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Modulating Dendritic Cells to Optimize Mucosal Immunization Protocols

Eilidh Williamson, Gina M. Westrich, and Joanne L. Viney

Oral administration of soluble protein Ag induces tolerance, a phenomenon that has hampered mucosal vaccine design. To provoke active immunity, orally administered Ag must be fed together with a mucosal adjuvant such as cholera toxin (CT). Unfortunately, CT is not suitable for clinical use because of its associated toxicity. There is, therefore, a need to develop alternative mucosal immunization regimens. Here we have attempted to alter the intrinsically tolerogenic nature of the intestine and improve immunization potential by expanding and activating intestinal APC in vivo. Previous studies have indicated that intestinal dendritic cells (DC) present oral Ag, but do so in a tolerogenic manner. In the present study we investigated whether DC can be converted from tolerogenic into immunogenic APC by treating mice with Flt3 ligand (Flt3L), a DC growth factor, and then immunizing with CT. We observed increased local and systemic responses to CT in the presence of elevated numbers of intestinal DC. In parallel, CT induced up-regulation of CD80 and CD86 on these Flt3L-expanded DC. In an attempt to develop a toxin-free adjuvant system, we investigated whether IL-1 could be used as an alternative DC-activating stimulus. Using a combination of Flt3L and IL-1α, we observed a potent active response to fed soluble Ag, rather than the tolerogenic response normally observed. These data suggest that Flt3L-expanded DC are well positioned to regulate intestinal responses depending on the presence or the absence of inflammatory signals. Flt3L may therefore be a reagent useful for the design of mucosal immunization strategies. *The Journal of Immunology*, 1999, 163: 3668–3675.

The intestinal immune system has developed sophisticated methods of immunoregulation to effectively discriminate between potentially harmful pathogens and essential dietary Ag (1, 2). A fundamental principle of mucosal immunology is therefore that most soluble protein Ags introduced via the mucosal route are poorly immunogenic and, unless administered in the presence of an appropriate adjuvant, induce a state of specific, long-lasting hyporesponsiveness termed mucosal tolerance (2–4).

The poor immunogenicity of mucosally administered proteins has been a major obstacle to the development of efficient oral vaccines. This has initiated many studies aimed at developing mucosal vaccine adjuvants. Of these, cholera toxin (CT) is arguably the most potent mucosal adjuvant yet described. Oral administration of CT elicits specific T and B cell-mediated immunity that protects against subsequent intestinal challenge (5–10). Moreover, feeding CT prevents mucosal tolerance to coadministered soluble protein Ags, (5–7, 11). However, although CT is generally very effective as a mucosal adjuvant in research animals, it is not well tolerated in humans and induces diarrhea in healthy volunteers (12). Mutant CT molecules have been generated that retain adjuvant activity in the absence of toxicity (13–16), and these have provided a useful standard for the characterization of additional safe and effective mucosal immunization regimens. Gaining a better understanding of the events that lead to tolerance vs active immunity in the mucosa is therefore clearly required.

Years of studying peripheral immune responses has revealed that the manner in which Ags are presented to T cells can critically determine the outcome of an immune response. Dendritic cells (DC) are thus reported to be the most potent immunostimulatory APC (17) and are classically associated with the induction of active immune responses. However, the majority of the studies providing this information have been performed using in vitro culture conditions, making it difficult to draw conclusions about the function of these cells in their natural in vivo state. The recent identification of Flt3 ligand (Flt3L), a growth factor that dramatically expands DC in vivo without inducing their activation (18, 19), has provided an important tool with which to study DC in situ. In an earlier study, we found that treating mice with Flt3L enhanced the level of mucosal tolerance elicited by feeding the soluble protein Ag OVA (20). Although these studies indicated that intestinal DC may act as tolerogenic APC, they raised the question of whether both tolerogenic and immunogenic responses can be elicited by DC in the gut. Can both types of response be mediated by this single APC type, and at what level is this regulated?

One mechanism by which adjuvants are assumed to promote T cell activation and prevent tolerance is by stimulating the production of proinflammatory cytokines such as IL-1, IL-6, and TNF-α, which, in turn, up-regulate costimulatory molecule expression on APC (21, 22). Because the intestinal DC expanded by Flt3L express minimal levels of the costimulatory molecules CD80 and CD86 in vivo (20), these cells may be well equipped to preferentially favor tolerance in the absence of any exogenous inflammatory signals. Interestingly, the mucosal immunogenicity and adjuvanticity of CT are known to be associated with IL-1 induction (23, 24), and the adjuvancy effects of CT can be reversed by anti-IL-1 treatment (23).

The focus of the present study was therefore to investigate whether intestinal DC expanded by Flt3L treatment are able to support the initiation of active mucosal responses and whether this
property could be used to advance mucosal immunization regimens. Specifically, we analyzed the effect of DC expansion on the local and systemic responses induced by oral immunization with CT. In addition, we sought to identify a means to reverse the profound tolerance usually induced by feeding soluble protein Ag. Using a combination of Flt3L and IL-1α, we were able to effectively immunize mice given soluble OVA perorally, thereby overcoming the default tolerogenic response induced by feeding Ag. These results demonstrate that intestinal DC can be either tolerogenic or immunogenic APC depending on their activation status. Furthermore, our findings highlight a potential alternative strategy for effective mucosal vaccines.

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 Immunix (Seattle, WA) in accordance with approved ethical guidelines. BALB/c DO11.10 OVA TCR transgenic (Tg) mice (25) were bred and maintained in the specific pathogen-free facility at Immunix.

In vivo treatment of mice with Flt3L

Flt3L-treated mice were injected i.p. once daily with purified CHO-derived human Flt3L (10 μg in 100 μl of PBS) for the number of days indicated. Control mice received 100 μl of PBS i.p. for the same period. Flt3L was produced and purified at Immunix as previously described (18).

Oral immunization with CT

Mice were immunized with three oral doses of 2.5, 10, or 20 μg of CT (Sigma, St. Louis, MO) in 200 μl of saline by gavage. Control mice were fed 200 μl of saline alone. Mice were treated with Flt3L or PBS throughout the period of CT immunization, starting 5 days before the first CT feed and ending on the day after the final CT feed. Immunizations were given at 10-day intervals. Analyses of CT-specific protection (ligated loop test; see below) as well as measurement of serum and mucosal anti-CT Ab levels were performed 1 wk after the final immunization.

Ligated loop test for CT-specific protection

The ligated loop test was performed as previously described (6). Injecting CT into a ligated intestinal loop induces dramatic secretion of electrolytes and fluid. Oral immunization with CT before intestinal loop challenge protects against these CT-mediated effects. Thus, mice that have been orally immunized with CT exhibit reduced fluid accumulation after challenge compared with unimmunized animals. Briefly, to perform the ligated loop test, mice were anesthetized, the abdomen was opened, and a 5- to 8-cm loop was ligated in the mid-ileum. Six micrometers of CT in 0.2 ml of saline was then injected into the loop, and the abdomen was closed with sutures. Four hours after intestinal CT challenge, the mice were sacrificed, and the CT-induced fluid accumulation was determined by weighing the ligated loop with its fluid content and measuring its length. The weight per length ratio in milligrams per centimeter was then calculated, and the protection afforded by prior immunization with CT was expressed as the percent protection relative to that in control unimmunized mice.

Collection of intestinal fluid for analysis of CT-specific Abs

Intestinal fluid for the measurement of CT-specific mucosal IgA Ab titers was obtained using a modification of a published technique (26). Mice were deprived of food for 18 h before being fed 0.2 ml of 16% (w/v) polyethylene glycol MW 3550 (Sigma) in protein-free PBS by gavage four times at 15 min intervals. Mice were then sacrificed, and the entire small intestine was removed and flushed through with 10 ml of PP-PBS containing 400 μl of protease inhibitors (Complete Protease Inhibitor Mixture tablets used according to the manufacturer’s instructions; Boehringer Mannheim, Indianapolis, IN). After vortex mixing vigorously, the resultant intestinal fluid was centrifuged at 2,500 rpm for 10 min at 4°C. Three milliliters of supernatant was transferred to a fresh tube, mixed with 100 μl of protease inhibitors, and centrifuged at 18,500 rpm for an additional 20 min at 4°C. Finally, 1 ml of clarified supernatant was removed, mixed with 50 μl of PBS (Life Technologies, Gaithersburg, MD), and transferred to an Eppendorf tube.

Measurement of CT-specific Abs in serum and intestinal fluid

To obtain serum from mice that had been orally immunized with CT, blood was collected by cardiac puncture. Intestinal fluid was collected as described above. Sera and intestinal fluid were assayed by ELISA using a modification of a previously published method (27). Briefly, to measure CT-specific IgA and IgG levels, 96-well ELISA plates (Maxisorp, Nunc, Naperville, IL) were coated with 0.5 nmol/ml GM1 ganglioside (Sigma) in PBS at 4°C overnight and washed twice with PBS/0.1% Tween-20. Plates were then coated with 0.5 μg/ml CT (Sigma) in PBS overnight at 4°C, blocked with PBS containing 5% FBS for 1 h at 4°C, and washed with PBS/0.1% Tween-20. Serum or intestinal fluid samples were diluted in PBS (starting dilutions, 1/50 or 1/10, respectively, for IgA; 1/200 for serum IgG), and serial 3-fold dilutions were made. Plates were incubated at 4°C overnight, washed, and incubated with alkaline phosphatase-conjugated anti-IgA or anti-IgG (1/1000 or 1/3000, respectively; Sigma) detecting Ab for an additional 2 h at room temperature. Plates were washed again, and enzyme activity was detected with p-nitrophenyl phosphate disodium (Sigma). The amount of reaction product was assessed on an ELISA plate reader at OD405 nm using the Deltasoft program (DeltaPoint, Monterey, CA).

In vitro effect of CT on Flt3L-expanded DC

Mesenteric lymph nodes (MLN) and Peyer’s patches (PP) were harvested from mice that had been pretreated with Flt3L for 10 days. Single cell suspensions were prepared by teasing the tissues apart in RPMI 1640 medium containing 10% FBS, followed by passage over nylon mesh. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin/streptomycin, and 2-ME (complete RPMI) at 37°C in a humidified 5% CO2 incubator at a density of 2 × 106 cells/ml. Cells were cultured in 24-well flat-bottom plates in a total volume of 1 ml, either alone or in the presence of 5 μg of CT. After 20 h the cells were aspirated and washed twice in complete RPMI, and the expression of costimulatory molecules on the CD11c+ DC population was analyzed by flow cytometry (see below).

Preparation of RNA from mice intestinally challenged with CT and measurement of CT-induced cytokine mRNA

To examine CT-induced cytokine levels in vivo, mice were anesthetized, intestinal loops were ligated as described above, and 6 μg of CT (or PBS control) was injected. Four hours after this challenge, the mice were sacrificed, and the intestinal loops were removed. After carefully removing the fecal contents, the intestinal loops were homogenized in guanidinium isothiocyanate buffer (4.5 M guanidinium isothiocyanate, 50 mM sodium citrate, and 0.5% (w/v) sodium sarcosyl) containing 2% 2-ME (Life Technologies), and the RNA was isolated over a cesium cushion. CT-induced IL-1β and IL-6 levels were then measured in each of the RNA samples using the Riboquant Multiprobe RNase Protection Assay kit mCK-2b (PharMingen, San Diego, CA) according to the manufacturer’s instructions.

Activation of Flt3L-induced DC in vivo

Mice that had been pretreated with Flt3L for 10 days received two i.p. injections of 1 μg of IL-1α, 100 μg of LPs (Sigma), or saline, given 12 h apart. MLN and PP were harvested 4 and 24 h after the final injection, and single-cell suspensions were prepared by teasing tissues apart in complete RPMI, followed by passage over nylon mesh. The expression of costimulatory molecules on the CD11c+ DC population was analyzed by flow cytometry (see below).

Flow cytometric analysis of isolated cells

Isolated cells were incubated for 30 min with primary Ab at 5 μg/ml at 4°C in the presence of 30% mouse serum, washed twice with PBS/2% FBS, then incubated with 10 μg/ml allophycocyanin-labeled streptavidin (10 μg/ml Molecular Probes, Eugene, OR) for an additional 20 min. Samples were washed three times, resuspended in PBS supplemented with 1% paraformaldehyde, and stored at 4°C until analysis on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). At least 30,000 cells were analyzed per sample.

Monoclonal Abs

The following mAbs were used: anti-CD11b (M1/70, rat IgG2b), anti-CD11c (HL3, hamster IgG), anti-CD80 (1G10, rat IgG2a), anti-CD86 (NCO, rat IgG2a), anti-CD40 (3/23, rat IgG2a), anti-type I IL-1R (12A6, rat IgG2a), rat IgG2b isotype control (R35-38), hamster IgG isotype control (G235-2356), and rat IgG2a isotype control (R35-95; all purchased from PharMingen).
DC AND INTESTINAL IMMUNOREGULATION

Assessment of Ag-specific T cell responses in adoptive transfer mice

For adoptive transfer of OVA TCR Tg T cells, syngeneic BALB/c mice were injected i.v. with 2.5 × 10^6 clonotypic TCR^+ (CD4^+ KJ1-26^+) Tg cells from DO11.10 mice, as described (28). Groups of 5 BALB/c mice were treated with Flt3L or PBS for 8 days before, and 2 days after, adoptive transfer of Tg cells. Mice were fed a single dose of 25 mg OVA in 0.2 ml saline 2 days after transfer of Tg T cells, and immunized s.c. in the footpad with 100 µg OVA in RIBI adjuvant 5 days later. After another 4 days, draining PLN were removed and the proportion of Tg cells in individual mice was determined by FACS analysis as described above. To determine the proportion of OVA specific CD4^+ Tg T cells in adoptive transfer mice, cells were incubated with mAb KJ1-26 FITC, which detects the clonotypic Tg TCR, and anti-CD4 PE (PharMingen) in 50 µl of blocking buffer containing 10 µg/ml anti-CD16 (PharMingen), 10% normal goat serum, and 1% normal mouse serum.

Assessment of functional 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 high dose feed of 25 mg of OVA (fraction V; Sigma) in 200 µl 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 Immunoc- hemicals, Hamilton, MT). Three weeks after immunization, mice were assayed for systemic 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 previously described (29). Mice were then bled by cardiac puncture, and OVA-specific serum Ab titers were measured (see below).

Analysis of OVA-specific serum Ab titers

OVA-specific serum IgG levels were measured by ELISA as previously described (20).

Statistical analysis

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

Results

Expanding DC with Flt3L alters the anti-CT response following mucosal immunization

To determine whether Flt3L-induced DC in the gut-associated lymphoid tissues (GALT) can promote active mucosal responses, we orally immunized Flt3L-treated mice with CT. Dose-response studies using the technique of CT challenge in ligated intestinal loops were conducted to optimize conditions for CT immunization. In our hands, three oral doses of 20 µg of CT afforded maximal CT-specific protection in PBS-treated control mice (68 ± 7% protection; Fig. 1A). When the immunizing dose of CT was lowered, the level of protection observed in PBS-treated CT immunized mice was concordantly reduced. Thus, three oral doses of 10 µg of CT afforded only 44% protection in PBS-treated mice (Fig. 1B), while 2.5 µg of CT was insufficient to protect most mice from CT challenge (Fig. 1C).

Flt3L-treated mice which had been orally immunized with a high dose of 20 µg of CT showed similar resistance to intestinal CT challenge to PBS-treated, CT-immunized mice (Fig. 1A). However, more striking was the effect on suboptimal doses of CT, which afforded only modest or no protection in PBS-treated mice, but still provided maximal protection in Flt3L-treated mice. Thus, we found that Flt3L-treated mice immunized with a 10-µg dose of CT were significantly more resistant (p < 0.001) than equivalent PBS-treated mice immunized with this dose of CT (Fig. 1B). Strikingly, Flt3L-treated mice immunized with a very low dose of 2.5 µg of CT were also resistant to subsequent CT challenge (Fig. 1C). This was in direct contrast to equivalently immunized PBS-treated animals, the majority of which were not resistant to CT challenge (32 vs 7% protection). These results clearly indicate that Flt3L-treatment dramatically enhanced the local anti-CT response.

Effect of Flt3L treatment on local and systemic anti-CT humoral responses following mucosal immunization

A prerequisite for effective mucosal vaccines is the stimulation of local mucosal IgA production (8, 30). Previous studies have indicated a strong association between intestinal CT-specific protection and local IgA Ab levels (9, 31). We therefore next examined the effect of Flt3L on anti-CT IgA Ab production. As shown in Fig. 2, the increased resistance against intestinal fluid accumulation following CT challenge observed in Flt3L-treated mice was accompanied by dramatically enhanced anti-CT humoral responses. Flt3L-treated mice that had been orally immunized with 10 µg of CT exhibited significantly elevated levels of CT-specific IgA Ab titers in both intestinal fluid (Fig. 2A) and serum (Fig. 2B) compared with similar PBS-treated mice. Similar results were achieved with both the 20- and 2.5-µg doses of CT (data not shown). The fact that CT-specific IgA Ab levels were increased in both intestinal secretions and serum samples from Flt3L- vs PBS-treated mice, clearly demonstrates that Flt3L can enhance both local and systemic responsiveness to an orally administered immunogen.

Both PBS- and Flt3L-treated mice exhibited similarly high serum levels of CT-specific IgG following CT immunization (Fig. 2C).

CT activates Flt3L-expanded DC in GALT

The above results indicate that Flt3L-expanded intestinal DC not only support, but actually enhance, mucosal immunity. Our previous work demonstrated that expanding DC with Flt3L could also enhance the induction of mucosal tolerance (20). This raised the intriguing question of how both immunogenic and tolerogenic responses can be heightened in the presence of increased DC numbers. The intestinal DC expanded by Flt3L express very low levels of the costimulatory molecules CD80 and CD86 in situ (20), a phenotype consistent with resting, unactivated DC (32, 33). There-fore, one possibility is that DC in the GALT preferentially favor tolerance induction in the absence of exogenous signals to promote their activation. To test the hypothesis that CT activates GALT DC, we examined CD80/86 levels on these cells following stimulation with CT in vitro. The procedure of culturing DC in vitro promoted a degree of CD80/86 up-regulation, consistent with previous reports (32, 33). However, the addition of CT to these cultures for 20 h clearly promoted a further marked up-regulation of

![FIGURE 1. Expanding DC with Flt3L enhances mucosally induced responses against CT. Effect of Flt3L on protection against CT challenge in ligated intestinal loops. Groups of 8–10 PBS- or Flt3L-treated mice were orally immunized three times with 20 µg (A), 10 µg (B), or 2.5 µg (C) of CT at 10-day intervals. One week after the final immunization, mice were challenged with 6 µg of CT in ligated intestinal loops, and 4 h later, the CT-induced fluid accumulation was measured as weight per centimeter of intestinal loop. The protection afforded by prior oral immunization with CT was calculated and expressed as the percent protection relative to that in control unimmunized mice. The data presented are the mean percent protection ± 1 SEM and are representative of four separate experiments. *, p < 0.005; **, p < 0.001 (Flt3L- vs PBS-treated mice).](http://www.jimmunol.org/DownloadedFrom/WWW/jimmunol.org/download/13.2017/3670)
CD80 and CD86 on CD11c⁺ DC from both the PP (Fig. 3) and MLN (data not shown) of Flt3L-treated mice. CT did not affect the levels of CD40 expressed on CD11c⁺ DC (data not shown). These findings demonstrate that CT can induce increased CD80/86 expression on GALT DC, thereby initiating their activation and potentially increasing their immunogenicity.

CT induces proinflammatory cytokine production in the intestine

What is the mechanism by which CT activates DC in the GALT and converts them from tolerogenic APC to immunogenic APC? Adjuvants are thought to promote T cell activation at least in part by inducing proinflammatory cytokine production, which may then up-regulate costimulatory molecule expression on APC (21, 22). We therefore examined whether CT can induce the production of proinflammatory cytokines in the intestine. Using RNase protection assays, we found that the expression levels of both IL-1 (3- to 4-fold increase) and IL-6 (2- to 3-fold increase) RNA were significantly increased following intestinal challenge with CT (Fig. 4). As such, it appears that CT is capable of inducing an inflammatory response in the intestine, during which both IL-1 and IL-6 are up-regulated.

Flt3L-expanded DC can be activated by proinflammatory cytokines in vivo

We and others (23, 24) have shown that CT induces the secretion of proinflammatory cytokines such as IL-1, IL-6 and TNF-α. We therefore next assessed whether Flt3L-expanded DC in the GALT can be activated to express increased CD80/86 levels when such inflammatory cytokines are provided in vivo. We decided to focus these studies on IL-1, particularly since it has been demonstrated previously that the adjuvancy effects of CT can be reversed by anti-IL-1 treatment in vitro (23). Furthermore, as shown in Fig. 5,
The histograms represent the levels of CD80/CD86 costimulatory molecules injection and stained with anti-CD11c and either anti-CD80 or CD86 Abs. or saline, 12 h apart. MLN and PP were harvested 4 h after the final representative of four separate experiments.

Flt3L-expanded DC express high levels of the type I IL-1R, providing indirect evidence that they may be responsive to this cytokine. Interestingly, myeloid-derived DC (CD11b^+CD11c^-) expressed higher levels of type I IL-1R than their lymphoid-derived (CD11b^-CD11c^+) counterparts, which may reflect differential responsiveness to IL-1 between the different putative DC subsets. This possibility is currently being examined in detail.

To examine the ability of IL-1 to activate DC in vivo, Flt3L-treated mice were injected i.p. with IL-1α or LPS, and the levels of CD80/86 on CD11c^- DC in PP and MLN were examined 4 h later. Both IL-1 (Fig. 6) and LPS (data not shown) treatments greatly increased the levels of CD80 and CD86 molecules expressed on CD11c^- cells isolated from the GALT of Flt3L-treated mice. This increase was maintained at 24 h (data not shown). Thus, Flt3L-expanded DC are fully responsive to the inflammatory cytokines that can be elicited by CT. These mediators promote DC maturation and activation, as demonstrated by CD80/86 costimulatory molecule up-regulation.

Effect of DC activation on Ag-specific T cell responses in vivo

A major obstacle in the development of mucosal vaccines is that most mucosally administered proteins induce tolerance, highlight-
OVA feeding we observed a dramatic increase in the number of CD4<sup>+</sup> KJ1-26<sup>+</sup> Tg T cells in the draining lymph nodes (Fig. 7). This increase was evident in terms of both the relative proportion (Fig. 7A) and absolute number (Fig. 7B) of CD4<sup>+</sup> Tg T cells and exceeded the number of Tg T cells observed in mice treated with Flt3L (with or without IL-1<sub>0</sub>) in the absence of OVA Ag. The above results clearly show that IL-1<sub>0</sub> is able to prevent tolerance induced by the oral route, as evidenced by the accumulation of Ag-specific Tg T cells in the draining lymph nodes of transfer mice, and can be used in combination with Flt3L as a means of inducing mucosal immunization.

**DC can be converted from functionally tolerogenic APC into immunogenic APC following activation with IL-1**

To further evaluate the ability of IL-1 to prevent oral tolerance and act in combination with Flt3L to induce active immunity, we assessed whether functional tolerance could be abrogated in Flt3L-treated mice by activating DC in vivo with IL-1α before OVA feeding in a conventional mouse model.

PBS-treated mice fed 25 mg of OVA before immunization with OVA in adjuvant had strikingly reduced DTH responses when rechallenged with OVA in vivo compared with saline-fed immunized control mice (Fig. 8). In addition, these animals showed significantly decreased levels of Ag-specific total IgG titers in their serum compared with saline-fed controls (Fig. 9). Mice treated with Flt3L to expand DC before OVA feeding exhibited more profound tolerance than equivalent PBS-treated mice fed OVA as assessed by these in vitro and in vivo parameters (Figs. 8 and 9), consistent with previous findings (20).

In contrast, we found that administering IL-1<sub>0</sub> before OVA feeding not only abrogated oral tolerance in PBS-treated mice fed OVA, but also reversed the more profound tolerance exhibited by Flt3L-treated OVA-fed mice (Figs. 8 and 9). The ability of IL-1<sub>0</sub> to reverse tolerance was evident in terms of both cell-mediated (Fig. 8) and humoral (Fig. 9) immunity. These findings clearly demonstrate that IL-1<sub>0</sub> is able to prevent the induction of oral tolerance in both PBS- and Flt3L-treated mice and to induce an active systemic response.

**Discussion**

Our studies provide direct in vivo evidence that DC are pivotally involved in directing the balance between tolerance and active immunity at mucosal sites. We found that CT activates Flt3L-expanded DC in the GALT to express high levels of the costimulatory molecules CD80 and CD86. In parallel, protective responses to CT were enhanced in Flt3L-treated mice, indicating that these highly activated intestinal DC were capable of supporting and enhancing an effective stimulatory mucosal response. We also found that CT elicits the production of the proinflammatory cytokines IL-1<sub>0</sub> and IL-6 in the intestine. Furthermore, the ability of CT to activate intestinal DC can be duplicated by administering IL-1<sub>0</sub> in vivo. Administration of IL-1<sub>0</sub> promoted activation of Flt3L-induced DC and prevented mucosal tolerance. Thus, the combination of Flt3L and IL-1<sub>0</sub> resulted in active immune responsiveness to orally administered soluble protein Ag. Taken together, our findings suggest that Flt3L-expanded DC in the intestine may have the potential to regulate mucosal immune responses and that inflammatory mediators modulate their function, converting them from tolerogenic into immunogenic APC. As such, our data demonstrate that Flt3L may have properties pertinent to the design of mucosal adjuvants and vaccines.

The intestinal environment clearly allows for the development of both tolerogenic and immunogenic responses, and in the healthy individual, this is tightly regulated. Our previous studies showed that DC in the intestine are centrally involved in the induction of mucosal tolerance (20), leading us to question whether intestinal DC expanded by Flt3L are intrinsically different from the classical stimulatory DC present in peripheral lymphoid organs (17). As such, we questioned whether these intestinal DC might be unable...
to initiate active immune responses. The data presented here indicate that this is not the case. Compared with PBS-treated controls, Flt3L-treated mice that had been orally immunized with a suboptimal dose of CT showed markedly increased CT-specific protection against subsequent intestinal challenge. Most strikingly, Flt3L-treated animals exhibited significant anti-CT protective responses when immunized with very low doses of CT that were essentially ineffective in PBS-treated controls. We believe that the fact that Flt3L compensates for low dose (2.5 and 10 μg) CT immunization can be explained by the greatly increased number of DC available to process and present CT to naïve T cells. As such, there may be an increased probability of an efficient immunogenic encounter between an Ag-loaded, activated DC and a naïve T cell. However, this Flt3L-induced enhancement is not observed at the higher 20-μg dose, at which maximal CT protection is already observed.

Our results also show that the increased resistance to CT challenge afforded by Flt3L treatment was accompanied by enhanced CT-specific IgA Ab levels, underlining the strong association between intestinal CT-specific protection and local production of neutralizing IgA Abs (9, 31). Notably, both intestinal and serum CT-specific IgA Ab levels were increased, indicating that Flt3L enhanced both local and systemic responses to orally administered CT. Flt3L-expanded intestinal DC are therefore not preferentially concerned with tolerance induction, but can initiate a potent active immune response against CT.

How can both immunogenic and tolerogenic responses be heightened in the presence of increased DC numbers? Recent reports have suggested that the levels of the costimulatory molecules CD80 and CD86 play a pivotal role in determining the regulation of immune responses (35, 36). APC that expresses low levels of CD80/86 appear to preferentially promote T cell tolerance, while APC expressing high levels of CD80/86 deliver positive, stimulatory signals to the T cell (36). Our results demonstrate that CT enhanced CD80/86 expression on intestinal DC, thereby inducing their activation. Furthermore, anti-CT immunity was greatly increased in Flt3L-treated mice following mucosal immunization. Thus, the augmented anti-CT protection observed in Flt3L-treated mice immunized perorally with CT can most likely be attributed to the increased number of DC able to provide an efficient stimulatory signal to mucosal T cells via the CD82 receptor (36). As such, these findings support the hypothesis that the levels of CD80/86 on APC determine whether an antigenic encounter with a naïve T cell promotes tolerance or active immunity. Moreover, we show that the distinct outcomes of tolerance vs active immunity in the intestine in particular may be regulated by the levels of CD80/86 expression on the resident DC (Fig. 10). Our finding that CT up-regulates both CD80 and CD86 levels contrasts with the work of Cong et al. (37), who showed that CT selectively up-regulates CD86 expression. However, it should be noted that these previous authors examined CD80/86 levels on bone marrow-derived macrophages and not on in vivo expanded DC, as in the present study, and this major difference could explain this apparent discrepancy.

A major obstacle in the development of mucosal vaccines is that most soluble Ag encountered via the oral route promote Ag-specific tolerance, unless administered with an appropriate adjuvant. In rodents, CT has long been recognized as a potent mucosal immunogen, and adjuvant that can stimulate protective immunity and prevent tolerization to unrelated soluble protein Ags, such as OVA (5–7, 11). However, the fact that even minute quantities of CT elicit diarrhea in healthy human volunteers (12) precludes its use in humans. This has prompted research aimed at identifying alternative safe and effective mucosal adjuvants.

The mechanism(s) responsible for the adjuvanticity of CT is not fully understood, although it is thought to be associated with the induction of proinflammatory cytokines (23, 24, 38). Because we found that anti-CT immunity was increased in the presence of elevated numbers of activated DC, we looked toward identifying alternative methods of activating DC in the intestine of Flt3L-treated mice. Using RNase protection assays, we found that intestinal challenge with CT significantly increases intestinal IL-1 and IL-6 mRNA. Furthermore, Flt3L-expanded DC express high levels of the type 1 IL-1R. We therefore investigated the potential of using IL-1 to activate DC in vivo. Our results indicate that IL-1 treatment can directly mimic the effect of CT by promoting DC activation in Flt3L-treated mice in vivo. More importantly, we found that activation of DC with IL-1α was able to functionally skew a normally tolerogenic response toward activation. Using an adoptive transfer system, we showed that when Flt3L-expanded DC were activated by IL-1 before OVA feeding, this effect was completely reversed; instead, we observed a striking increase in the number of Tg T cells in the draining lymph nodes, indicating that active immunization had occurred. Similarly, in a conventional model of oral tolerance, we found that low levels of CD80/86 on the surface of Flt3L-expanded DC promoted a high level of functional T and B cell tolerance following oral administration of soluble OVA. However, when these same DC were activated by IL-1 to express high levels of CD80/86 in vivo, feeding
soluble OVA promoted an active systemic response, similar to that seen in immunized mice. Interestingly, when this manuscript was under review, another group also highlighted the potential of IL-1 to act as an effective mucosal adjuvant (39). Although we believe that our results suggest an important role for resting vs activated DC in the induction of oral tolerance vs active mucosal immunity, the exact mechanism by which IL-1 is abrogating oral tolerance in our system has yet to be definitively characterized. It is therefore important to note that IL-1 is a pleiotropic cytokine with demonstrable effects on a number of different cell types, including intestinal epithelial cells, T cells, B cells, and other APC types (23, 40–46). Thus, IL-1 has many properties that could explain its influence on tolerance, including alterations in intestinal architecture and Ag uptake. To this end, we are currently investigating the potential of other DC-activating molecules to similarly abrogate oral tolerance.

In summary, our data indicate that Flt3L-induced DC are well positioned to regulate the qualitative nature of intestinal immune responsiveness depending on the presence or the absence of appropriate inflammatory signals. Furthermore, our studies suggest that Flt3L, used in conjunction with an inflammatory mediator such as IL-1, may have potential use as a mucosal vaccine adjuvant.

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