ as compared with Foxp3− CD4SP cells does not result from conditions unique to the neonatal thymus.

We also examined the kinetics of Foxp3+ Treg cell development in the CD4SP stage. We observed that most Foxp3+ CD4SP cells are HSAbright at early time points (e.g., 3–3.5 wk), but mostly HSAlow at steady-state (Fig. 5D). Although this time-course analysis cannot prove precursor-product relationships, these data support the notion that Foxp3+ cells can arise from the Foxp3− HSAbright CD4SP subset. Furthermore, Foxp3+ CD25+ CD4SP cells, which have been suggested to be enriched in thymic Treg cell precursors (9), are found initially at much higher frequency than Foxp3+ cells, but decrease over time as the frequency of Foxp3+ correspondingly increases (Fig. 5E). Although correlative, these data are consistent with a model in which Treg cell development occurs at the HSAbright CD4SP stage through a Foxp3− CD25− intermediate.

Discussion

These data highlight one circumstance in which identifying rare cell populations by flow cytometry is problematic due to the congruence of several factors. First, the gating criteria are based only on positive staining, facilitating the inclusion of doublets. In contrast, identifying rare Foxp3+ CD8SP cells requires a CD4+ gate, excluding doublets to everything but another CD8SP cell, which is statistically much less likely assuming random interactions. Second, Foxp3+ DP cells represents the vast majority of thymocytes, making it likely that random doublet formation would include this subset, regardless of whether there are additional attractive forces between Foxp3+ CD4SP and Foxp3− DP cells. Third, the actual Foxp3+ DP cell population appears to be rare, allowing infrequent doublets of Foxp3+ CD4SP with Foxp3− DP to be relatively more prominent. The low frequency of Foxp3+ DP cells may further encourage staining under more concentrated cell conditions, facilitating doublet formation (supplemental Fig. 2). Moreover, the rarity of the cells may prompt more relaxed gating to include as many events as possible, diminishing the effectiveness of strategies designed to improve doublet discrimination (Fig. 3, B and C, and supplemental Fig. 4). We think that relaxed gating resulted in our initial inability to sort these rare Foxp3+ DP cells with high purity. The use of stringent gating criteria is therefore required for more specific identification of Foxp3+ DP cells.
These data demonstrate that the induction of Foxp3 in the DP stage is a relatively rare occurrence. Although the use of a small gate may result in the exclusion of larger Foxp3+ cells that are not doublets, we estimated the frequency of Foxp3+ cells that are DP cells by flow cytometry to be ~1%, which is considerably lower than a recent estimate (20). While we cannot exclude that TCR interactions with self-Ags at the DP stage condition thymocytes to undergo Treg cell development in a multistep process (9), these data suggest that Foxp3 acquisition occurs at the immature HSAhigh stage of CD4SP development (Fig. 5), and they favor a model in which Treg cell development in the DP and CD4SP stage occur in parallel, rather than serially (18).

In addition to the enumeration of Foxp3+ DP cells, these results illustrate the difficulty of studying this rare Foxp3+ population. While the use of a small gate may result in the exclusion of larger Foxp3+ cells that are not doublets, we estimated the frequency of Foxp3+ cells that are DP cells by flow cytometry to be ~1%, which is considerably lower than a recent estimate (20). While we cannot exclude that TCR interactions with self-Ags at the DP stage condition thymocytes to undergo Treg cell development in a multistep process (9), these data suggest that Foxp3 acquisition occurs at the immature HSAhigh stage of CD4SP development (Fig. 5), and they favor a model in which Treg cell development in the DP and CD4SP stage occur in parallel, rather than serially (18).

In addition to the enumeration of Foxp3+ DP cells, these results illustrate the difficulty of studying this rare Foxp3+ population. While a difference of 1% vs 3% Foxp3+ cells in the DP gate may be of little importance with regard to understanding the role of Treg cell development in the DP stage, analysis of a cell population in which the majority is comprised of doublets would be problematic. For example, TCR sequences from Foxp3+ DP events would have been difficult to interpret due to contamination with CD4SP Treg cells. Thus, great care should be taken with functional, developmental, or phenotypic evaluation of Foxp3+ DP cells to avoid the potential for bias by inclusion of Foxp3+ CD4SP cells due to doublets.

Acknowledgments
We thank Nicole Santacruz and Jeremy Hunn for expert technical assistance, and Chan-Wang Lio, Jhoanne Bautista, and Jason Fontenot (Biogen Idec) for helpful discussions and critical review of the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References


