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Delayed Goblet Cell Hyperplasia, Acetylcholine Receptor Expression, and Worm Expulsion in SMC-Specific IL-4Rα–Deficient Mice

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Interleukin 4 receptor α (IL-4Rα) is essential for effective clearance of gastrointestinal nematode infections. Smooth muscle cells are considered to play a role in the type 2 immune response–driven expulsion of gastrointestinal nematodes. Previous studies have shown in vitro that signal transducer and activator of transcription 6 (STAT-6) signaling in response to parasitic nematode infection significantly increases smooth muscle cell contractility. Inhibition of the IL-4Rα pathway inhibits this response. How this response manifests itself in vivo is unknown. In this study, smooth muscle cell IL-4Rα–deficient mice (SM-MHC⁺/⁻IL-4Rα⁻/α⁻) were generated and characterized to uncover any role for IL-4/IL-13 in this non–immune cell type in response to Nippostrongylus brasiliensis infection. IL-4Rα was absent from α-actin–positive smooth muscle cells, while other cell types showed normal IL-4Rα expression, thus demonstrating efficient cell-type–specific deletion of the IL-4Rα gene. N. brasiliensis–infected SM-MHC⁺/⁻IL-4Rα⁻/α⁻ mice showed delayed ability to resolve infection with significantly prolonged fecal egg recovery and delayed worm expulsion. The delayed expulsion was related to a delayed intestinal goblet cell hyperplasia, reduced T helper 2 cytokine production in the intestine, the precise role of IL-4Rα in coordinating the immune and physiological response remains unclear [6]. IL-13/IL-4Rα/STAT-6 signaling is required for the host to produce an effective goblet cell hyperplasia [7]. Disruption of this response impairs the host ability to resolve an N. brasiliensis infection. Additionally, acetylcholine-driven contractions of longitudinal smooth muscle in the intestine are

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Murine infection with N. brasiliensis induces a strong protective host T helper 2 (T_h2) response for which IL-13 production and signaling through IL-4Rα are essential for successful clearance of infection [3,4]. Infective third-stage N. brasiliensis larva penetrate the skin and migrate via the blood system, to the lungs. Larva emerge from blood vessels and enter the airways, from which they are coughed up and swallowed. Upon reaching the intestine, larva develop into egg-producing adult worms that attach to the small intestine epithelium. BALB/c mice clear N. brasiliensis infection after approximately 9 d [5].

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Author Summary

Intestinal parasitic worm infections are a major public health concern, with more than 1 billion people infected worldwide. Symptoms associated with these infections are similar to that of other intestinal illnesses, including irritable bowel syndrome. It is likely that the immune response required to expel the worm can also, when activated inappropriately, cause the symptoms of irritable bowel syndrome. This makes understanding parasitic worm infections important in their own right and also as a model for other intestinal illnesses. In previous studies, we demonstrated the crucial importance of interleukin 4 receptor (IL-4R) responsiveness for worm expulsion in global IL-4R-deficient mice. In this study, we specifically addressed the role of IL-4R responsiveness in a novel smooth muscle cell–specific IL-4R-deficient mouse model. These mice showed decreased ability to control the worm infection, with delayed expulsion and reduced protective immune responses. These data provide compelling evidence for smooth muscle cell IL-4R being an important coordinator of both the immune and physiological responses to intestinal worm infections. A proposed model is suggested with IL-4R responsiveness on smooth muscle cells coordinating T helper 2 cytokine responses, goblet hyperplasia, and acetylcholine-driven smooth muscle contractions for optimal worm expulsion.

also implicated in playing a role in worm expulsion [8]. A number of in vitro studies have shown that intestinal segments and intestinal smooth muscle cells previously exposed to infection by murine nematode models have increased contractile ability. This contractile ability of intestinal segments and smooth muscle cells is abrogated in STAT-6−/− mice. Therefore, the IL-13/IL-4Rα/STAT-6 pathway is necessary for elevated smooth muscle cell contractility required to aid worm expulsion [6,9,10]. Additionally, IL-13/IL-4Rα/STAT-6-dependent smooth muscle cell signaling can induce responses in surrounding tissues [11], as well as inducing smooth muscle cell release of chemokines, such as thymus- and activation-regulated chemokine [12], in order to coordinate early host responses to pathogens. From these studies, it is apparent that both goblet cell and smooth muscle cell responses to nematode infections are coordinated by the host immune response to infection and that this coordination is essential for optimal disease resolution [13].

To date, no studies have been able to demonstrate in vivo the effect of a cell-specific inhibition of the IL-13/IL-4Rα/STAT-6 pathway in smooth muscle cells. Using smooth muscle myosin heavy chain (SM-MHC)CreIL-4Rαlox/lox mice, we demonstrate that disrupted IL-4Rα expression in smooth muscle cells influences host immunity to an intestinal nematode infection. The absence of smooth muscle IL-4Rα delays worm expulsion and goblet cell hyperplasia. Furthermore, induction of Th2 cytokines is delayed and/or reduced, as is intestinal expression of the M3 acetylcholine receptor, in response to infection with N. brasiiliensis.

Results

Transgenic mice, expressing Cre recombinase under the control of the smooth muscle cell–specific myosin heavy chain promoter (SM-MHCCre), were backcrossed to the BALB/c genetic background for nine generations and then intercrossed with IL-4Rα−/− and “floxed” IL-4Rαlox/lox BALB/c mice to establish smooth muscle cell–specific IL-4Rα-deficient BALB/c mice (SM-MHCCreIL-4Rαlox), with one deleted and one floxed IL-4Rα allele (SM-MHCCreIL-4Rαlox) to increase the efficiency of Cre-mediated site-specific recombination. Mutant mouse strains were identified by PCR genotyping (Figure 1A), and cell specificity of disrupted IL-4Rα expression was confirmed by fluorescence-activated cell sorting analysis (FACS).

IL-4Rα expression was analyzed on α-actin–positive cells derived from aortic cells (Figure 1B). Surface expression of IL-4Rα on α-actin–positive cells was equivalent in SM-MHCCreIL-4Rαlox (geometric mean fluorescence [GMF]: 11.02) and global IL-4Rα−/− (GMF: 11.2) mice (Figure 1B).

Low levels of expression were present in IL-1Rxlox mice (GMF: 18.37). IL-4Rα expression on α-actin–positive smooth muscle cells isolated from small intestine and lung was too low to detect using FACS analysis (unpublished data). However, CRE mRNA was highly expressed in tracheal and intestinal tissue in the SM-MHCCreIL-4Rαlox mice. As expected, IL-4Rαlox mice demonstrated no CRE expression. In agreement with the smooth muscle specificity of the deletion, IL-4Rα mRNA expression was substantially depressed in both tracheal and intestinal tissue in SM-MHCCreIL-4Rαlox mice compared to IL-4Rαlox mice (Figure 1C). Importantly, IL-4Rα expression was maintained on CD3+ T cells, CD19+ B cells (Figure 1D), and macrophages (unpublished data) in smooth muscle cell–specific IL-4Rα knockout mice and equivalent to levels expressed in transgenic CRE-negative IL-4Rαlox control littermates. Functional analysis confirmed IL-4Rα responsiveness in these cell types (unpublished data). Together, these results provide convincing support for the specificity of smooth muscle cell disruption of IL-4Rα in SM-MHCCreIL-4Rαlox mice, in agreement with previously published data on the characterization of SM-MHCCre transgenic mice [14].

To investigate a possible role of IL-4/IL-13–stimulated smooth muscle cells in nematode infections, comparative infection studies with the gastrointestinal nematode N. brasiliensis were performed. Worm fecundity in the host was followed by determination of egg production in a time kinetic (Figure 2A). As previously demonstrated [2], control IL-4Rαlox mice behaved as BALB/c mice with peak fecal egg production found at day 7 and subsequently declining thereafter due to a functional host protective immune response [1,3]. Both the IL-4Rα−/− and SM-MHCCreIL-4Rαlox mice demonstrated prolonged egg production, with SM-MHCCreIL-4Rαlox mice having eggs present in their feces until day 12 postinfection (PI). As expected, IL-4Rαlox mice demonstrated a chronic infection with eggs present in feces at day 14 PI. Determining the number of worms in the intestine at various time points following infection with N. brasiliensis resulted in comparable worm burdens between IL-4Rαlox, IL-4Rα−/−, and SM-MHCCreIL-4Rαlox mice at days 4 and 7 PI. However, at day 10 PI, IL-4Rαlox control mice, but not SM-MHCCreIL-4Rαlox or IL-4Rα−/− mice, had cleared the worm (Figure 2B), explaining the extended worm fecundity. SM-MHCCreIL-4Rαlox mice responded like the IL-4Rαlox mice. Together, these results demonstrate increased susceptibility to N. brasiliensis immunity in smooth muscle cell
specific IL-4Rα-deficient mice with increased parasite burden and delayed worm expulsion.

TH2 cytokines drive protective mechanisms following N. brasiliensis infection [4]. Therefore, cytokine production by anti-CD3-stimulated CD4^+ T cells purified from mesenteric lymph nodes (MLNs) was analyzed at days 4, 7, and 10 PI. A reduction (p < 0.05) of TH2 cytokine responses was observed from CD4^+ T cells of SM-MHCCreIL-4Rα/^lox mice at all time points compared to IL-4Rα^+/lox control mice, including IL-4, IL-5, IL-9, and IL-13 (Figure 3). Impairment was comparable to mesenteric CD4^+ T cell from IL-4Rα^+/lox mice at day 7 PI.

Whereas global IL-4Rα^+/lox mice shifted to a polarized T_{H1} cytokine response, indicated by the production of interferon γ, this was not observed in infected SM-MHCCreIL-4Rα/^lox mice, which had similar interferon γ levels as IL-4Rα^+/lox control mice. In order to ascertain any compensatory cytokine production in the intestine, we examined IL-13 levels from small intestine tissue at days 4, 7, and 10 PI (Figure 4). At days 4 and 7 PI, IL-13 levels were significantly elevated in IL-4Rα^+/lox mice compared to IL-4Rα^+/lox and SM-MHCCreIL-4Rα/^lox mice (p < 0.05). By day 10 PI, intestinal IL-13 levels were reduced in IL-4Rα^+/lox mice but still
significantly higher than those in IL-4Rα−/− mice (p, 0.05). SM-MHC CreIL-4Rα−/lox mice, however, also showed significantly higher levels of IL-13 than did IL-4Rα−/− mice at day 10 PI (p, 0.05) in accordance with the delayed worm expulsion.

Reduced TH2 responses in the MLNs had no influence on systemic type 2 antibody responses, as there were similar total serum IgG1 (unpublished data) and IgE (Figure 2) concentrations in infected SM-MHC CreIL-4Rα−/lox and IL-4Rα−/− mice. Effective clearance of *N. brasiliensis* is associated with a CD4+ T cell-driven TH2 cytokine response with IL-13 playing an essential role [1]. In order to confirm a requirement for CD4+ T cells in conferring protection, we carried out a CD4+ antibody–driven depletion of these cells. Depletion was confirmed using FACS analysis (unpublished data). As expected [15], IL-4Rα−/lox–treated mice were unable to clear infection, and CD4+ T cells were also essential for clearance in SM-MHC CreIL-4Rα−/lox mice, as depletion resulted in increased adult worm burdens in SM-MHC CreIL-4Rα−/lox mice (Figure 5).

TH2 cytokine–driven expulsion of *N. brasiliensis* infections is associated with a concomitant increase in IL-4Rα−/lox-dependent intestinal goblet cell hyperplasia and mucus production, a process impaired in IL-4Rα−/− mice [3]. Interestingly, impairment of goblet cell hyperplasia was observed in SM-MHC CreIL-4Rα−/lox mice. At day 7 PI, where SM-MHC CreIL-4Rα−/lox mice showed comparable worm burdens and egg production as Cre-negative IL-4Rα−/lox control mice (Figure 2), qualitative analysis of intestine histology sections, stained with periodic acid Schiff reagent to visualize goblet cell mucus production, indicated abrogated mucus production in global IL-4Rα−/− mice and a transient reduction of goblet cell hyperplasia in SM-MHC CreIL-4Rα−/lox mice, compared to IL-4Rα−/lox control mice (Figure 5). The mucus production was delayed in SM-MHC CreIL-4Rα−/lox mice as by day 10 PI goblet cell hyperplasia was comparable to levels observed in IL-4Rα−/lox control mice at day 7 PI (Figure 6).

In addition to goblet cell hyperplasia, another proposed mechanism of expulsion of *N. brasiliensis* from the host is an increased contractile ability of smooth muscle cells [6]. Induction of such contractility is primarily mediated through an acetylcholine-driven cholinergic response mediated by the M3 muscarinic receptor [6,16,17]. We examined mRNA expression levels of the M3 receptor in the intestine of mice at days 4, 7, and 10 PI (Figure 7). At day 4 PI, no significant difference was noted between groups, although a trend for

**Figure 2.** SM-MHC CreIL-4Rα−/lox Mice Have a Delayed Adult Worm Expulsion from the Intestine

(A) *N. brasiliensis* egg production in infected mice was assessed daily from day 5 PI using the modified McMaster technique. (B) Worm burden was established on days 4, 7, and 10 PI by counting worms in intestines removed from infected mice. (C) Serum IgE antibody responses in IL-4Rα−/lox and SM-MHC CreIL-4Rα−/lox mice are equivalent. Serum from infected mice was taken on days 7 and 10 PI and analyzed for antibody production by ELISA as described in Materials and Methods. *Significant differences from IL-4Rα−/lox mice (p < 0.05); data are representative of four separate experiments.

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**Figure 3.** CD4+ Lymphocytes Are Essential for *N. brasiliensis* Clearance

MLNs were removed from infected mice on days 4, 7, and 10 PI. CD4+ cells were isolated and stimulated with CD3 for 72 h. Supernatants were then analyzed for cytokine production by ELISA as described in Materials and Methods. *Significant difference (p < 0.05) from IL-4Rα−/lox mice. Data are representative of four repeated experiments.

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higher expression in IL-4Rα−/−lox mice was noted. We found that at peak infection (day 7 PI), IL-4Rα−/−lox mice had significantly higher (p < 0.05) expression levels of M3 than both IL-4Rα+/+ and SM-MHCCreIL-4Rα−/lox mice. By day 10 PI, IL-4Rα+/+ mice still showed a significantly lower level of M3 mRNA expression compared to IL-4Rα−/−lox mice. However, SM-MHCCreIL-4Rα−/lox mice showed increased M3 expression compared to that on day 7 PI. This important result is the first report of IL-4Rα expression having an effect on the expression of acetylcholine receptors in vivo.

Together, these results show smooth muscle IL-4Rα plays an important role in the regulation of both draining lymph and intestinal cytokine production, goblet cell hyperplasia, and acetylcholine responsiveness. Disruption of these responses in the SM-MHCCreIL-4Rα−/lox mice results in delayed expulsion of the parasites.

**Discussion**

This work provides the first description of the generation, characterization, and functional analysis of a smooth muscle cell–specific IL-4Rα−/−lox mouse model. Disruption of IL-4Rα expression in smooth muscle cells was applied to a disease model where smooth muscle cells are proposed to play an important role in the resolution of infection, namely, a gastrointestinal nematode infection [6].

Clearance of nematode pathogens from the intestine is considered to require a number of physiological and immunological responses by the host. Increased intestinal contractions [6], increased mucus production [18], and elevated levels of Tg2-associated antibodies and cytokines [3] are all mechanisms induced by nematode infection. Wild-type mice infected with *N. brasiliensis* cleared the infection at day 9 PI, while SM-MHCCreIL-4Rα−/lox mice had an impaired ability to clear the nematode until day 12 PI. We demonstrated this impairment to be associated with a delay in goblet cell hyperplasia and the subsequent influx of mucus into the lumen of the host intestine. These physiological disruptions were related to an inability of the host to amplify appropriate cytokine production both locally and by CD4+ T cells from the draining MLNs.

A number of authors have demonstrated nematode-induced amplification of intestinal smooth muscle contractions to be dependent on IL-13/IL-25/STAT-6 signaling. Isolated strips of smooth muscle from the small intestine of *N. brasiliensis*–infected STAT6−/− mice have a significantly decreased tensile potential in vitro [6]. Depressed contractile ability was also observed in other nematode models in the absence of STAT-6 [6,9]. The significance of these nematode-induced contractions in the resolution of infection remains unclear. Recent work has demonstrated that the serotonin receptor 5-HT2a is a potent inducer of IL-13– and *N. brasiliensis*–dependent intestinal contractions. However, specific inhibition of 5-HT2a failed to affect the ability of the host to resolve infection [19]. We demonstrate a striking reduction in the expression of the acetylcholine M3 receptor in SM-MHCCreIL-4Rα−/lox and IL-4Rα+/+ mice following *N. brasiliensis* infection. The M3 expression data we present here.
are similar to those of 5-HT₂a in response to *N. brasiliensis* infection. However, the potential role of M3 in mediating expulsion of intestinal parasites is more compelling. M3⁻/⁻ mice are incapable of eliciting smooth muscle contractions [16]; this is not the case in 5-HT₂a⁻/⁻ mice [20]. Previous studies have demonstrated IL-13– and STAT-6–dependent increases in acetylcholine-induced smooth muscle contractions in tissue from *N. brasiliensis*–infected mice [6]. M3 is the principal acetylcholine receptor in smooth muscle and drives 75% of the contractile response in the small intestine [16]. As such, our demonstration of significant inhibition of M3 expression in IL-4Rα-deficient mice is compelling in vivo evidence of IL-4Rα–muscarnic receptor interactions contributing to proposed muscle hypercontractility–aided nematode expulsion [8].

In addition to contractile responses, host epithelial responses constitute a second major physiological response to the parasite. This response varies according to parasite; in the case of the intraepithelial nematode *Trichuris muris*, expulsion is driven by epithelial cell turnover [21]. The principal aspect of this response to the luminal dwelling *N. brasiliensis* is induction of goblet cell–driven mucus production. Goblet cell–derived mucus is essential for clearance of *N. brasiliensis* infection [22,23]. Secreted mucus directly affects viability of the worms through inhibition of parasite motility [24,25] and ability to feed [26]. Pathogen-induced mucus production is strongly influenced by the host immune response. A deficiency in T_{H}2 polarization severely impairs the ability of goblet cells to secrete mucus and expel *N. brasiliensis* [18]. Mucus production is also modulated by the enteric nervous system via innervation of mucosal mast cells [27] and goblet cells [28]. Innervation of epithelial mucus-producing cells is also important for the host mucosal response to *N. brasiliensis* infection [29,30]. Previous studies have established the importance of this epithelial response, the most significant cells for effective expulsion being the mucus-producing goblet cells. This body of work combined with the data we present suggests that smooth muscle cells may represent an intermediate zone of signal transduction between the epithelium and MLNs. Disruption of the ability of the smooth muscle cells to respond to IL-4Rα ultimately results in a delayed mucosal response and depressed MLN cytokine production.

Prolonged *N. brasiliensis* infection, due to a deficiency in smooth muscle cell expression of IL-4Rα, may therefore be a result of the host’s inability to mount an effective mucosal response. Delayed mucus responses to *N. brasiliensis* infection are associated with an impaired T_{H}2 response [18]. The delayed expulsion we report here is then explained by the reduced MLN CD4⁺ T_{H}2 response (Figure 3), delaying mucus production (Figure 6) through inhibition of smooth muscle responsiveness to neurotransmitters (Figure 7) and cytokines. The depressed T_{H}2 response we suggest to be a result of smooth muscle cells being unable to react effectively to the key smooth muscle contraction amplifying cytokine IL-13 and the neurotransmitter acetylcholine [6] sufficiently to stimulate rapid cytokine production in the MLNs. Parasite clearance would then be more reliant on local effector lymphoid tissue responses [31]. The resulting recovery in response to infection and its eventual clearance in the SM-MHC^Cre^IL-4Rα^lox^ mouse may then be explained by local responses in the intestine providing a sufficient, albeit delayed and reduced compensatory response which induces the eventual disease-resolving response (Figure 4).

In conclusion, we have demonstrated in vivo a significant role for smooth muscle cell IL-4Rα in the optimal resolution of a gastrointestinal nematode infection. Deletion of smooth muscle cell IL-4Rα expression in *N. brasiliensis* infection. Deletion of smooth muscle cell IL-4Rα expression in *N. brasiliensis* infection.
muscle IL-4Rα significantly disrupts the host ability to resolve infection with *N. brasiliensis*. We demonstrate severe disruption of both known and proposed mediators of expulsion. Depressed M3 receptor expression, delayed goblet cell hyperplasia, disruption of CD4+ MLNs, and intestinal cytokine production provide compelling evidence for an important role in the induction of both physiological effector mechanisms and immunological mediators of expulsion. Together, these data are suggestive of smooth muscle IL-4Rα being an important inducer of T₃₂ cytokine signaling from the lymph node and tissue and goblet cell hyperplasia and having a striking effect on the key smooth muscle contraction–inducing M3 muscarinic receptor (Figure 8).

Materials and Methods

**Generation and genotyping of conditional IL-4Rα-deficient mice.** SM-MHCCre mice were a kind gift from Gary K. Owens, Charlotteville, Virginia, United States [14,32]. SM-MHC^Cre^ mice were backcrossed to C57BL/6J for 40 cycles on an MJ thermocycler (Biozym Diagnostik, http://www.biozym.com). Cell purity was approximately greater than 98%. CD4+ T cells were restimulated for 72 h with anti-CD3 (clone 145–2C11; 20 μg/ml). Supernatants were then collected and stored at −80 °C until analysis.

**ELISA analysis.** Cytokines in supernatants and serum antibody isotype levels from infected animals were determined as previously described [35].

**Histology.** Tissue samples were fixed in a neutral buffered formalin solution. Following embedding in paraffin, samples were cut into 5-μm sections. Sections were stained with hematoxylin and eosin or periodic acid–Schiff reagent. The number of positively stained cells per five villi were counted by light microscopy. All samples were randomized and counted in a blinded fashion.

**RT-PCR.** RNA was extracted from the intestine of infected mice with the use of Tri-reagent (Sigma), and cDNA was synthesized using the ImProm-II Reverse Transcription System (Promega, http://www.promega.com). M3 cDNA was amplified using the following primers: 5’-CCGG AAA AGG ATG TCG-3’ and 5’-GGG ACT CCG GTG TGA A-3’. Data were normalized using the β-actin housekeeping gene.

**Statistics.** Values are given as mean ± SD, and significant differences were determined using the Mann-Whitney U test.

**Supporting Information**

**Accession Numbers**

Accession numbers for the genes and gene products discussed in this paper are IL-13 (16163), IL-4 (16190), STAT-6 (20852), M3 (12671), smooth muscle myosin heavy chain (17880), and α-actin (11475).

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**Authors/Contributors.** WGC and FB conceived and designed the experiments. WGC, AJC, CJH, HM, EM, and BA performed the experiments, WGC, AJC, and CJH analyzed the data. FDE, GKO, and DE contributed reagents/materials/analysis tools. WGC and AJC wrote the paper.

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**Competing interests.** The authors have declared that no competing interests exist.

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