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 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-MHCcreIL-4Rαlox/lox) 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-MHCcreIL-4Rαlox/lox 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 mesenteric lymph node, and reduced M3 muscarinic receptor expression during infection. Together, these results demonstrate that in vivo IL-4Rα–responsive smooth muscle cells are beneficial for N. brasiliensis expulsion by coordinating T helper 2 cytokine responses, goblet cell hyperplasia, and acetylcholine responsiveness, which drive smooth muscle cell contractions.

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Introduction

The interleukin (IL)-13/IL-4 receptor α (IL-4Rα)/signal transducer and activator of transcription 6 (STAT-6) signaling pathway is essential in the control of a number of infectious diseases as well as being a key factor in the induction of allergic responses. Signaling through this pathway can either confer protective immunity or mediate tissue damage depending on the antigenic stimuli and the cell-specific response [1]. Previously, our laboratory provided the first description of the effect of a cell-specific deletion of IL-4Rα from macrophages and neutrophils on the host’s ability to respond to two parasitic infections [2]. It was demonstrated that such a deletion failed to affect resolution of infection by the nematode Nippostrongylus brasiliensis, while mice demonstrated an increased susceptibility to infection by the trematode Schistosoma mansoni. The work presented here describes the effect of a smooth muscle–specific disruption of IL-4Rα expression on the immune response to N. brasiliensis.

Murine infection with N. brasiliensis induces a strong protective host T helper 2 (Th2) 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].

Although essential for expulsion of N. brasiliensis from 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...
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 α (IL-4Rα) responsiveness for worm expulsion in global IL-4Rα-deficient mice. In this study, we specifically addressed the role of IL-4Rα responsiveness in a novel smooth muscle cell–specific IL-4Rα-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-4Rα being an important coordinator of both the immune and physiological responses to intestinal worm infections. A proposed model is suggested with IL-4Rα 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/or smooth muscle cells is abrogated in STAT-6lox/lox 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-MHCCre) IL-4Rαlox/lox mice, we demonstrated 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 TGFβ cytokines is delayed and/or reduced, as is intestinal expression of the M3 acetylcholine receptor, in response to infection with *N. brasilensis*.

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-4Rαlox/lox and “floxed” IL-4Rαlox/lox BALB/c mice to establish smooth muscle cell–specific IL-4Rα-deficient BALB/c mice (SM-MHCCreIL-4Rαlox/lox, with one deleted and one floxed IL-4Rα allele (SM-MHCCreIL-4Rαlox/lox) to increase the efficiency of Cre-mediated site-specific recombination. Mutant mice 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-4Rαlox (GMF: 11.2) mice (Figure 1B). Low levels of expression were present in IL-4Rαlox 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 on transgenic Cre-negative IL-4Rαlox control littersmates. 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. brasilensis* 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-4Rαlox 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. brasilensis* resulted in comparable worm burdens between IL-4Rαlox, IL-4Rαlox, 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-4Rαlox mice, had cleared the worm (Figure 2B), explaining the extended worm fecundity. SM-MHCCreIL-4Rαlox mice, but not IL-4Rαlox mice, showed complete worm expulsion at day 14 PI (Figure 2A). Examination of total serum IgE antibody (Figure 2C) levels showed that SM-MHCCreIL-4Rαlox mice responded like the IL-4Rαlox mice. Together, these results demonstrate increased susceptibility to *N. brasilensis* in smooth muscle cell–specific IL-4Rα-deficient BALB/c mice.
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-4Rαlox/lox mice at all time points compared to IL-4Rαlox/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-4Rαlox/lox mice at day 7 PI. Whereas global IL-4Rαlox/lox mice shifted to a polarized T11 cytokine response, indicated by the production of interferon γ, this was not observed in infected SM-MHCCreIL-4Rαlox/lox mice, which had similar interferon γ levels as IL-4Rαlox/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-4Rαlox/lox mice compared to IL-4Rαlox/lox and SM-MHCCreIL-4Rαlox/lox mice ($p < 0.05$). By day 10 PI, intestinal IL-13 levels were reduced in IL-4Rαlox/lox mice but still
significantly higher than those in IL-4Rα/C0/lox mice (p, 0.05).
SM-MHCCreIL-4Rα/C0/lox mice, however, also showed significantly higher levels of IL-13 than did IL-4Rα/C0/lox 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-MHCCreIL-4Rα/C0/lox and IL-4Rα/C0/lox mice. Effective clearance of _N. brasiliensis_ is associated with a CD4+-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-4Rx/C0/lox–treated mice were unable to clear infection, and CD4+ T cells were also essential for clearance in SM-MHCCreIL-4Rα/C0/lox mice, as depletion resulted in increased adult worm burdens in SM-MHCCreIL-4Rα/C0/lox mice (Figure 5).

T_{H2} cytokine–driven expulsion of _N. brasiliensis_ infections is associated with a concomitant increase in IL-4Rx–dependent intestinal goblet cell hyperplasia and mucus production, a process impaired in IL-4Rα/C0/lox mice [3]. Interestingly, impairment of goblet cell hyperplasia was observed in SM-MHCCreIL-4Rα/C0/lox mice. At day 7 PI, where SM-MHCCreIL-4Rα/C0/lox mice showed comparable worm burdens and egg production as Cre-negative IL-4Rα/C0/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/C0/lox mice and a transient reduction of goblet cell hyperplasia in SM-MHCCreIL-4Rα/C0/lox mice, compared to IL-4Rα/C0/lox control mice (Figure 5). The mucus production was delayed in SM-MHCCreIL-4Rα/C0/lox mice as by day 10 PI goblet cell hyperplasia was comparable to levels observed in IL-4Rx/C0/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
higher expression in IL-4R<sup>-/lox</sup> mice was noted. We found that at peak infection (day 7 PI), IL-4R<sup>-/lox</sup> mice had significantly higher (p < 0.05) expression levels of M3 than both IL-4R<sup>-/-</sup> and SM-MHC<sup>Cre</sup>IL-4R<sup>-/lox</sup> mice. By day 10 PI, IL-4R<sup>-/-</sup> mice still showed a significantly lower level of M3 mRNA expression compared to IL-4R<sup>-/lox</sup> mice. However, SM-MHC<sup>Cre</sup>IL-4R<sup>-/lox</sup> mice showed increased M3 expression compared to that on day 7 PI. This important result is the first report of IL-4R<sup>-</sup> expression having an effect on the expression of acetylcholine receptors in vivo.

Together, these results show smooth muscle IL-4R<sup>-</sup> 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-MHC<sup>Cre</sup>IL-4R<sup>-/lox</sup> 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-4R<sup>-</sup>-deficient mouse model. Disruption of IL-4R<sup>-</sup> 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 Th2-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-MHC<sup>Cre</sup>IL-4R<sup>-/lox</sup> 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<sup>+</sup> 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-13/4R<sup>-</sup>/STAT-6 signaling. Isolated strips of smooth muscle from the small intestine of *N. brasiliensis*-infected STAT6<sup>–/-</sup> 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-HT<sub>2a</sub> is a potent inducer of IL-13– and *N. brasiliensis*–dependent intestinal contractions. However, specific inhibition of 5-HT<sub>2a</sub> 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-MHC<sup>Cre</sup>IL-4R<sup>-/lox</sup> and IL-4R<sup>-/-</sup> mice following *N. brasiliensis* infection. The M3 expression data we present here...
are similar to those of 5-HT₂₃ in response to N. brasilienis 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-HT₂₃⁻⁻ mice [20]. Previous studies have demonstrated IL-13– and STAT-6–dependent increases in acetylcholine-induced smooth muscle contractions in tissue from N. brasilienis–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–muscariic 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. brasilienis is induction of goblet cell–driven mucus production. Goblet cell–derived mucus is essential for clearance of N. brasilienis 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 Th2 polarization severely impairs the ability of goblet cells to secrete mucus and expel N. brasilienis [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. brasilienis 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 results in a delayed mucosal response and depressed MLN cytokine production.

Prolonged N. brasilienis 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 mucus response. Delayed mucus responses to N. brasilienis infection are associated with an impaired Th2 response [18]. The delayed expulsion we report here is then explained by the reduced MLN CD4⁺ Th2 response (Figure 3), delayed mucus production (Figure 6) through inhibition of smooth muscle responsiveness to neurotransmitters (Figure 7) and cytokines. The depressed Th2 response suggest we 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-MHCcreIL-4Rαlox 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 is associated with reduced mucus production and delayed expulsion. Deletion of smooth muscle cell IL-4Rα ultimately results in a delayed mucosal response and depressed MLN cytokine production.
muscle IL-4Rα significantly disrupts the host ability to resolve infection with *N. brasilensis*. 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 immunomediatory mediators of expulsion. Together, these data are suggestive of smooth muscle IL-4Rα being an important inducer of T_{H2} 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-MLC–Cre mice were kindly provided by Gary K. Owens, Charlottesville, Virginia, United States [14,32]. SM-MHC-Cre mice were backcrossed to BALB/c for nine generations and intercrossed with IL-4Rα−/− mice (n = 30). These mice were then mated with IL-4Rα−/− mice (n = 2) to generate SM-MHCCre/IL-4Rα−/− mice. Transgene negative littermates (IL-4Rα−/−) were used as controls in all experiments. Mice were housed under specific pathogen-free barrier conditions in the University of Cape Town animal facility. All work was approved by the University of Cape Town animal ethics board.

**Genotyping.** Specific PCR primer pairs were for the IL-4Rα−/−, 5′-GTACGCGGACCATTTTCTC-3′ and 5′-CATCGGCGCAGTCACC CCTCT-3′; deletion, 5′-GCTTGCCCCGAAATTATC ACC-3′ and 5′-CCCTTGAGAACTGCGGGCT-3′; LoxP, 5′-CCCTTTGTCCG CCTCGTAAATT-3′ and 5′-GTTCCTCCTACCGTTAG-3′; and Cre, 5′-ATGCCCAAAAGAACAGAGAGGT-5′ and 5′-GAATATT CAGTCGC CCAAAGGCTGAGA-5′. PCR conditions were as follows: 94°C for 1 min, 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min for 40 cycles on an MJ thermocycler (Biozym Diagnostik, http://www.biozym.com).

**Analysis of IL-4Rα expression by FACS.** A single cell suspension of smooth muscle cells was prepared as previously described [33] along with lymph node cells. For the intracellular stain, cells in single cell suspension were incubated with 1% normal rat serum and stained with rat anti-mouse IL-4Rα PE (mIL-4R-M-I; BD Biosciences, http://www.bdbiosciences.com). Stained cells were then washed, fixed in 2% paraformaldehyde, permeabilized with saponin, preblocked with 2% NRS and stained with anti-actin–FITC (Abcam, http://www.abcam.com) or isotype control IgG2a–FITC (BD Biosciences). For lymphocyte staining, anti-CD3–FITC, anti–CD19–PE, and anti-IL-4Rα–biotin in combination with SA-APC were used to identify lymphocyte subsets (BD Pharmingen, http://www.bdbiosciences.com). Neighboring cells were stained with 7-AAD and excluded from analysis (Sigma, http://www.sigmadiich.com). Acquisition was performed using FACS Calibur and cells analyzed using CellQuest (Becton Dickinson, http://www.bd.com).

**Infection studies.** Mice were infected subcutaneously with 750 *N. brasiliensis* L3 larvae (kindly provided by Klaus Erb, Wurzburg, Germany). Analysis of parasite eggs in feces was carried out using the modified McMaster technique [34]. Adult worm burdens were determined as previously described [3]. CD4+ T-cell depletion. CD4+ T cells were depleted from mice by intraperitoneal injection of 0.5 mg of anti-CD4+ monoclonal antibody (clone GK1.5) 3 d prior to infection. Mice received booster injections every 3 d to maintain depletion. Effective depletion was confirmed by FACS analysis.

**Ex vivo restimulation of lymphocytes.** CD4+ T cells were purified from pooled MLNs at days 7 and 10 PI. Enrichment was carried with a negative selection. Briefly, cells in single cell suspension were stained with anti-CD8, CD11b, Gr-1, B220, and CD16/32. Stained cells were depleted using goat anti-rat IgG-coated magnetic beads (Bioแมง). Staining was confirmed by FACS analysis.

**Statistics.** Differences were determined using the Mann-Whitney U test.

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