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Dependence of IL-4, IL-13, and Nematode-Induced Alterations in Murine Small Intestinal Smooth Muscle Contractility on Stat6 and Enteric Nerves

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IL-4 and IL-13 promote gastrointestinal worm expulsion in part through effects on nonlymphoid cells, such as intestinal smooth muscle cells. The roles of Stat6 in IL-4-, IL-13- and parasitic nematode-induced effects on small intestinal smooth muscle contractility were investigated in BALB/c wild-type and Stat6-deficient mice treated with a long-lasting formulation of recombinant mouse IL-4 (IL-4C) or IL-13 for 7 days. Separate groups of BALB/c mice were infected with Nippostrongylus brasiliensis or were drug-cured of an initial Heligmosomoides polygyrus infection and later reinjected. Infected mice were studied 9 and 12 days after inoculation, respectively. Segments of jejunum were suspended in an organ bath, and responses to nerve stimulation and to acetylcholine and substance P in the presence and absence of tetradoxotoxin, a neurotoxin, were determined. Both IL-4 and IL-13 increased smooth muscle responses to nerve stimulation in wild-type mice, but the effects were greater in IL-13-treated mice and were absent in IL-13-treated Stat6-deficient mice. Similarly, hypercontractile responses to nerve stimulation in H. polygyrus- and N. brasiliensis-infected mice were dependent in part on Stat6. IL-13, H. polygyrus, and N. brasiliensis, but not IL-4, also increased contractility to acetylcholine by mechanisms that involved Stat6 and enteric nerves. These studies demonstrate that both IL-4 and IL-13 promote intestinal smooth muscle contractility, but by different mechanisms. Differences in these effects correlate with differences in the relative importance of these cytokines in the expulsion of enteric nematode parasites. The Journal of Immunology, 2003, 171: 948–954.

The cytokines produced in response to an infectious agent provoke and regulate the immune and inflammatory responses that typically protect the host against that agent (1). Infection of mice with intestinal nematode parasites, such as Heligmosomoides polygyrus and Nippostrongylus brasiliensis, induces a strong Th2 cytokine response (1) that includes two related Th2 cytokines, IL-4 and IL-13, that induce worm expulsion through an IL-4Rα-activated, Stat6-dependent mechanism. Although the mechanism(s) by which IL-4Rα-activated Stat6 signaling induces worm expulsion remains unknown, recent studies demonstrated that non-bone marrow-derived cells mediate the principal IL-4Rα-dependent host-protective effect against N. brasiliensis (2). Expulsion is associated with changes in jejunal physiology (3–5), because adult worms normally reside in and are expelled from the jejunum. Thus, we evaluated the effects of IL-4 and IL-13 on intestinal cell types to identify mechanisms that may lead to cytokine-induced expulsion. Recent studies demonstrated that IL-4 and IL-13 have potent Stat6-dependent effects on intestinal epithelial cell ion flux, mucosal permeability, and mucus production (3, 4); however, preliminary results with transgenic mice that express Stat6 solely in intestinal epithelial cells suggest that these effects are insufficient to expel N. brasiliensis (L. Gildea, J. Urban, S. Morris, G. Smulian, and F. Finkelman, unpublished observations). This result prompted an examination of additional cell types that might contribute to worm expulsion by their responses to IL-4 and IL-13. In this regard, Akioh et al. (6) have shown that IL-4 and IL-13 induce cultured smooth muscle cells to develop a Stat6-dependent hypercontractile response to the cholinergic agonist carbachol, and that infection of mice with the gastrointestinal nematode Trichinella spiralis also induces a Stat6-dependent increase in smooth muscle contractility. In addition, we have demonstrated that IL-4 amplifies smooth muscle responses to nerve stimulation (7). Our current hypothesis is that IL-4 and/or IL-13 are responsible for the nematode-induced effects on smooth muscle, and that these effects are critical for the host resistance to enteric infection. We also propose that these effects are dependent on interactions between smooth muscle and enteric nerves. Observations that enteric nerves regulate intestinal smooth muscle contractility (8–10) and contribute to IL-4/IL-13-induced alterations in intestinal epithelial cell function (3) suggest that the effects of intestinal worm infection on intestinal smooth muscle contractility might be nerve dependent. To evaluate this hypothesis, we have now studied 1) the contributions of enteric nerve-dependent and -independent effects on IL-4/IL-13-enhancement of intestinal smooth muscle contractility, 2) the role of cholinergic and noncholinergic neurotransmitters in these

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effects, 3) the role of nematode parasite-induced IL-4 and IL-13 in the enhancement of intestinal smooth muscle contractility that is associated with *N. brasilienis* and *H. polygyrus* infection, and 4) the Stat6 dependence of the increases in intestinal smooth muscle contractility induced in vivo by IL-4, IL-13, and infection with either *H. polygyrus* or *N. brasilienis*.

**Materials and Methods**

**Administration of IL-4 or IL-13**

The effects of IL-13 and IL-4 were studied in female 8- to 12-wk-old BALB/c mice (National Cancer Institute, Frederick, MD) and Stat6-deficient mice (Stat6<sup>−/−</sup>) that were bred on a BALB/c background. Animals were injected i.v. with vehicle, IL-13 (Wyeth Research, Cambridge, MA) or IL-4 (2, 3). Briefly, a long-lasting IL-4 formulation (IL-4/anti-IL-4 complexes (IL-4C))<sup>3</sup> was prepared consisting of 10 μg of IL-4 (PeproTech, Rocky Hill, NJ) mixed with 50 μg of 11B11, a neutralizing rat IgG1 anti-mouse IL-4 mAb (Verax, Lebanon, NH). The anti-IL-4/IL-4 complexes (IL-4C) increase the in vivo half-life of IL-4 from <30 min to ~24 h (11). All the effects of IL-4C have been shown to result from slow release of IL-4 from the complex, rather than to any effect of the anti-IL-4 mAb or to IgGFc- or complement-mediated effects (11). To control the amount of Ab protein in the IL-4 complexes, a separate group of BALB/c mice (n = 3–4) was given 60 μg (the total amount of protein in the complexes) of GL113, a rat IgG1 isotype control, every 3 days (days 0, 3, and 6) and studied on day 7. Because the physiological measurements of GL113-treated and those given normal saline did not differ significantly (data not shown), all vehicle-treated control mice were subsequently given normal saline as a control. A single injection of IL-4C that contains 10 μg of IL-4 stimulated IL-4-dependent effects for at least 3 days. The amount of cytokine administered was selected based on the observation that injection of immunocompetent BALB/c mice with these doses of IL-4C or IL-13 daily enhances worm expulsion (5, 12). In our current experiments mice were injected i.v. on days 0, 3, and 6 with IL-4C in 0.1 ml of normal saline, with 10 μg of IL-13 daily for 7 days, or with an equal volume of normal saline (n = 7). All mice were studied 7 days after the initial injection.

**Nematode infection**

 Infective, ensheathed, third-stage *H. polygyrus* larvae (L3; specimens on file at the U.S. National Parasite Collection, U.S. National Helminthological Collection, Collection 81930, Beltsville, MD) were propagated and stored at 4 °C until used. Wild-type (WT) and Stat6<sup>−/−</sup> mice were inoculated orally with 200 *H. polygyrus* L3 using a ball-tipped feeding needle and subsequently cured with the antihelminthic drug, pyrantel tartrate, 3 wk after inoculation. These groups were reinfected orally with 200 *H. polygyrus* L3 (day 0) 12–20 days later and were studied 12 days after the second *H. polygyrus* inoculation. Separate groups of WT and Stat6<sup>−/−</sup> mice were inoculated i.c. with 500 *N. brasilienis* infective third-stage larvae (L3) and studied 9 days later. Appropriate age-matched controls were performed for each infection.

**In vitro contractility**

Segments of jejunum (1 cm) were flushed of their intestinal contents and suspended longitudinally in organ baths. One end of the tissue was attached to an isometric tension transducer (model FT03; Grass Medical Instruments, Quincy, MA), and the other to the bottom of the bath. Tissues were stretched to a load of 9.9 mN (2 g). Preliminary experiments showed that this load stretched tissues to their optimal length for active contraction. Tissues were then allowed to equilibrate for at least 30–45 min in Krebs buffer solution. The bath solution was replaced every 10 min throughout each study. Tension was recorded using a polygraph (model 79; Grass Medical Instruments) and was expressed as force per cross-sectional area (13).

Frequency-dependent responses were constructed to electrical field stimulation (EFS; 1, 2.5, 5, 10, and 20 Hz; 1-ms duration; 80 V). Responses were determined in the presence and the absence of atropine (1 μM). In addition, concentration-dependent response curves to acetylcholine (1 nM to 1 mM) were constructed. Strips in organ baths were challenged with noncumulative additions of acetylcholine, and when responses reached a maximum, the strips were washed. The next concentration was added after a 10-min interval. Responses to the tachykinin, substance P (1 μM), were also examined by adding appropriate dilutions of substance P to the organ bath. The contribution of enteric nerves to the contractions induced by acetylcholine (1 μM) and substance P (1 μM) were determined by comparing the maximal responses in the presence and the absence of the neuromodulator, tetradotoxin (TTX; 1 μM).

**Solutions and drugs**

Krebs buffer contained 4.74 mM KCl, 2.54 mM CaCl<sub>2</sub>, 118.5 mM NaCl, 1.19 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.19 mM MgSO<sub>4</sub>, 25.0 mM NaHCO<sub>3</sub>, and 11.0 mM glucose. All drugs were obtained from Sigma–Aldrich (St. Louis, MO) unless indicated otherwise. Stock solutions were prepared as follows: substance P was dissolved in distilled water (0.1 mM) and stored at ~70°C; TTX was dissolved in citrate buffer to a stock solution of 1 mM, and acetylcholine was dissolved in ultrapure water (1 μM) and frozen. On the day of the experiment, appropriate dilutions of acetylcholine and TTX were made using distilled water.

**Data analysis**

Statistical analysis was performed using one-way ANOVA, and maximal responses were compared in the presence and the absence of TTX. Concentration-dependent responses were compared using MANOVA (Systat 5.2) with post hoc analysis for multiple comparisons. A value of p < 0.05 was considered significant. Appropriate vehicle and time- and age-matched controls were performed for each group; however, there were no significant differences among control groups, and therefore, only one control group is shown for comparison.

**Results**

**Administration of IL-4C or IL-13 for 7 days to WT and Stat6<sup>−/−</sup> mice**

**Responses to nerve stimulation.** EFS (1–20 Hz, 80 V, 1 ms) evoked a frequency-dependent contraction of longitudinal smooth muscle from small intestine (Fig. 1). The frequency and voltage of EFS were based on the results of preliminary experiments that established the optimum frequencies and voltage necessary to induce contractions that were mediated entirely by nerves rather than through a direct effect on smooth muscle. Responses to EFS under these conditions, therefore, were fully blocked by TTX (1 μM). In vehicle-treated groups, responses were similar in WT and Stat6<sup>−/−</sup> mice. In WT mice, responses to EFS were increased significantly by both IL-4C and IL-13 (Fig. 1A), but the effects were greater in IL-13-treated mice. In Stat6<sup>−/−</sup> mice, contractions to EFS in IL-4- and IL-13-treated mice were nearly identical. Compared with WT mice, however, responses were significantly lower only in IL-13-treated mice, indicating a primary dependence of this cytokine on Stat6.

The cholinergic muscarinic receptor antagonist, atropine (1 μM), significantly reduced responses to EFS (Fig. 1, C and D vs A and B) by almost 80% in vehicle-treated WT and Stat6<sup>−/−</sup> mice, showing that the responses to nerve stimulation are mediated primarily by cholinergic nerves. Responses to EFS in IL-4- or IL-13-treated WT mice were also reduced by atropine, but remained elevated above those in vehicle-treated mice, indicating that these Th2 cytokines alter both cholinergic and noncholinergic nerve-mediated smooth muscle contraction. The elevated responses to EFS in the presence of atropine were not observed in IL-13-treated Stat6<sup>−/−</sup> mice, but persisted in IL-4-treated Stat6<sup>−/−</sup> mice (Fig. 1D), indicating that the effect of IL-13, but not IL-4, on noncholinergic nerves is Stat6 dependent.

**Responses to acetylcholine and substance P.** Smooth muscle from the small intestine of mice exhibited concentration-dependent contractions in response to acetylcholine, an endogenous cholinergic ligand (Fig. 2). In vivo treatment with IL-4C had no effect on responses to acetylcholine, while IL-13 dramatically increased contractions compared with the vehicle treatment (Fig. 2A and Table 1). The enhanced contractions to acetylcholine in IL-13-treated WT mice were not observed in Stat6<sup>−/−</sup> mice, indicating a dependence on Stat6 activation (Fig. 2B).

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3 Abbreviations used in this paper: IL-4C, IL-4/anti-IL-4 complexes; EFS, electrical field stimulation; LTD<sub>4</sub>, leukotriene D<sub>4</sub>; TTX, tetradotoxin; WT, wild type.
Responses to acetylcholine were compared in the presence and the absence of the neurotoxin TTX to determine their neural dependence. TTX had little effect on the maximum response to acetylcholine in WT mice (Table I), but reduced responses to acetylcholine in IL-13-treated mice to the level seen in vehicle-treated WT mice. Thus, while the smooth muscle contractile response to acetylcholine was independent of nerves in vehicle-treated mice, IL-13-induced hypercontractility to acetylcholine was mediated by enteric nerves. TTX had no effect on responses to acetylcholine in Stat6−/− mice in any treatment group (data not shown).

One of the major noncholinergic excitatory neurotransmitters is substance P. IL-4C had no effect on responses to substance P in WT (Table II) or Stat6−/− mice (data not shown), but IL-13 significantly increased the responses in WT mice (Table II). The IL-13-induced enhancement of substance P contractility was not observed in Stat6−/− mice (20743 ± 1212 vs 8156 ± 1209 mN/cm²), showing the dependence of the IL-13 effect on Stat6.

To determine whether the hypercontractile responses to substance P in IL-13-treated WT mice were due to an effect on nerves or to a direct effect on smooth muscle, responses were compared in the presence and the absence of TTX (1 μM). TTX had no effect on responses to substance P in vehicle-treated WT mice, indicating that contraction was due to a direct action of these agonists on smooth muscle (Table II). TTX, however, diminished the IL-13-induced increase in contractile responses to substance P in WT mice, indicating that the effects of IL-13 on these responses were nerve dependent (Table II).

Nematode-infected WT and Stat6−/− mice

Responses to nerve stimulation. To determine the effects of endogenously produced IL-4 and IL-13 on smooth muscle contractility, BALB/c mice were infected with H. polygyrus or N. brasiliensis, each of which considerably increases the production of both cytokines (14–16). Responses to EFS in WT mice were elevated significantly by infection with either nematode (Fig. 3A); however, the effect was greater in N. brasiliensis-infected mice, and only N. brasiliensis infection significantly elevated responses in Stat6−/− mice (Fig. 3B). Atropine significantly inhibited contractions in response to EFS in all mice; however, responses in N. brasiliensis-infected, but not H. polygyrus-infected, mice remained elevated above those in uninfected mice (Fig. 4). These data indicate that the enhanced responses to H. polygyrus infection are mediated primarily by cholinergic nerves, while those to N. brasiliensis infection are mediated by both cholinergic and noncholinergic nerves and involve Stat6-dependent and independent pathways.

Responses to acetylcholine and substance P. H. polygyrus and N. brasiliensis infection uniformly enhanced the response to acetylcholine in WT mice (Fig. 5A). Responses were attenuated, but not abrogated, in Stat6−/− mice (Fig. 5B), suggesting only partial dependence on Stat6. Although TTX had no effect on responses to acetylcholine in WT mice, it attenuated contractility in N. brasiliensis-infected mice (Table I), indicating that the hypercontractile effects of N. brasiliensis infection are dependent in part on enteric nerves. In contrast, TTX had no effect on the enhanced contractions to H. polygyrus (Table I), suggesting that the primary effect of this nematode is directly on smooth muscle.

Because IL-13 significantly increased contractions in response to substance P by a Stat6-dependent mechanism, the ability of intestinal nematode infection to alter tachykinin responses was also assessed. Compared with vehicle-treated mice, contractions in response to substance P were unaltered by H. polygyrus infection, indicating that the hypercontractility does not involve noncholinergic...
nerves (Table II). In contrast, responses to substance P were increased significantly by *N. brasiliensis* infection (Table II), indicating that both cholinergic and noncholinergic pathways mediate nematode-induced hypercontractility. The difference in responses to substance P between the two infections is consistent with the findings of an enhanced response to nerve stimulation in the presence of atropine in *N. brasiliensis*-infected, but not in *H. polygyrus*-infected, WT mice (Fig. 4). The enhanced contractions to substance P in *N. brasiliensis*-infected WT (Table II) mice were not observed in Stat6<sup>−/−</sup> infected mice (18,800 ± 1,497 vs 6,089 ± 1,225 mN/cm²).

**Discussion**

The protective immune response to enteric parasitic nematode infection requires the production of type 2 cytokines, particularly IL-4 and IL-13 (1, 14), which induce worm expulsion through...
processes that require IL-4R ligation and activation of the transcription factor, Stat6. Although IL-4R ligation with Stat6 activation appears to be a consistent requirement for intestinal worm expulsion, the precise mechanisms through which IL-4 and IL-13 promote expulsion differ for different worm species. Host-protective effects may include IL-4/IL-13-induced increases in smooth muscle contractility as well as changes in epithelial and vascular endothelial cell function that increase fluid and mucus secretion into the gut lumen and interfere with fluid absorption. The enteric nervous system also plays a major role in the coordination of mucosal and smooth muscle function in host defense, including helminth infection (8–10). The present data clearly show the important contributions of IL-4 and IL-13 to host defense through activation of Stat6-dependent pathways in vivo that induce alterations in the function of nonimmune cells such as smooth muscle.

Although both IL-4 and IL-13 bind to the type 2 IL-4R (IL-4Rα/IL-13Rα1) and activate Stat6, and both cytokines enhance smooth muscle contractility, our data demonstrate quantitative and qualitative differences in the effects of these cytokines on contractility. IL-13 has a much greater effect than IL-4 when smooth muscle contractions are evoked by stimulation of nerves (EFS). The difference between the effects of IL-4 and IL-13 can be explained almost entirely by a Stat6-dependent effect that accounts for most of the response to IL-13 and little or none of the response to IL-4 (compare A and B in Fig. 1). The Stat6-dependent effect is manifested by an increased contractility in response to acetylcholine, the neurotransmitter released by muscarinic cholinergic nerves. Thus, atropine, which blocks muscarinic cholinergic nerve stimulation, attenuates the effects of IL-13, but not the effects of IL-4, on contractility (Fig. 1C). Consistent with these observations, IL-4 has no effect on the smooth muscle response to acetylcholine, while IL-13 has a marked effect that is entirely Stat6 dependent (Fig. 2). Moreover, enteric nerves mediate the Stat6-dependent effect of IL-13 on responses to acetylcholine, because hypercontractility is not observed in the presence of TTX (Table I).

The observation that increased responses to nerve stimulation persist, even in the presence of atropine, in mice treated with IL-4 and IL-13 or infected with N. brasiliensis suggests an effect on noncholinergic nerves as well. Substance P is an important noncholinergic excitatory neurotransmitter as well as a sensory neuropeptide in the enteric nervous system. In addition to enhancing responsiveness to cholinergic nerve stimulation, IL-13 enhances responses to substance P (Table II). This effect of IL-13 on substance P probably explains that portion of enhanced response to nerve stimulation that is both atropine resistant and Stat6 dependent (Fig. 1). In contrast to the complete neural dependence of the IL-13-induced hypercontractility in response to acetylcholine, the effect of IL-13 on responses to substance P is only partly dependent on nerves (Table II).

The mechanism of IL-4 enhancement of smooth muscle contractility appears to be totally different from that of IL-13. In the absence of both Stat6 signaling and cholinergic nerves (Fig. 1D), IL-4 has a greater effect than IL-13 on EFS-induced smooth muscle contractility. Moreover, the hypercontractile responses in IL-4-treated mice do not involve increased sensitization to acetylcholine or substance P. We showed previously that IL-4 has effects on epithelial cell function that are dependent on mast cells and that are not shared by IL-13 (4). Studies using the leukotriene D₄ (LTD₄) receptor antagonist Wy-48,252 showed that LTD₄, a 5-lipoxygenase product of mast cells, contributes to the contractile response to Ag challenge (17). Mast cell release of LTD₄ increases contractility by enhancing the sensitivity of nerves to stimulation (18), and IL-4-induced hypercontractility of smooth muscle in response to EFS was not observed in mast cell-deficient W/Wᵥ or 5-lipoxygenase-deficient mice (7). Taken together, our data indicate that...
IL-4 enhances smooth muscle contractility through an effect of mast cell-produced LTD₄ on enteric nerves acting to enhance their sensitivity to neurotransmitters, rather than to a direct effect of LTD₄ on smooth muscle. This hypothesis is consistent with the ability of IL-4, but not IL-13, to induce intestinal mastocytosis and the Stat6 independence of this IL-4 effect (4). This hypothesis is also consistent with evidence that IL-13 has a greater effect than IL-4 in promoting hyper-responsiveness to pulmonary cholinergic stimulation in response to Ag challenge in mouse models of asthma (19, 20).

Responses to acetylcholine in WT mice were enhanced by H. polygyrus and, to a greater extent, by N. brasiliensis infection, an effect consistent with an increased contractility to carbachol in T. spiralis-infected mice (21). In the present study there was a significant Stat6 dependence of the contractions in both H. polygyrus and N. brasiliensis infected mice, implicating IL-4 and IL-13. The greater effect of IL-13 vs IL-4 on smooth muscle is consistent with previous studies showing that expulsion of T. spiralis was linked to an enhanced response to carbachol and that these effects were inhibited in Stat6⁻/⁻ mice, but were only slightly attenuated in IL-4⁻/⁻ mice (21). These data provide strong evidence of a major role for IL-13 in nematode-induced hypercontractility. These observations, however, do not explain how IL-13, but not IL-4, induces a Stat6-dependent increase in response to cholinergic and noncholinergic neurotransmitters. The type 2 IL-4R, the only receptor known to be responsive to IL-13, binds IL-4 and IL-13 with similar affinity and is activated by either cytokine to phosphorylate Stat6. IL-4 stimulates some cell types (e.g., B cells, T cells, and mast cells) that are not stimulated by IL-13 because these cells exclusively express the type 1 IL-4R, which binds IL-4, but not IL-13. It is possible, therefore, that IL-4 stimulation of these cell types induces an effect that blocks the ability of IL-4 to directly enhance the responsiveness of smooth muscle cells to neurotransmitters. Alternatively, IL-4 and IL-13 may have opposite effects on the expression of IL-4Rα1, one of the constituents of the type 2 IL-4R, with IL-4 decreasing, and IL-13 increasing the expression of this polypeptide. Such differences could lead to decreased type 2 IL-4R expression (and Stat6 signaling) following IL-4 exposure, but increased type 2 IL-4R expression following IL-13 exposure. A third possibility is that IL-13Rα2, a membrane polypeptide that binds IL-13 with high affinity, but does not bind IL-4 and that has been hypothesized to have a purely inhibitory effect on IL-13 function, may actually promote signaling by IL-13, but not IL-4, by maintaining a high IL-13 concentration at the cell membrane and transporting IL-13 to the type 2 IL-4R (22). Any of these possibilities might explain the ability of high concentrations of either IL-4 or IL-13 to increase the responsiveness of isolated smooth muscle cells to the cholinergic compound carbachol that has been described in a short term in vitro culture system (6).

An alternate possibility is that the differences in IL-4 vs IL-13 effects on smooth muscle contractility result from differences in effective in vivo concentration. We believe that this possibility is unlikely, because both cytokines were administered at concentrations sufficient to induce N. brasiliensis expulsion by T cell-deficient mice. In addition, we have previously shown that IL-4 actually has a greater effect than IL-13 on intestinal epithelial cell function at the doses administered (4). Furthermore, it could be argued that the complexes used to deliver IL-4 to mice maintain IL-4 levels better than IL-13 levels can be maintained by the daily injections of uncomplexed IL-13. Additional support for differences between IL-4 and IL-13, however, comes from studies in another organ system, showing that transgene-mediated increased pulmonary expression of IL-13 has been associated with increased responsive-


