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IL-12 Is an Effective Adjuvant for Induction of Mucosal Immunity

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We addressed the effects of two cytokines, IL-6 and IL-12, derived from APCs, for the development of mucosal IgA Ab responses following their nasal delivery with the protein vaccine tetanus toxoid (TT). Mice treated nasally with IL-6 and TT showed higher TT-specific serum IgG (mainly IgG1 and IgG2b) Ab responses than did control mice, but exhibited no IgE and negligible secretory IgA (S-IgA) Ab responses. In contrast, IL-12 administered nasally with TT not only induced sharp increases in TT-specific serum IgG (mainly IgG1 and IgG2b) and IgA, but also elevated mucosal S-IgA Ab responses. Coadministration of IL-6 and IL-12 with TT did not enhance the mucosal or serum Ab responses over those seen with IL-12 alone. TT-specific CD4+ T cells from mice given TT with IL-6 or IL-12 produced higher levels of IFN-γ, IL-6, and IL-10 than did those from control mice, but only negligible levels of IL-4 and IL-5. In summary, both intranasal IL-6 and IL-12 induced serum Abs that protected mice from systemic challenge with TT, whereas only IL-12 induced mucosal S-IgA Ab responses. The significance of IL-12-induced Th1-type responses for regulation of both mucosal and systemic immunity is discussed.

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Materials and Methods

Mice

C57BL/6 mice were obtained from the Charles River Laboratories (Wilmington, DE) at 5–6 wk of age and were maintained in horizontal laminar flow cabinets in sterile cages in the Animal Facility of the University of Alabama Immunobiology Vaccine Center (Birmingham, AL). Sterile food and water were provided according to guidelines proposed by the committee for the Care of Laboratory Animal Resources, Commission of Life Sciences, National Research Council. The mice were pathogen free as determined by routine Ab-screening against common mouse pathogens and histological analysis of major organs and tissues. The mice were used at 8–12 wk of age in all experiments described here.
immunization and cytokine treatment

Mice were intranasally immunized on days 0, 7, and 14 with 15 μl of preparation (7.5 μl per nostril) consisting of 20 μg of tetanus toxoid (TT) (kindly provided by Dr. Stanley Wolf; Genetics Institute, Cambridge, MA) and/or human rIL-6 (Genzyme, Cambridge, MA) complexed with cationic liposomes (DOTAP, Boehringer Mannheim, Indianapolis, IN) as described previously (11). Ear thickness measurements showed that optimal IL-12 effects required intranasal administration of 1 μg/dose on days 0, 7, 10, 14, and 17 (11) and established that murine rIL-6 or human rIL-6 induced comparable in vivo effects in C57BL/6 mice (data not shown). On days when mice were given intranasal cytokine-liposomes and TT, the protein TT was administered first followed 15 min later by cytokine-liposomes or liposomes only.

Cell isolation and purification

Spleen (SP) and cervical lymph nodes (CLN) were aseptically removed and single cell suspensions were obtained by gently teasing small fragments through sterile wire screen. The cells from lungs and lamina propria were obtained as described in previous studies (10, 26, 27) and were >95% viable as determined by trypsin blue dye exclusion. Single cell preparations were resuspended in complete medium (RPMI 1640, Cellgro Mediatech, Washington, DC) containing 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 μM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. Enriched CD4+ T cells were obtained from CLN and SP cell suspensions by panning on anti-L3T4 (GK 1.5) mAb-coated petri dishes as described elsewhere (28). This procedure resulted in CD4+ T cell-enriched cultures, which were >97% CD3+ CD4+ and contained <1% CD3+, CD8+ T cells. The CD3+, CD4+, CD8+ T cell population was >98% viable as determined by trypsin blue dye exclusion.

Analysis of Ab isotypes and IgG subclasses

Vaginal washes, fecal pellets, and blood samples for serum were collected at weekly intervals (days 0, 7, 14, and 21) and saliva was collected on day 21. Samples were processed as previously described and stored at −70°C until assayed for TT-specific Abs (11, 27). Briefly, ELISA plates (Milltest III; Becton Dickinson, Oxnard, CA) were coated with a 100-μl solution of TT (5 μg/ml; 1.25 μl units/ml), and serial twofold dilutions of serum or fecal extracts, saliva, or vaginal washes were added to individual wells. Titers of IgM, IgG, or IgA Abs were determined by addition of a horseradish peroxidase conjugated-goat anti-mouse -μ-, -γ-, or -α- heavy chain-specific antisera (Southern Biotechnology Associates, Birmingham, AL). To determine serum IgG subclass titers, 100 μl of biotin-conjugated, rat anti-mouse-γ1 (G1; 1:75; 0.5 μg/ml), -γ2b (R12-3; 0.5 μg/ml), or -γ3 (R40-82; 0.5 μg/ml) heavy chain-specific Abs were used (PharMingen, San Diego, CA) as described previously (7, 11). Following incubation and washing, a 100-μl aliquot of HRP-conjugated streptavidin (Life Technologies, Grand Island, NY) was added. The absorbance at 415 nm was determined after 15 min and the data expressed as reciprocal endpoint titers of the dilution exhibiting an OD of >0.1 when compared with negative controls. Total IgG levels and Ag-specific IgE Abs were determined by ELISA and a passive cutaneous anaphylaxis assay, respectively, as previously described (7, 11).

B cell ELISPOT for IgA Ab-forming cells (AFCs)

An enzyme-linked immunospot (ELISPOT) assay was used to quantify numbers of AFCs in the intestinal lamina propria, SP, CLN, and lungs of mice immunized and challenged with TT in the presence or absence of IL-6 and/or IL-12. TT-specific IgG, IgM, and IgG AFCs were determined by ELISPOT assay as described previously (26). Briefly, 96-well nitrocellulose-based plates were coated with a 100-μl solution of TT (5 μg/ml) diluted in PBS, while control wells received only PBS. Following washing, the wells were blocked with 1% BSA in PBS. Fivefold serial dilutions of cell suspensions (starting at 1 × 106 cells) were added to the wells in duplicate and incubated for 6 h. AFCs were detected with peroxidase-labeled anti-mouse -μ-, -γ-, or -α-chain-specific Ab (Southern Biotechnol-
treatment in the absence of TT resulted in the induction of serum anti-TT Ab responses (data not shown). Serum TT-specific IgM Ab responses were enhanced by intranasal IL-12 or IL-12 plus IL-6 but not by IL-6 alone (Fig. 2). In contrast, IgG Ab titers were significantly increased in all mice receiving nasal cytokines, although the administration of IL-12 alone or with IL-6 resulted in higher TT-specific Ab responses than administration of TT with IL-6 (Fig. 2). Interestingly, nasal IL-12 also resulted in significant titers of TT-specific IgA Abs, which were not further increased by coadministration of IL-6. In contrast, nasal administration of IL-6 and TT did not induce significant titers of TT-specific serum IgA Abs (Fig. 2). Administration of liposomes without cytokine after nasal immunization with TT did not enhance anti-TT Ab responses (Fig. 2).

We further characterized serum Ab responses by analyzing the pattern of IgG subclasses present in sera from mice intranasally immunized with TT in the presence (or absence) of IL-6 or IL-12. Anti-TT IgG1 and IgG2b titers were low after nasal administration of TT alone but increased after nasal IL-6 treatment (Fig. 2). When sera from mice intranasally immunized with TT and IL-12 were analyzed, a significant increase in all IgG subclasses was noted. Specifically, comparable titers of IgG1, IgG2a, and IgG2b Abs with low but significant IgG3 Ab responses were noted (Fig. 2). Codelivery of IL-6 and IL-12 resulted in an identical TT-specific IgG subclass distribution and titers comparable with those measured following delivery of IL-6 alone. None of the nasal cytokine treatments induced detectable levels of TT-specific IgE (data not shown).

Secretory IgA Ab responses following nasal TT and IL-12 or IL-6

Since intranasal administration of cytokines resulted in increased titers of TT-specific serum Abs, we next investigated whether intranasal administration of IL-6 or IL-12 supported S-IgA Ab responses. Nasal administration of TT and IL-6 resulted in modest IgA Ab titers in fecal extracts and negligible IgA Ab titers in saliva and vaginal washes (Fig. 4), while nasal administration of TT and IL-12 resulted in high TT-specific IgA Ab titers in all mucosal secretions that were not further increased by coadministration of IL-6 (Fig. 4). The source of mucosal TT-specific IgA Abs measured in external secretions was confirmed by enumeration of IgA AFCs in both mucosal immune sites such as the lamina propria and lungs and in systemic immune sites such as the SP and CLNs. As shown in Table I, intranasal administration of IL-6 alone slightly increased the frequency of IgA AFC in the intestinal lamina propria. On the other hand, intranasal delivery of IL-12 or IL-12 plus IL-6 resulted in high frequencies of TT-specific IgA AFC in all mucosal compartments analyzed.

Cytokine profiles of TT-specific CD4+ T cells

We studied the array of Th1-type and Th2-type cytokines secreted by TT-specific CD4+ T cells following in vitro restimulation. CD4+ T cells from CLN and SP of mice nasally immunized with TT failed to respond to TT (data not shown) and produced low or negligible levels of cytokines (Fig. 5). When SP or CLN CD4+ T cells from mice receiving nasal TT and IL-6 were restimulated in vitro, high proliferative responses were seen, accompanied by high levels of IFN-γ, IL-6, and IL-10 synthesis (Fig. 5). No significant differences in profiles of TT-specific CD4+ T cell cytokines were observed between mice that received intranasal IL-12 or IL-12 plus IL-6. In fact, these treatments resulted in large increases in IFN-γ, IL-6, and IL-10 secretion by TT-specific CD4+ T cells (Fig. 5). Furthermore, TT-specific CD4+ T cells from mice that received IL-12 or IL-12 plus IL-6 produced higher levels of IFN-γ and IL-6 than did T cells from mice treated with IL-6 only (Fig. 5).
Nasal TT with IL-6 or IL-12 induces protective immunity

It was important to determine whether the immune responses induced by mucosal cytokine treatment resulted in protective immunity. Therefore, we tested mice that received nasal IL-6 and/or IL-12 treatment with TT to determine sensitivity to a systemic challenge with tetanus toxin. Naive mice and those nasally immunized with either TT alone or with cytokine alone did not survive the challenge. In contrast, mice receiving TT plus IL-6 or TT plus IL-12 or TT plus both IL-12 and IL-6 were completely protected, demonstrating that protective immunity is induced by these mucosal cytokine treatments (Table II).

Discussion

To date, few studies have addressed the role of APCs in the induction of mucosal immune responses. A major role for APCs is their contribution to the cytokine environment for growth and development of Th1- or Th2-type cells. Two cytokines secreted by APCs, e.g., IL-6 and IL-12, can influence the development of Th cell subsets and subsequent immune responses (4, 22, 24). Despite the potential of IL-6 and IL-12 to bias the development of Th2- and Th1-type cells, respectively, it is still unclear whether the presence of these cytokines in a mucosal inductive site can trigger the development of S-IgA Ab responses. Here we report that both IL-6 or IL-12 are mucosal adjuvants for induction of systemic immunity. However, only IL-12 provides necessary signals for mucosal S-IgA Ab responses.

Early studies using in vitro systems suggested a role of IL-6 as a key factor for B cell differentiation into plasma cells (12, 13, 18). However, the role of IL-6 in Ag-specific responses remains controversial. It has been suggested that IL-6 is not required for Ag-specific Ab responses (28) and both normal and impaired Ab responses have been reported in mice lacking IL-6 (19, 20). Here we
show that nasally administered IL-6 can act as adjuvant for systemic immunity to coadministered vaccine protein. Our results are consistent with observations by others that parenteral administration of IL-6 enhances Ag-specific Ab responses in mice (29). Interestingly, although IL-6 was reported to selectively stimulate mIgA+ B cells residing in the Peyer’s patches to secrete IgA in vitro (8), no significant Ag-specific S-IgA Ab responses were induced by nasal IL-6 treatment. This observation is in contrast with the study by others in which oral treatment with IL-6 before oral infection Campylobacter jejuni induced Campylobacter-specific serum and mucosal S-IgA Ab responses (30). It is unlikely that induction of S-IgA Abs was due to the oral route of IL-6 delivery in these studies. In this regard, bacterial LPS is known to be a trigger for a large array of effects on B lymphocytes and APCs (31), and thus, this molecule could have exerted a costimulatory effect for the induction of Campylobacter-specific S-IgA Ab responses.

In contrast to IL-6, which is also produced by B and T cells, the production of IL-12 is mainly restricted to APCs (i.e., macrophages and dendritic cells) (23). IL-12 is primarily involved in the induction of IFN-γ and Th1-type responses. However, IL-12 was also reported to stimulate B cells growth (32) and Ig secretion in vitro (25). We have previously reported that IL-12 administered by mucosal routes (i.e., either oral or nasal) could redirect Th2-type immune responses induced by the mucosal adjuvant CT toward a Th1 type (11, 40). In the present study, nasal treatment with IL-12 increased serum Ab responses to co-administered TT vaccine, clearly showing an adjuvant effect by this cytokine when administered by a mucosal route.

A striking observation in our previous studies was the fact that CT-induced S-IgA Ab responses were not affected by mucosal administration of IL-12 (11, 40). Interestingly, we report here that nasal treatment with IL-12 could also trigger S-IgA Ab responses. Our observation supports a recent report by others that IL-12 displays mucosal adjuvant activity when delivered by the nasal route (33). Even though IL-6 is a stimulatory factor for B cell differentiation (13, 18), the absence of an enhancing effect when IL-6 was coadministered with IL-12 suggests that IL-12 does not require additional signals for its mucosal adjuvanticity.

Since the adjuvant effects of IL-6 and IL-12 are likely mediated by Th cell-derived cytokines (4, 22), we further characterized serum IgG subclasses and IgE Ab responses. Indeed, Th cytokines control the pattern of IgG subclass Ab responses (34), and IgE Abs were shown to correlate with IL-4 production (11, 35). The adjuvant effect of IL-6 resulted in Ag-specific serum IgG1 and IgG2b Abs followed by IgG2a Abs, while nasal IL-12 induced comparable levels of IgG1, IgG2a, and IgG2b as well as high IgG3 Ab responses. Thus, IL-6 induced a pattern of IgG subclasses corresponding to a mixed Th1- and Th2-type response. Despite this difference in the profiles of IgG subclasses, no IgE Ab response was induced by either IL-6 or IL-12 as nasal adjuvant.

In order to clearly establish that Th cell-derived cytokine responses are involved in the adjuvanticity of IL-6 and IL-12, we analyzed cytokine secretion patterns by TT-specific CD4+ T cells from mice that received nasal IL-6 and/or IL-12 as adjuvant. Our results show that the adjuvant effect of both IL-6 and IL-12 involved Th1- and Th2-type cytokines. In fact, increased IFN-γ secretion was measured in culture supernatants of TT-specific CD4+ T cells from mice treated with either IL-6 and/or IL-12. This observation is consistent with the ability of IL-12 to induce Th1-type cells (23) and the reported impaired Th1-type development seen in...
induced IL-6 and IL-10 secretion by TT-specific CD4+ T cells. Furthermore, coadministration of IL-6 enhanced the IL-12 production, that IL-12 was a better inducer of Th2-type responses than was IL-6, confirming the greater ability of IL-12 to promote Th1-type responses. Similar patterns of Th2-type cytokines were observed after IL-6 and IL-12 treatment with increased IL-6 and IL-10 secretion with no significant levels of IL-4 and IL-5. Interestingly, IL-6-deficient mice (36). Furthermore, mice that received IL-12 treatment secreted larger amount of IFN-γ than those treated with IL-6, confirming the greater ability of IL-12 to promote Th1-type responses. Similar patterns of Th2-type cytokines were observed after IL-6 and IL-12 treatment with increased IL-6 and IL-10 secretion with no significant levels of IL-4 and IL-5. Interestingly, the IL-12 treatment resulted in significantly higher IL-6 secretion in culture supernatants than the IL-6 treatment itself, suggesting that IL-12 was a better inducer of Th2-type responses than was IL-6. Furthermore, coadministration of IL-6 enhanced the IL-12-induced IL-6 and IL-10 secretion by TT-specific CD4+ T cells, demonstrating an absence of interference between these two cytokines even though a subunit of IL-12 shares homologies with the IL-6R (23). It was reported that IL-12 promotes differentiation of Th2-type cells in the absence of IFN-γ (37) and enhances rather than suppresses ongoing Th2-type responses (38, 39). On the other hand, we have shown that serum elevated serum IFN-γ occurs 12 h following nasal IL-12 administration (40). Thus, it is possible that Th2 cells were the first stimulated following nasal administration of IL-12 and TT and that Th1 cells were later induced through IFN-γ production. In this regard, we have reported that administration of IL-12 from a site distant from that where the immune responses is initiated could both enhance CT-induced Th2-type response and promote Th1-type responses (40).

It is important to note that the ability of IL-12 (but not IL-6) to display a mucosal adjuvant activity correlates with a greater ability of IL-12 to induce IL-6 and IFN-γ secretion by Ag-specific CD4+ T cells. These results are consistent with the ability of Salmonella vectors to induce mucosal S-IgA Ab responses with Th responses characterized by IFN-γ, IL-6, and IL-10 secretion (9). Thus, strong Th1 (i.e., IFN-γ)-type responses together with IL-6 might provide necessary signals for S-IgA Ab responses. An alternative explanation for the mucosal adjuvanticity of IL-12 is a specific effect of IL-12 on B cells. Indeed, while the role of IL-6 is restricted to stimulation of Th2 cells producing IL-6 and IL-10 are associated with mucosal immunoglobulin A responses (4). We thank Dr. Karan Singh for help with the statistical analysis. References


### Table II. Intranasal administration of IL-6, IL-12, or IL-6 plus IL-12 together with TT induces protective immunity to systemic challenge with tetanus toxin

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Intranasal Cytokine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
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</tr>
<tr>
<td>IL-6</td>
<td>0</td>
</tr>
<tr>
<td>IL-12</td>
<td>0</td>
</tr>
<tr>
<td>IL-6 + IL-12</td>
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</tr>
<tr>
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<td>(&lt;5) (&lt;5) (&lt;5) (&lt;5)</td>
</tr>
<tr>
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</tr>
<tr>
<td>IL-12</td>
<td>3</td>
</tr>
<tr>
<td>IL-6 + IL-12</td>
<td>3</td>
</tr>
</tbody>
</table>

*The minimum lethal dose (MLD) was established as 1 μg/mouse in naive mice in previous studies (26). Intranasal Immunization with TT in the presence or absence of cytokines was performed on days 0, 7, and 14. All mouse groups were challenged s.c. on day 28 with 100 MLDs of toxin in 0.5 ml of PBS-0.2% gelatin. Serum IgG titers (log, titers) in each group are indicated in parentheses. The rates of survival after intranasal cytokine treatments were significantly different compared with the group that received the vaccine alone (p < 0.05).*


