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Expanding Dendritic Cells In Vivo Enhances the Induction of Oral Tolerance

Joanne L. Viney,1* Allan M. Mowat,*† Jamie M. O’Malley,∗ Eilidh Williamson,* and Neil A. Fanger‡

The intestine is under perpetual challenge from both pathogens and essential nutrients, yet the mucosal immune system is able to discriminate effectively between harmful and innocuous Ags. It is likely that this selective immunoregulation is dependent on the nature of the APC at sites where gut Ags are processed and presented. Dendritic cells (DC) are considered the most potent of APC and are renowned for their immunostimulatory role in the initiation of immune responses. To investigate the role of DC in regulating the homeostatic balance between mucosal immunity and tolerance, we treated mice with Flt3 ligand (Flt3L), a growth factor that expands DC in vivo, and assessed subsequent systemic immune responsiveness using mouse models of oral tolerance. Surprisingly, mice treated with Flt3L to expand DC exhibited more profound systemic tolerance after they were fed soluble Ag. Most notably, tolerance could be induced in Flt3L-treated mice using very low doses of Ag that were ineffective in control animals. These findings contrast with the generally accepted view of DC as immunostimulatory APC and furthermore suggest a pivotal role for DC during the induction of tolerance following mucosal administration of Ag. The Journal of Immunology, 1998, 160: 5815–5825.

Oral administration of soluble proteins is one of the most effective means of inducing specific systemic immunologic unresponsiveness, a phenomenon known as mucosal tolerance (1–3). The mechanisms responsible for determining whether orally administered Ags induce tolerance or immunity remain poorly defined, but it is likely that the manner in which Ags are presented to T cells is critical. Although the intestine is a rich source of many types of professional APC, including dendritic cells (DC),2 B cells, and macrophages, little is known of how each contributes to the presentation of individual Ags.

DC are reported to be the most potent of APC, characterized by an apparent constitutive ability to present Ag to naive T cells in an immunostimulatory manner (4). DC are present in the different compartments of the gut, including the diffuse and organized lymphoid tissues (5–9). Previous functional studies have indicated that intestinal DC can be strongly immunogenic after the oral administration of Ags (10–12), suggesting that DC in the intestine may be similar to those in the periphery, with a preferential ability to promote active immunity.

Studies of DC function have been complicated, however, by the low frequency of DC in normal tissues. As a result, much of the past work studying DC function has used DC in vitro or DC transferred into recipient mice. Since it is well documented that co-stimulatory molecules such as CD80 and CD86 are up-regulated on DC following removal from the local tissue microenvironment (13, 14), the in vitro manipulation of DC may have altered the intrinsic functional properties of the DC used in those studies. Thus, the possibility that DC may play a role in the induction of mucosal tolerance in vivo may have been missed. One approach for circumventing these problems originates from the recent observation that treating mice with the hemopoietic growth factor Flt3 ligand (Flt3L) can dramatically increase the numbers of functionally mature DC in peripheral lymphoid tissues (15). Here we have investigated whether Flt3L treatment has a similar effect on the DC populations in the gut-associated lymphoid tissues (GALT) of mice and have examined how increasing the number of DC in vivo influences the induction of systemic immune tolerance following oral administration of protein Ag.

Materials and Methods

Mice

Female C57BL/6 or BALB/c mice (6–10 wk of age) were obtained from Taconic Laboratories (Germantown, NY) and maintained in a specific pathogen-free facility at Immunex (Seattle, WA) in accordance with approved ethical guidelines. DO11.10 OVA-TCR transgenic mice (16) were bred at the specific pathogen-free facility at Immunex.

In vivo treatment of mice with Flt3L

Flt3L-treated mice were injected i.p. once daily with purified CHO-derived human Flt3L (10 µg) for 10 to 12 days as indicated. Control mice were injected i.p. with either human IgG (10 µg; Sigma, St. Louis, MO) or PBS (100 µl) for the same period.

Cell isolations

Gut-associated lymphoid tissues. Single cell suspensions were prepared from mesenteric lymph nodes, spleen, Peyer’s patches (PP), and Peyer’s patch lymph nodes (PLN) by teasing tissues apart in complete medium, followed by mashing through nylon mesh.

Lamina propria. Small intestine lamina propria (SILP) and large intestine lamina propria (LILP) cell suspensions were prepared from intestines opened longitudinally and cut into 1-cm segments. Tissues were incubated at 37°C in 1 mM EDTA in Ca2+ - and Mg2+-free HBSS (Life Technologies, Gaithersburg, MD) for three sequential 15-min incubations to remove the epithelial layer. Denuded tissues were digested with collagenase (90
U/mmol (Sigma) in Ca\(^{2+}\) and Mg\(^{2+}\)-free HBSS/FCS, and the resulting suspension of cells was washed and passed over a prewet glass wool column before centrifugation over a discontinuous Percoll (Pharmacia, Piscataway, NJ) density gradient as previously described (17, 18). This procedure did not influence the cell populations obtained from identically treated LN and spleen cell preparations (data not shown).

**Monoclonal Abs**

The following mAbs were used: anti-CD11b (M170, rat IgG2b), anti-CD11c (HL-3, hamster IgG), anti-CD19 (1D3, rat IgG2a), anti-CD40 (3/23, rat IgG2a), anti-CD80 (1G10, rat IgG2a), anti-CD86 (GL1, rat IgG2a), anti-I-A\(^d\) (25-9-17, mouse IgG2a), anti-I-A\(^d\) (145.2C11, hamster IgG), anti-B220 (RA3-6B2, rat IgG2a), mouse IgG2a isotype control (G155-178), hamster IgG isotype control (G235-2356), rat IgG1 isotype control (R3-34), rat IgG2b isotype control (R35-38), all purchased from Pharmingen (San Diego, CA).

**Immunofluorescence analysis of frozen sections**

Tissues were frozen in liquid nitrogen. Cryostat sections were cut at 6 μm, air-dried, and acetone fixed before staining using conjugated Abs. Sections were stained with anti-CD11c biotin and anti-I-A\(^d\)-FITC mAbs in PBS/30% mouse serum, followed by streptavidin-Texas Red (Molecular Probes, Eugene, OR). Sections were visualized using a confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA).

**Flow cytometric analysis of isolated cells**

Isolated cells were incubated at 4°C for 1 h in primary Ab at 5 μg/ml in the presence of 30% mouse serum, washed with PBS-BSA (2 mg/ml), then incubated with 40 μl of APC-labeled streptavidin (10 μg/ml; Molecular Probes) for an additional 1 h. Samples were washed three times, resuspended in PBS-BSA supplemented with 1% paraformaldehyde, and stored at 4°C until analysis on a FACStar flow cytometer (Becton Dickinson, San Jose, CA). Fifty thousand cells were analyzed per sample. To determine the proportion of OVA-specific CD4\(^+\) cells expressing the clonotypic TCR, cells were stained with mAb KJ1.26 FITC, which detects the clonotypic TCR, and with anti-CD4 PE in 50 μl of blocking buffer containing 10 μg/ml anti-CD16 (PharMingen), 10% normal goat serum, and 1% normal mouse serum.

**In vivo BrdUrd labeling**

**Cell suspension analysis.** For identification of cycling cells, mice were injected i.p. with 1 mg of BrdUrd (Sigma) twice (24 and 12 h) before tissue harvest. Single cell suspensions were prepared from each tissue, and cells were stained with PE-conjugated mAb to CD11c, B220, or CD3 surface Ags before fixing and permeabilizing them for BrdUrd-FITC staining. PE-labeled cells were resuspended in 0.15 M NaCl before being fixed in 95% ethanol (4°C 30 min) and permeabilized with 1% paraformaldehyde/0.01% Tween-20 (at room temperature for 30 min followed by 4°C for 30 min). Cells were then treated with 50 U/ml DNase I (Sigma) in 0.15 M NaCl/4 mM MgCl\(_2\) (at room temperature for 10 min, then at 4°C for 30 min), washed, and stained with anti-BrdUrd FITC mAb (Sigma) for 30 min at room temperature.

**Immunohistologic analysis.** For localization of cycling cells in tissues in situ by immunohistology, mice were injected i.p. once with 1 mg of BrdUrd (Sigma) 2 h before tissue harvest. Dissected tissues were formalin fixed, paraffin embedded, sectioned, and stained using the immunoperoxidase technique. Positive cells were identified by the presence of a brown reaction product.

**DC activation in vivo**

Mice treated with Flt3L for 10 days were injected i.p. with saline only or with 100 μg of LPS solubilized in 0.9% pyrogen-free NaCl. Tissues were harvested 6 h after LPS injection, and single cell suspensions were prepared. The expression of costimulatory molecules on the CD11c\(^+\) cells was analyzed by FACS.

**Induction and assessment of oral tolerance**

Mice were injected with PBS or Flt3L for 10 days before and for 2 days after oral administration of Ag given as a single feed of OVA (fraction V, Sigma) in 0.2 ml of saline by gavage. Ten days after OVA feeding, all mice were immunized s.c. in the footpad with 100 μg of OVA in 50 μl of adjuvant (Ribi adjuvant, Ribi Immunocorulents). Two weeks after immunization half of the mice were sacrificed, and the draining PLN were removed for assessment of Ag-specific proliferative capability (see below). After an additional 7 days, the remaining mice were assayed for systemic delayed-type hypersensitivity (DTH) responses by measuring the increase in footpad thickness 24 h after challenge with 100 μg of heat-aggregated OVA in 50 μl of saline as described previously (19). Mice were then exsanguinated, and Ag-specific serum Ab titers were measured (see below).

**Ag-specific proliferation assay**

Cells isolated from PLN were cultured in complete RPMI 1640 medium supplemented with 10% FCS, penicillin/streptomycin, and β-ME at 37°C in a humidified 6% CO\(_2\) incubator at a density of 2 × 10\(^5\) cells/well for 48 to 96 h. All cultures were performed in quadruplicate in 96-well flat-bottom plates in a total volume of 200 μl either alone or in the presence of 1 μg/ml Con A (Sigma) or 1 mg/ml OVA. Proliferation was assessed by addition of 1 μCi/well [\(^{3}H\) thymidine (Amersham, Aylesbury, U.K.) 1 h before harvest. (See the amount of radioactivity incorporated into DNA was measured using a Matrix-96 cell harvester (Inotech, Lansing, MI) and a direct beta counter (Packard, Meridan, CT). The data are reported as the mean counts per minute ± 1 SEM of quadruplicate wells.

**Analysis of Ag-specific serum Ab titers**

Ninety-six-well ELISA plates (Maxisorp, Nunc, Naperville, IL) were coated overnight with 1 μg/ml OVA in PBS at 4°C, quenched with PBS/5% FCS and washed with PBS/0.1% Tween-20. Serum samples were diluted in PBS/5% FCS (starting at 1:100), and threefold dilutions were made. Plates were incubated for 2 h at room temperature, washed, and incubated with alkaline phosphatase-conjugated anti-IgG1 (1/3000; Sigma), anti-IgG1 (1/2000; PharMingen), or anti-IgG2a (1/1000; PharMingen) detecting Abs for an additional 2 h at room temperature. Plates were washed again, and enzyme activity was detected with p-nitrophenyl phosphate di-sodium (Sigma). The amount of reaction product was assessed on an ELISA plate reader at an OD of 405 nm using the DeltaSoft program (DeltaPoint, Monterey, CA).

**Assessment of tolerance in transfer mice**

For adoptive transfer of OVA TCR transgenic T cells, syngeneic BALB/c mice were injected i.v. with 2.5 × 10\(^5\) clonotypic TCR\(^+\) (CD4\(^+\), KJ1.26\(^+\)) transgenic cells from DO11.10 mice, essentially as previously described (20, 21). BALB/c mice were treated with Flt3L or PBS for 8 days before and for 2 days after adoptive transfer of transgenic cells. Mice were fed a single dose of OVA in 0.2 ml of saline 2 days after transfer and immunized s.c. in the footpad 5 days later with 100 μg of OVA in RIBI adjuvant. After an additional 4 days, draining PLN were removed, and the proportion of transgenic cells in individual mice was determined by FACS analysis. Isolated cells from individual mice were also assessed for Ag-specific proliferative capability as described above.

**Statistical analysis**

Student’s t test was used to compare data from different groups.

**Results**

**CD11c\(^+\) cells are expanded in GALT following Flt3L treatment**

We first investigated whether treating mice with Flt3L could expand DC in the intestine as well as in peripheral lymphoid tissues. Flow cytometric analysis of both the organized lymphoid tissues (PP and mesenteric lymph nodes) and the diffuse lymphoid tissues (small and large intestine lamina propria) of the intestine revealed a large preferential increase in cells expressing CD11c, an Ag expressed by cells of the DC lineage (13, 22), following 10 days of Flt3L treatment (Fig. 1 and Table I). The increase in CD11c\(^+\) cells in the GALT was comparable to that observed in the spleen (Fig. 1). Similar results were seen in C57BL/6 and BALB/c mice (data not shown).

**All putative CD11c\(^+\) DC populations are present in the GALT of Flt3L-treated mice**

DC have been divided into putative subpopulations based upon the relative coexpression of CD11c with other cell surface molecules, notably CD11b (15, 23–28). CD11c\(^+\)/CD11b\(^{low}\) cells often coexpress DEC205 and CD80 and represent a putative subset derived from lymphoid precursors. Conversely, CD11c\(^+\)/CD11b\(^{high}\) cells correspond to a putative myeloid-derived DC subset (15, 23–28).

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Having observed the increase in CD11c+ DC using cells isolated from the intestinal lamina propria, we localized the CD11c+ cells in frozen sections of small intestine using immunofluorescence microscopy. Dual color staining was used to analyze CD11c+ cells for expression of MHC class II, I-A (MHCI). Occasional CD11c+/MHCI+ cells with DC morphology were observed in sections of small intestine from control PBS-treated mice (Fig. 3). This population was greatly expanded in mice treated with Flt3L, with large numbers of CD11c+/MHCI+ cells visible in the lamina propria of both the villus and crypt regions (Fig. 3). DC were also observed in the dome and interfollicular regions of PP from control mice as previously described (9, 12), and these populations were expanded following Flt3L treatment (not shown).

### CD11c+ DC in the GALT proliferate following Flt3L-treatment, but B and T cells are not affected

Previous reports show that treating mice with Flt3L expands CD11c+ DC in the spleen without influencing mature B or T cell numbers (15). To determine whether this would also apply to cells in the intestine, the absolute number of each of the various cell types in the GALT was calculated. As expected, tissues from mice treated with Flt3L contain significantly elevated numbers of DC (Table I). There was a small, but not significant, increase in the number of B cells in some tissues from Flt3L-treated mice compared with those from PBS-treated mice and no change in the number of T cells (Table I).

To further analyze the effects of Flt3L treatment, mice were injected with BrdUrd to label cycling cells, and two-color FACS analysis was used to identify recently divided DC, B cells, or T cells (Fig. 4A). There were few CD11c+ cells in SILP from PBS-treated mice, none of which incorporated BrdUrd. In contrast, a much greater proportion of BrdUrd+ CD11c+ cells was evident in SILP from mice that were treated with Flt3L for 10 days before BrdUrd injection; indeed, almost half the CD11c+ cells detected were BrdUrd+. The relative proportion of B and T cells was smaller in the Flt3L-treated mice, owing to the massive increase in CD11c+ cells, but the absolute number of B and T cells did not significantly differ between Flt3L-treated and control mice. In both PBS- and Flt3L-treated mice, the proportion of dividing B and T cells was low, and the absolute numbers of dividing B and T cells were similar in the two groups. These data indicate that Flt3L treatment preferentially stimulates CD11c+ DC to divide without influencing mature B or T cells.

The dividing cells in PBS- and Flt3L-treated mice were also analyzed in tissue sections following BrdUrd injection. In PBS-treated mice, only the proliferating crypt epithelial cell population of the small intestine was labeled 2 h after BrdUrd injection (Fig. 4B). In Flt3L-treated mice, there were many BrdUrd+ cells obvious in the lamina propria in addition to the labeled crypt epithelial cells (Fig. 4C). The BrdUrd+ cells could be seen throughout the

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Table 1. Flt3L treatment selectively expands CD11c+ DC in vivo, but does not affect mature B or T cells

<table>
<thead>
<tr>
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<th>SILP</th>
<th>PP†</th>
<th>MLN</th>
<th>SP</th>
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<td>DC</td>
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</tr>
<tr>
<td>PBS treated</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.2</td>
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<tr>
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<td>3.8 ± 1.0*</td>
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<td>B cells</td>
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<tr>
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<td>9.7 ± 2.2</td>
<td>7.3 ± 4.3</td>
<td>89.8 ± 19.2</td>
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<tr>
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<td>15.3 ± 2.4</td>
<td>22.3 ± 7.1</td>
<td>136.8 ± 30.7</td>
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<tr>
<td>T cells</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PBS treated</td>
<td>1.5 ± 0.7</td>
<td>3.1 ± 0.2</td>
<td>14.3 ± 3.9</td>
<td>57.0 ± 4.5</td>
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<tr>
<td>Flt3L treated</td>
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<td>3.2 ± 0.1</td>
<td>23.2 ± 2.9</td>
<td>57.0 ± 6.7</td>
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* The mean cell yield per mouse is shown (n = 7 mice).
† The value attributed to PP yields are per small intestine, representative of 8 to 10 individual follicles. There were no obvious changes in the number of PP per intestine in mice treated with Flt3L.

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### Localization of CD11c+ DC in the gut of control and Flt3L-treated mice

Having observed the increase in CD11c+ DC using cells isolated from the intestinal lamina propria, we localized the CD11c+ cells in frozen sections of small intestine using immunofluorescence microscopy. Dual color staining was used to analyze CD11c+ cells for expression of MHC class II, I-A (MHCI). Occasional CD11c+/MHCI+ cells with DC morphology were observed in
crypt-villus axis of the lamina propria. These observations together with the FACS data indicating that the BrdUrd\(^1\) cells were CD11c\(^1\) DC suggest that DC in all regions of the mucosa can be expanded by Flt3L. The overall gross architecture of the mucosal tissues did not appear to be affected by Flt3L treatment.

CD11c\(^1\) cells expanded by Flt3L are resting DC, but can be activated in vivo

To determine the activation status of CD11c\(^+\) DC expanded in the gut by Flt3L treatment, CD11c\(^+\) cells were analyzed for the expression of MHCII, CD80 (B7-1), and CD86 (B7-2). Multicolor flow cytometry demonstrated that the majority of freshly isolated CD11c\(^+\) cells from both GALT and spleen expressed moderately high levels of MHCII (Fig. 5A). These CD11c\(^+\)/MHCII\(^+\) cells expressed little or no CD80 and only low levels of CD86 (Fig. 5A). This phenotype is consistent with unactivated, resting DC (13, 14), suggesting that Flt3L can expand DC in vivo without activation.

Since classical DC are known to up-regulate MHCII and other costimulatory molecules upon activation (13, 29), alterations in the expression levels of MHCII, CD80, and CD86 were examined in CD11c\(^+\) DC from Flt3L-treated mice following in vivo treatment with LPS (Fig. 5B). CD11c\(^+\) cells isolated from Flt3L-treated mice injected with LPS exhibited increased levels of MHCII, CD80, and CD86 compared with CD11c\(^+\) cells from Flt3L-treated mice injected with saline alone. Thus, the DC expanded by Flt3L are in a resting state, but can be activated by appropriate inflammatory stimuli.

Expansion of DC by Flt3L is associated with enhanced induction of oral tolerance

The massive expansion of DC in the GALT induced by Flt3L allowed us to examine the potential role of DC in determining immune responsiveness following oral administration of Ag. We decided to investigate whether the increased numbers of DC would influence oral tolerance induction using a well-established model in which mice are fed soluble OVA before parenteral challenge (1, 19). As expected, PBS-treated mice fed 25 mg of OVA before immunization with OVA plus adjuvant displayed markedly reduced DTH responses when rechallenged with OVA in vivo compared with saline-fed immunized control mice (Fig. 6A). Cells from the draining LN of these animals also showed suppressed Ag-specific proliferative reactivity after restimulation with OVA in vitro (Fig. 6B). T cell responses in vivo and in vitro were essentially normal in PBS-treated mice fed 0.5 or 0.01 mg of OVA (Fig. 6). These results are consistent with many previous reports (1, 19, 30) and illustrate the dose-dependent tolerogenic effects of OVA feeding on T cell-dependent immune responses.

Unexpectedly, mice treated with Flt3L to expand DC before being fed OVA had more profound tolerance than equivalent OVA-fed control mice, as determined by both in vivo and in vitro parameters (Fig. 6). The enhanced unresponsiveness induced by Flt3L treatment was illustrated most clearly in mice that were fed low doses of Ag. Mice pretreated with Flt3L before being fed 0.5 or 0.01 mg of OVA displayed significantly decreased T cell responses, unlike PBS-treated animals fed the same doses (Fig. 6, A and B). The tolerance induced by low doses of fed OVA was generally of a magnitude comparable to that seen in PBS-treated mice fed the highest dose of Ag.

A similar pattern of enhanced tolerance induction in Flt3L-treated mice was seen when humoral immunity was examined. Ag-specific total IgG, IgG1, and IgG2a levels in the sera of PBS-treated mice fed 25 mg of OVA were significantly decreased compared with those in immunized mice, while feeding low doses (0.5 or 0.01 mg) had no effect (Fig. 7). In contrast, humoral immune
responses were significantly decreased in mice that were fed all doses of OVA after treatment with Flt3L (Fig. 7). Notably, the levels of both IgG1 and IgG2a isotypes were decreased to similar extents in these mice, suggesting that Th2- and Th1-dependent immune responses were equally diminished in the Flt3L-treated OVA-fed mice.

Evidence of enhanced tolerance of Ag-specific T cells in Flt3L-treated mice following Ag feeding

To evaluate further the ability of Flt3L-mediated DC expansion to enhance the induction of tolerance in specific T cells by feeding Ag, we used an adoptive transfer model in which OVA-specific transgenic T cells from D011.10 mice are transferred into normal syngeneic BALB/c hosts. Previous studies have shown this to be an appropriate means of studying peripheral and oral tolerance of T cells in a relatively physiologic manner (20, 21), and transgenic T cells can be easily detected with the clonotypic Ab (KJ1.26) to the transgenic TCR.

When adoptively transferred PBS-treated mice were fed saline and immunized with OVA in adjuvant, there was a marked expansion of transgenic cells in the draining LN. The relative proportion and the absolute number of transgenic T cells were significantly decreased in PBS-treated mice fed 25 mg of OVA before immunization compared with saline-fed PBS-treated mice (Fig. 8). The expansion of transgenic T cells in Flt3L-treated adoptive transfer mice fed saline before immunization was comparable to
that of PBS-treated mice fed saline. As in PBS-treated mice, the relative proportion and the absolute number of transgenic cells were decreased in Flt3L-treated mice fed 25 mg of OVA before immunization (Fig. 8). The major difference between PBS-treated and Flt3L-treated mice was most evident in animals fed a low dose of OVA. Feeding 1 mg of OVA to PBS-treated mice did not prevent the expansion of Ag-specific T cells, whereas feeding 1 mg of OVA to Flt3L-treated mice did result in a decrease in both the relative proportion and absolute number of transgenic T cells (Fig. 8).

Enhanced functional tolerance in Flt3L-treated OVA-fed adoptive transfer mice

The reduced expansion of transgenic T cells in OVA fed Flt3L-treated adoptive transfer mice was accompanied by enhanced functional tolerance (Fig. 9). LN cells from both PBS- and Flt3L-treated mice that were fed saline before immunization proliferated vigorously in response to OVA stimulation in vitro, whereas cells isolated from PBS- and Flt3L-treated mice fed 25 mg of OVA showed significantly decreased proliferative responses. Again, the differences between PBS- and Flt3L-treated mice were most evident in animals fed a low dose of Ag. Cells from the Flt3L-treated mice fed 1 mg of OVA had significantly decreased Ag-specific proliferative capability compared with equivalently fed PBS-treated mice and saline-fed animals (Fig. 9).

Discussion

The balance between active immunity and tolerance is critical at sites such as the intestine, where mucosal lymphoid tissues are only separated from the huge antigenic load in the gut lumen by a single layer of epithelial cells (31). Although it is essential that effective immunity be maintained to protect the mucosa from invasion by harmful pathogens, it is also important that immune responses are not mounted against potentially beneficial Ags such as food and commensal bacteria. The potential consequences of aberrant intestinal responses of this type are illustrated by the findings that ablation of any one of a number of immunoregulatory components can give rise to active gut inflammation (31–33). However, it is still unclear how this balance is regulated.

The way in which intestinally derived Ags are processed and presented to T cells is likely to be a critical factor in determining how the mucosal immune system can distinguish pathogens from harmless Ags. Ag presentation in the intestine has been the focus of many studies, and the gut contains many different types of conventional APC, including DC, B cells, and macrophages, as well as other putative APC, such as MHCII-expressing epithelial cells (reviewed in Ref. 31). The relative contributions of these different APC types are likely to be an important factor in determining whether active immunity or tolerance is induced at any particular time. The data presented here

mice. There were no obvious differences in the number of proliferating B or T cells in Flt3L-treated vs PBS-treated mice. The data shown are representative of three experiments, and similar results were obtained in all tissues from both C57BL/6 and BALB/c mice. B and C, PBS- or Flt3L-treated mice were injected with BrdUrd 2 h before tissue harvest. Tissues were fixed and paraffin embedded, and immunoperoxidase staining was used to localize proliferating cells. BrdUrd+ cells can be identified by the presence of a brown reaction product. In PBS-treated mice, only the proliferating crypt epithelial cells were labeled (B). In Flt3L-treated mice, there was an increase in labeled cells throughout the lamina propria in addition to the labeled crypt epithelial cells (C). The data shown are representative of five mice per group.
show clearly that increasing the numbers of DC in vivo, by treating mice with Flt3L, enhances the induction of tolerance in two models induced by feeding soluble protein. In the first, we found that conventional mice fed OVA developed a more profound systemic tolerance when treated with Flt3L before feeding, and this was particularly notable in mice fed low doses of...
Ag. In the second model, we used a recently characterized adoptive transfer system to assess directly the behavior of Ag-specific T cells following the induction of oral tolerance (21). In these experiments we confirmed that the expansion of Ag-specific transgenic T cells in response to systemic challenge is reduced after oral tolerance induction (21) and observed that the effect is augmented by Flt3L.

At first sight these results are not consistent with the classical assumption that DC have an apparent constitutive ability to activate T cells or with previous findings that intestinal DC isolated from protein-fed mice can be potently immunogenic (11). These discrepancies may reflect the fact that most previous studies have used isolated DC, employing protocols likely to induce costimulatory molecule expression (13, 14). Since a role for DC in the induction of tolerance may have been overlooked previously due to the use of inadvertently activated cells, we decided to explore the function of DC under physiologic conditions in situ. As we confirm here, the DC expanded by Flt3L in vivo are apparently in a resting state, with low or no expression of CD80, CD86. However, these resting DC remain fully responsive to inflammatory stimuli, as administration of LPS to Flt3L-treated mice induces significant increases in the expression of MHCII, CD80, CD86, and CD40 on the DC. These cells are thus in a good position to regulate the nature of the immune response to intestinal Ags depending on the presence or the absence of appropriate inflammatory signals. We are currently testing the conjecture that the enhanced tolerance associated with expansion of DC will be reversed by converting the DC from a resting phenotype to an activated phenotype. Our hypothesis that DC may be involved in oral tolerance induction is further supported by studies which show that targeting DC with Abs in vivo does allow these cells to present Ag in a tolerogenic as well as an immunogenic fashion (34). In addition, it has been previously suggested that donor-derived hepatic DC may be involved in the maintenance of donor-specific unresponsiveness in liver allograft recipients (35, 36).
How does increasing the number of DC lead to enhanced tolerance? One possibility is that there are simply more DC accessible to pick up the available Ag, and therefore, there is an increased probability that more Ag-specific T cells can interact with a tolerogenic Ag/APC complex. However, this still leaves the question of whether DC can be tolerogenic APC. There is accumulating evidence that it is the qualitative nature of the interaction between APC and Ag-specific T cells that determines whether tolerance is induced. It has been hypothesized that T cell tolerance occurs when APC expressing low levels of CD80/86 interact with naive T cells via the high affinity CTLA4 receptor in preference to the CD28 receptor, which delivers an activating stimulus after interacting with APC expressing high levels of CD80/86. This hypothesis is supported by a recent study demonstrating that peripheral tolerance can be prevented by using neutralizing Abs directed against CTLA4 (37). Our data show that DC in the intestine normally express minimal levels of CD80/86 and therefore may be potential candidates for presenting intestinally derived Ags to T cells in a tolerogenic fashion. Studies are currently underway investigating the roles of CD80/86 and CTLA4 interactions in the induction of oral tolerance.

The intestine clearly provides a unique environment allowing for the development of both tolerogenic and immunogenic responses, but whether this is regulated purely at the level of co-stimulatory molecule expression on a homogeneous population of DC is unclear. An alternative explanation might be that discrete subsets of intestinal DC are responsible for different functions. Recent studies of DC have identified at least two distinct lineages of cells derived from different precursors, and some evidence indicates that these populations might be functionally distinct (15, 23–28). In our studies, all the putative lineages and subpopulations of DC were present in intestinal tissues of Flt3L-treated mice. Their proportions were relatively similar to those found elsewhere, and we were unable to identify any novel subsets of cells in the GALT, suggesting that the intestine is unlikely to harbor a unique regulatory DC population.

Although we observe enhanced tolerance associated with Flt3L treatment, the origin and the precise location of the DC involved in induction of oral tolerance remain to be clarified. Is there a population of tolerogenic DC present in the gut in normal mice, or are tolerogenic DC recruited de novo by Flt3L? Our immunohistologic analysis shows that the greatest increases in the numbers of CD11c+ cells in Flt3L-treated mice can be seen in the tissues of OVA-fed mice. Their proportions were relatively similar to those found elsewhere, and we were unable to identify any novel subsets of cells in the GALT, suggesting that the intestine is unlikely to harbor a unique regulatory DC population.

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suggesting that the mechanistic process of tolerance induction is comparable in Flt3L-treated mice following a single feeding of either a high or a low dose of Ag. Although these findings could reflect either clonal deletion or anergy of Ag-specific T cells following contact with fed Ag, it is tempting to speculate that the marked decrease in the absolute numbers of T cells after the induction of tolerance indicates that deletion may be the dominant mechanism in Flt3L-treated mice. Proof of this idea will require direct assessment of apoptosis in Ag-specific T cells in Ag-fed mice. In addition to the hypothesis that clonal deletion and anergy of Ag-specific T cells can give rise to tolerance, there is a great deal of evidence suggesting that tolerance induced by feeding multiple low doses of Ag is mediated by regulatory cells that secrete cytokines such as IL-4, IL-10, and TGF-β (3). Since we used only single dose feedings throughout this study, it is not clear whether the tolerance seen in Flt3L-treated mice following low dose feeding will be mediated by this type of active suppression. We are currently in the process of determining whether there is evidence for cytokine biasing and increased induction or recruitment of regulatory cells in Flt3L-treated mice following Ag feeding.

The ability to modulate oral tolerance induction with Flt3L in vivo highlights a potentially central role for DC in regulating mucosal immune responses. This has important implications for investigating how and where orally administered proteins are presented and processed to the presented immune system. Thus, it should now be possible to explore where the initial contact between APC and T cell occurs after Ag feeding, to determine the subsequent fate of Ag-reactive T cells, and to define the immunologic consequences of these interactions more precisely. These investigations will help in understanding the mechanisms of oral tolerance as well as increase our understanding of how the mucosal immune system discriminates between harmful and beneficial Ags. This information will assist vaccine design and aid in designing regimens aimed at applying mucosal tolerance as a treatment for human disease, where oral tolerance has been promoted as therapy for a number of clinical disorders, including rheumatoid arthritis, multiple sclerosis, and uveitis (reviewed in Ref. 3).

References
