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The Lung Is an Important Site for Priming CD4 T-Cell-Mediated Protective Immunity against Gastrointestinal Helminth Parasites▽‡

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The rodent hookworm *Nippostrongylus brasiliensis* typically infects its host by penetrating the skin and rapidly migrating to the lungs and gut. Following primary infection, immunocompetent mice become highly protected from reinfection with *N. brasiliensis*, with the numbers of worms gaining access to the lungs and gut being reduced by up to 90%. We used green fluorescent protein/interleukin-4 (IL-4) reporter mice and truncated infection studies to identify both the tissue site and mechanism(s) by which the host protects itself from reinfection with *N. brasiliensis*. Strikingly, we demonstrated that the lung is an important site for priming immune protection. Furthermore, a lung-initiated, CD4 T-cell-dependent, and IL-4- and STAT6-dependent response was sufficient to confer protection against reinfection. In conclusion, vaccination strategies which seek to break the cycle of reinfection and egg production by helminths such as hookworms can include strategies which directly stimulate Th2 responses in the lung.

Establishing protective immunity to helminth nematodes (whipworms, hookworms, pinworms, and ascarids) through vaccination is currently a major global health objective. It has long been recognized that increasing host age and exposure to infections result in a state of Th2-mediated immunity which protects the host from reinfection. However, the immune cells, signaling factors, and cytokines that mediate such immunity and how and where they act within the body remain largely undefined. The potential opportunities for the immune system to attack tissue-migrating helminths include the skin entry point, the phase of migration through the circulatory system and lungs, and the intestinal phase where maturation to an egg-laying adult occurs. To date, investigations of the immune mechanisms mediating protection against this group of helminths has focused mainly on the expulsion of adult worms from the gut; however, expulsion from the gut does not explain the observed increased resistance to reinfection seen with increasing age and exposure to infection. To better define the immune mechanisms that confer protection against reinfection with helminths, we used a rodent hookworm (*Nippostrongylus brasiliensis*) infection model in combination with G4 reporter mice and truncated infection techniques to study and compare the worm burden and immune responses at the tissue-specific stages of the infection cycle in mice. In primary infections, infective L3 (iL3) worms invade the mouse host by penetrating the skin, entering venous blood vessels, and being carried to the lung capillary bed, where they burst through to the lung parenchyma as L3 worms. In the lungs, worms mature to the L4 stage and, over a 24- to 48-h period, are swept to the esophagus by the mucociliary ladder, whereupon they are ingested and mature in the gastrointestinal (GI) tract. Once in the gut, the worms actively localize to the upper portion of the small intestine, where L4 worms mature to adult L5 worms, which mate and produce eggs until they are expelled from the immunocompetent host in a "self-cure" reaction (4). The mechanisms underlying the expulsion of adult stage worms have been very well defined (14), with CD4 T cells, interleukin-4 (IL-4), IL-13, and STAT6 (9, 28) playing the major roles in expulsion. IL-5 and IL-9 have also been shown to have additive roles in this expulsion phenomenon (7). Additional studies by Dent and colleagues utilizing an air pouch challenge infection model of *N. brasiliensis* demonstrated that boosting eosinophil numbers can confer resistance to reinfection, most particularly at the skin site and lungs (6, 17, 25).

It is clear that for the purposes of both global vaccination and understanding acquired resistance in humans, we need to identify the key immunological processes that underlie the development of protective immunity. Evidence supporting the skin as an important site has come from studies using skin-penetrating parasitic nematodes (6, 10, 11, 17). Vaccination experiments involving other species of helminth parasite have emphasized the importance of the lung as a site of protective immunity (5, 20, 21, 24), and protective roles for the gut have also been postulated (8).

It has been well established that IL-4-producing CD4 T cells are a critical component of the protective immune response to *N. brasiliensis* (8, 9, 14). Therefore, we developed assays that could detect and quantify the IL-4-producing cells that arise following primary infection and reinfection. IL-4/green fluorescent protein (GFP) reporter G4 mice (12) have proven

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themselves to be a highly accurate model for quantitatively measuring Th2 immune responses *in vivo*. When G4 mice are maintained as a heterozygous G4/IL-4 line, with one allele expressing GFP and the other allele expressing IL-4, GFP can be used as a faithful reporter of IL-4 production, while sufficient IL-4 production is provided by the other allele to allow the development of a normal Th2 immune response (12, 29). In this study, we infected G4 mice with different stages of the *N. brasiliensis* infection cycle (L3, L4, or L5 worms) via different routes to determine the tissue location where IL-4-producing Th2 immune responses are generated. Utilizing this sensitive *in vivo* technique combined with *in vitro* studies, we showed that key protective Th2 immune responses were generated in the lung. Furthermore, by using reinfection studies of mouse strains deficient in key cytokines and signaling pathways, we found that CD4 T-cell-dependent mechanisms are important for protection from reinfection with *N. brasiliensis*.

MATERIALS AND METHODS

Mice. The G4/IL-4 (on a BALB/c background), STAT6$^{-/-}$, G4/G4 (IL-4$^{-/-}$ in a BALB/c background), IL-5$^{-/-}$ B6Aao (MHC [major histocompatibility complex] class II$^{-/-}$), BALB/c, μMT (B cell$^{-/-}$), CD1$^{-/-}$, and C57BL/6J mice used in these experiments were bred by the Biomedical Research Unit, Malaghan Institute of Medical Research, Wellington, New Zealand. Six 10-week-old age- and sex-matched mice were used in all of the experiments. All of the experimental procedures described in this report were approved by the Victoria University Animal Ethics Committee and carried out in accordance with the guidelines of the Victoria University of Wellington, Wellington, New Zealand.

Maintenance, isolation, and use of the L3, L4, and L5 stages of *N. brasiliensis*. *N. brasiliensis* was originally sourced from Lindsay Dent (University of Adelaide) and has been maintained by monthly passage through Lewis rats for 15 years with additional selective passage in immunodeficient and immunocompetent mice at previous times in its life history to improve parasitism in the mouse host. iL3 worms were prepared from 2-week rat fecal cultures, with careful preparation of 100% viable iL3 worms being essential for consistent infection studies with mice (4). Viable motile fourth-stage larval forms of *N. brasiliensis* (L4 worms) were isolated by migration from *in vitro* cultures of 2-day postinfection lung tissues, followed by extensive washing in phosphate-buffered saline (PBS) (4). The homogeneity and viability of L4 worms were confirmed by microscopic analysis. Lung infection of mice with L4 worms was achieved by the intranasal (i.n.) administration of ~200 freshly harvested L4 worms in a 50-μl drop to the noses of mice which were lightly anesthetized with xylazine and ketamine for the duration of the inoculation procedure. Regular breathing of the mice causes the drops containing L4 worms to be inspired, with the viable L4 worms being detected in lung tissues following this procedure. L5 adult worms were prepared by migration from the intestines of infected mice, washed extensively in PBS, and counted, and their viability was determined by microscopy (4). Infection of mice with L5 worms was performed by gavage of ~300 freshly harvested L5 adult worms. The anthelminthic pyrantel embonate was administered where specified by oral gavage daily at 1 mg/mouse. Subcutaneous (s.c.) infections were performed by inoculating mice in the scruff of the neck with 600 dead or live iL3 worms in a volume of 200 μl (4). For intradermal (i.d.) infections, mice were anesthetized using xylazine and ketamine (Phoenix, New Zealand) and 30 μl containing 600 dead or live iL3 worms was injected into the ear pinnae using a BD Ultrafine 29-gauge needle and syringe (Becton Dickinson, New South Wales, Australia).

CFSE labeling of live parasites. iL3 worms were washed several times in PBS, incubated at room temperature for 8 min in 2.5 mM carboxyl fluorescein succinimidyl ester (CFSE), and then washed in PBS three times before infection of the host.

Preparation of dead iL3 worms. iL3 worms were harvested from fecal cultures and washed five times in sterile PBS. Larvae were made up to a concentration of 600 L3 worms per 30 μl in PBS and put through three freeze-thaw cycles. Larvae were then checked for loss of viability by microscopic analysis.

***In vitro* skin penetration assay.** The abdomen skin was shaved, and 2 cm^2 was harvested from naïve or previously infected mice. Skin was placed on 15-ml tubes containing PBS. Two hundred L3 worms were added to each tube and incubated overnight at 37°C. Larvae penetrating the skin accumulated at the bottom of the tube. Skin was then incubated in PBS to cause the larvae remaining on the skin to migrate out. This method is an adaptation of that of Brown et al. (3).

Imaging of larvae in ear tissue. CFSE-labeled L3 worms were injected i.d. into the ear pinnae. The ears were excised, and the dorsal and ventral sheets were separated. Images were captured using an Olympus BX51 microscope, an Olympus DP70 camera, and AnalySIS LS software.

Viable worm recovery assay. Tissue (lung or gut) was diced, placed on cheesecloth, and suspended in a 50-ml centrifuge tube containing PBS at 37°C for at least 2 h. For recovery of worms from inoculated ears, the dorsal and ventral sheets were separated, placed on cheesecloth, and suspended in PBS at 37°C. Viable worms migrated out and accumulated at the bottom of the tube before being counted on a gridded counting plate.

Cell preparation and flow cytometric analysis of lymphocytes. Mediastinal and mesenteric lymph nodes were isolated and pressed through a 70-μm cell strainer to make a single-cell suspension. Isolated cells were then resuspended in fluorescence-activated cell sorting buffer, and Fc receptors were blocked (2.4G2) before staining with monoclonal antibodies against CD4 (GK1.5) and CD3 (2C11) prior to collection on a FACScalibur flow cytometer (BD). Analysis was performed with Flowjo software (Treestar).

Lung histopathology. Lung tissues were harvested from naïve and *N. brasiliensis*-infected mice into 10% formalin before embedding in paraffin. Mucus-containing goblet cells were detected by staining of 4-μm-thick slice specimens with periodic acid-Schiff (PAS) and alcian blue. All bronchioles with a diameter of 200 to 600 μm were counted in both lungs, with the number of PAS-positive cells per bronchiole being reported. Further sections were stained with hematoxylin and eosin (H&E) to assess the inflammatory infiltrate.

Statistical analysis. Statistical analysis was performed with Prism software using two-tailed Mann-Whitney U testing.

RESULTS

Kinetics of larval elimination and Th2-type immunity development during primary and secondary *N. brasiliensis* infections. To determine if the induction of Th2-type immunity directly correlates with the worm burden, we used the viable worm recovery assay to compare the kinetics of larval recovery with Th2 development at different tissue sites of infected, naïve, or reinfected G4/IL-4 mice. iL3 worms were inoculated by the i.d. (Fig. 1A) or s.c. (Fig. 1C) route to ensure a more uniform infection than experimentally possible by natural penetration of the skin by iL3 worms. Indeed, the kinetics of infection and fecundity (unpublished observation, data not shown) of adult L5 worms did not appear to be affected when different injection routes were used (Fig. 1C, s.c. exposure; see Fig. S1A in the supplemental material for i.n. exposure) and a linear relationship between the number of L3 worms inoculated and the number of viable worms which could be recovered from the lungs was achieved in the dose range used in these experiments (see Fig. S2 in the supplemental material for s.c. inoculation). It is important to note that the degree of worm recovery from either lung or gut tissue never exceeded 50 to 60% of the input inoculum, even in completely immunodeficient mice, indicating that there is a natural attrition of worm numbers, probably due to nonhost factors (see Fig. S2 in the supplemental material; data not shown). Our attempts to enumerate worm migration and trapping by histological analysis proved problematic due to the relatively long, hollow, tubular shape of the parasite. Therefore, we developed a CFSE staining (Fig. 1B) and viable worm recovery protocol adapted from techniques of Camberis et al. (4) for the accurate quantification of viable versus trapped worms in tissues. In naïve mice, the majority (>80%) of the L3 worms injected into the ear pinnae rapidly disappeared (5 to 60 min) from the i.d. injection site during a primary infection (Fig. 1A). Worms were observed in the lungs from 24 to 72 h after s.c. infection

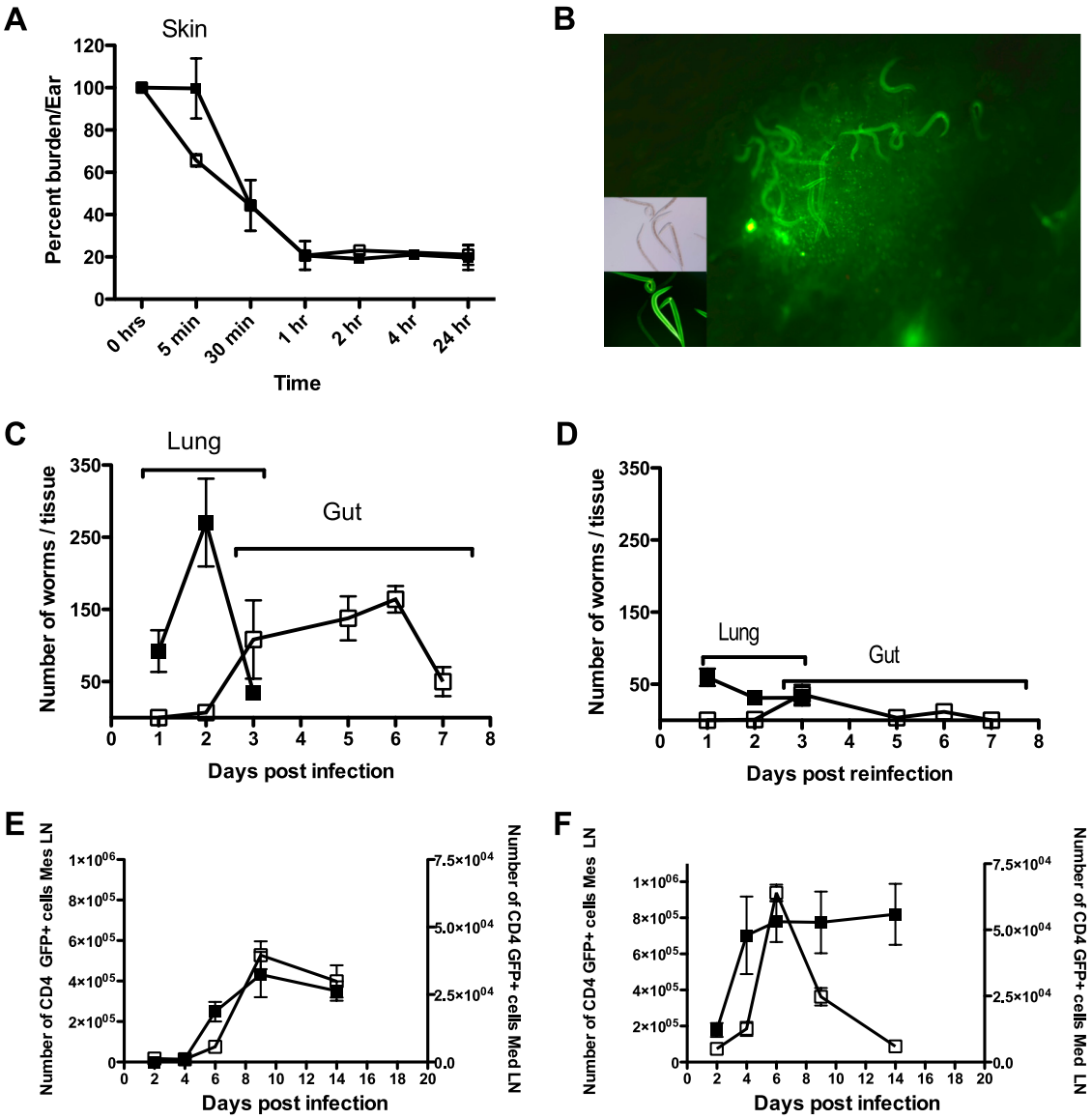

FIG. 1. Reduction in worm burden correlates with induction of CD4 GFP$^+$ Th2 immune response in mice reinfected with *N. brasiliensis*. (A) Number of larvae migrating from site of skin infection. G4/IL-4 mice ($n = 3$ per time point) were infected i.d. in the ear pinna with 600 L3 *N. brasiliensis* worms (open squares) or left uninfected (closed squares), rested, and challenged by i.d. reinfection with 600 L3 *N. brasiliensis* worms. At the time points indicated, viable larvae that migrated out of excised ears were enumerated. Values are expressed as percent burden ± the standard error of the mean (SEM) and are representative of three experiments. (B) Representative photomicrograph of a mouse ear 5 min after the injection of CFSE-labeled L3 *N. brasiliensis* worms. Inset, differential interference contrast versus fluorescence microscopy of the same worms. The worms pictured are 0.5 mm in length. (C) Parasite burden during s.c. primary *N. brasiliensis* infection. G4/IL-4 mice ($n = 4$) were primed with 600 L3 *N. brasiliensis* worms s.c. Lung (closed squares) and gut (open squares) tissues were harvested, and viable worms that migrated out were enumerated. The values shown represent the mean number of worms recovered ± the SEM and are representative of three independent experiments. (D) Parasite burden during *N. brasiliensis* reinfection. G4/IL-4 ($n = 4$) mice were primed with 600 L3 *N. brasiliensis* worms s.c., rested, and reinfected s.c. with 600 L3 *N. brasiliensis* worms. Lung (closed squares) and gut (open squares) tissues were harvested, and viable worms that migrated out were enumerated. The values shown represent the mean number of worms recovered ± the SEM and are representative of three independent experiments. (E) GFP$^+$ CD4 T-cell responses during primary s.c. *N. brasiliensis* infection. Mediastinal (closed squares) and mesenteric (open squares) lymph nodes from s.c. *N. brasiliensis*-infected animals ($n = 4$) were harvested and surface stained for CD3 and CD4. The values shown represent the mean number of cells ± the SEM and are representative of at least three independent experiments. (F) GFP$^+$ CD4 T-cell responses during *N. brasiliensis* reinfection. Mediastinal (closed squares) and mesenteric (open squares) lymph nodes from s.c. *N. brasiliensis*-infected animals ($n = 4$) were harvested and surface stained for CD3 and CD4. The values shown represent the mean number of cells ± the SEM and are representative of at least three independent experiments.

(kinetics were identical after i.d. infection, data not shown) and then accumulated in the gut, with the worm burden peaking at day 6 postinfection (Fig. 1C). When the activation of GFP$^+$ Th2 CD4 T cells in the draining lung and gut lymph nodes was followed during the same primary infection, the numbers of GFP$^+$ Th2 cells per lymph node peaked at day 9 postinfection, after 90% of the L5 worms had been expelled from the intestine of the host (Fig. 1E). To follow the fate of worms during

reinfection, previously s.c. infected mice were rested for a total of 30 days postinfection to allow the cellular inflammatory response, indicated by the number of $CD4^+$ GFP^+ cells in the draining lymph nodes of the lungs (see Fig. S3B in the supplemental material), to subside and then reinfected s.c. with *N. brasiliensis* L3 worms. Surprisingly, despite an increase in cellularity of the previously infected ear tissue (data not shown), the iL3 worms were not trapped in the skin as we expected and were able to migrate from the skin site with a speed and efficiency equal to those of worms infecting a naïve mouse, with 80% of the larvae gone by 60 min (Fig. 1A). However, when the lungs of s.c. reinfected mice were analyzed for worm burdens (Fig. 1D), there was a dramatic 10-fold reduction in the number of recoverable L3/L4 worms throughout the 24- to 72-h postinfection period compared to the worm burdens in lungs of identically s.c. infected naïve mice (Fig. 1C). Similarly, in the guts of s.c. reinfected mice, a dramatic 10-fold reduction in the L5 worm burden was observed (Fig. 1D). The lack of evidence for trapping of worms in the skin of reinfected mice was confirmed by the finding that identical skin, lung, and gut worm recoveries were obtained in reinfected mice, whether the skin reinfection site was the same as, or distant from, the site of the primary skin infection (data not shown). Of the most significance was the finding that the reduction in worm burden seen in s.c. reinfected mice correlated with the rapid induction of a CD4 GFP^+ T-cell response in the lung draining mediastinal lymph nodes that was strikingly faster (detectable at 48 h) and 20-fold greater than the CD4 GFP^+ T-cell response seen in the lymph nodes of s.c. infected naïve mice (Fig. 1E and F). Similarly, faster and increased CD4 GFP^+ responses were detected in the mesenteric lymph nodes of s.c. reinfected mice, although the duration of the CD4 GFP^+ response was greatly reduced. This was most probably due to the significant decrease in L4 and L5 worms making it to the guts of the reinfected mice compared to those of naïve mice.

Immune skin does not protect against *N. brasiliensis* L3 worm invasion. The reduction in viable worm recovery from the lungs and guts of s.c. reinfected mice implied that adaptive immune-mediated mechanisms either reduced the viability of the L3, L4, and L5 worms or blocked their migration through the host to reach adulthood in the gut. Previous studies have already demonstrated that L3 worms can be trapped at the initial inoculation site and fail to migrate to the lung, especially in situations where there is an abundance of eosinophils accumulating from previous infections or aberrant IL-5 cytokine expression (6, 17). To determine whether skin of previously s.c. infected mice prevented the migration of L3 worms either through tissue remodeling or chronic accumulation of residual immune cells, we adapted an *in vitro* assay to follow the migration of L3 worms through skin (Fig. 2A). First we examined the ability of L3 worms to migrate through skin that has been isolated from either naïve or previously s.c. infected/immune mice. Worms were placed on either naïve or immune skin, a temperature gradient was applied, and the worms that migrated through the skin were enumerated. No difference was observed in the ability of worms to migrate through skin from previously s.c. infected, hence immune, animals or skin from naïve mice (Fig. 2B). In a separate experiment, it was observed that infection with living worms was necessary for the development of protection, as mice vaccinated i.d. with dead L3 worms were not protected from s.c. reinfection (Fig. 2C). A time course analysis of the duration of the protective effect in the lung and gut established that the reduction in worm burdens in the lung began to wane after 180 days but the ability to reduce the burden of adult worms in the gut was maintained essentially for the life of the mouse (see Fig. S3A in the supplemental material). Taken together with the results in Fig. 1, these data indicate that the reduction of lung and gut worm burdens in reinfected mice is not due to the worms being trapped in the skin but rather to largely undetermined tissue immune-mediated events leading to the failure of adult worms to establish an infection in the GI tract.

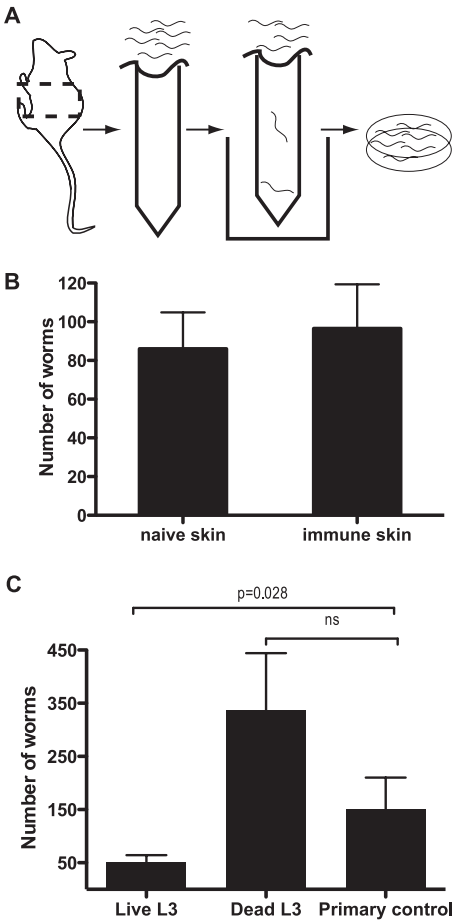

FIG. 2. The skin is not the site of protection from reinfection. (A) Schematic representation of *in vitro* skin migration assay. Skin harvested from naïve or previously s.c. infected mice ($n = 4$ per group) was suspended over PBS. iL3 worms were placed on the skin samples, and those that migrated through were enumerated. (B) L3 worm skin migration efficiency *in vitro*. L3 *N. brasiliensis* worms able to migrate through naïve or immune skin (as described for panel A) were enumerated. The values shown represent the mean number of larvae that migrated ± the SEM. (C) Priming with dead worms compromises protection. G4/IL-4 mice ($n = 3$ per group) were inoculated i.d. in the ear pinna with 600 dead or live L3 *N. brasiliensis* worms, rested, and challenged s.c. with 600 live L3 *N. brasiliensis* worms. Lungs were excised on day 2 postinfection, and the viable worms present were enumerated using the migration assay described in Materials and Methods. Data show the mean number of worms that migrated out ± the SEM. All experiments are representative of three experiments.

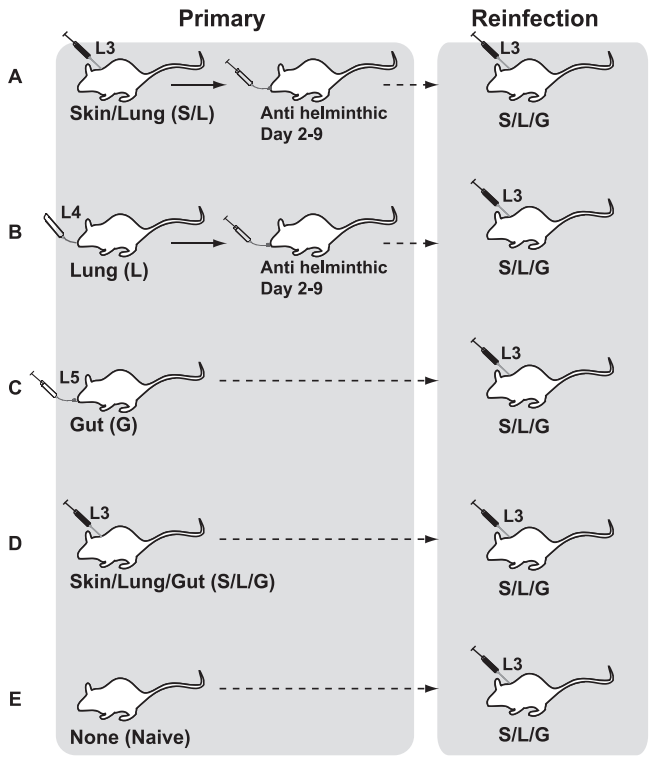

FIG. 3. Schematic representation of the truncated infection strategy used for selective infection of lung and gut tissues. (A) Skin and lung priming. Mice were infected with ~600 L3 *N. brasiliensis* worms s.c. and given an anthelminthic daily by gavage from day 2 to day 9 to prevent the establishment of L4/L5 worms in the gut and prevent the induction of a gut-localized immune response to *N. brasiliensis*. Mice were s.c. reinfected with 600 L3 *N. brasiliensis* worms at day 30 postinfection. (B) Lung priming. Mice were i.n. administered ~200 L4 *N. brasiliensis* worms and given an anthelminthic daily by gavage from day 2 to day 9 to prevent the establishment of L4/L5 worms in the gut and prevent the induction of a gut-localized immune response to *N. brasiliensis*. Mice were s.c. reinfected with 600 L3 *N. brasiliensis* worms on day 30 postinfection. (C) Gut priming. Mice were infected with ~300 L5 *N. brasiliensis* worms by gavage. Mice were s.c. challenged with 600 L3 *N. brasiliensis* worms on day 30 postinfection. (D) Skin, lung, and gut priming. Mice were infected with ~600 L3 *N. brasiliensis* worms s.c. Mice were s.c. reinfected with 600 L3 *N. brasiliensis* worms on day 30 postinfection. (E) No-priming (naive control) naïve mice were s.c. infected with ~600 L3 *N. brasiliensis* worms at the same time as the groups shown in panels A, B, C, and D were s.c. reinfected to enable comparison of worm burdens and calculation of the level of protection.

Priming of CD4 T cells in the lung is necessary for protective immunity against infective *N. brasiliensis* L3 worms. Having established that primary s.c. infection with *N. brasiliensis* generated long-lived adaptive responses which could significantly reduce the burden of viable of L3/L4 worms that could establish an infection in reinfected mice, we wished to determine in which tissues and with which immune mechanisms the protective immunity was induced and active. Therefore, we developed tissue-specific (skin, lung, or gut) priming models using distinct phases of the *N. brasiliensis* life cycle (illustrated in Fig. 3) to identify the site and source of protective immunity against reinfection. It was already apparent that skin-localized priming with dead *N. brasiliensis* was insufficient to confer protective immunity (Fig. 2C). To determine the role of the circulation, lung, and gut in the protective response, anthelminthic treatment was combined with inoculation with the respective skin, lung, and gut tissue stages of *N. brasiliensis* as follows. In group A, skin and lung (S/L) priming was achieved by s.c. infection with L3 worms and infection of the GI tract was prevented by anthelminthic treatment from day 2 to day 9 of infection. In group B, lung-only (L) priming was achieved by i.n. administration of L4 worms and infection of the GI tract was prevented by anthelminthic treatment from day 2 to day 9 of infection. In group C, gut-only (G) priming was achieved by gavage of adult L5 worms. In group D, skin, lung, and gut (S/L/G) priming was achieved by conventional s.c. infection with L3 worms. It is important to note that L4 worms introduced i.n. into non-drug-treated mice were able to complete their life cycle to the L5 stage and produce eggs (unpublished observation, data not shown), indicating that they were not affected by the extraction procedure (see Fig. S1 in the supplemental material). Also, adult L5 worms introduced into BALB/c mice by gavage were cleared with normal kinetics (expulsion day 1 to day 5 after gavage) while those introduced into class II MHC$^{-/-}$ or STAT6$^{-/-}$ mice by gavage failed to be cleared, indicating that the L5 worms were not affected by the extraction and gavage procedures (see Fig. S1B in the supplemental material).

Once the tissue-specific priming infections had cleared and cellular immune responses had dissipated (days 25 to 30 postinfection; see Fig. S3B in the supplemental material), all of the test groups and a group of naïve mice (group E) were challenged by s.c. reinfection using 600 iL3 worms and the worm burdens, cellular responses, and protective immune responses were compared (Fig. 4A). One limitation of this experimental model is that it cannot determine the specific priming and attrition of larvae after migration from the skin and prior to arrival in the lung. Evidence in Fig. 1C and D shows that the same number of worms arrived in the lungs of naïve and protected mice at 24 h after inoculation, but there was no additional accumulation of viable worms after this point in previously s.c. infected mice (Fig. 1D) compared to naïve mice (Fig. 1C). This suggests that protective mechanisms are initiated after 24 h within the lung; however, this experiment does not exclude the possibility that protective mechanisms could exist before entry into the lungs.

Mice whose primary infection was restricted to the skin and lungs (S/L and L) significantly reduced the number of recoverable L4 worms in the lungs compared to the high numbers found in naive s.c. infected mice (Naïve). In contrast, mice whose primary infection with *N. brasiliensis* was restricted to the gut using L5 worms (G) introduced by gavage failed to significantly reduce their lung worm burdens when s.c. reinfected with iL3 worms. Analysis of the lymph nodes draining the tissues selectively infected with *N. brasiliensis* (s.c., i.n., or by gavage) revealed that the presence of more than 10^4 CD4 GFP$^+$ cells per mediastinal lymph node, which occurred in mice primed in the skin and lung, the lung only, or the skin, lung, and gut (S/L, L, and S/L/G), correlated with a significant reduction in the worm burden (Fig. 4C). By comparison, significantly lower numbers of CD4 GFP$^+$ cells were found in the mediastinal lymph nodes of gut-primed (G) and naive mice (Naive).

In contrast to the apparent need for lung-specific priming by

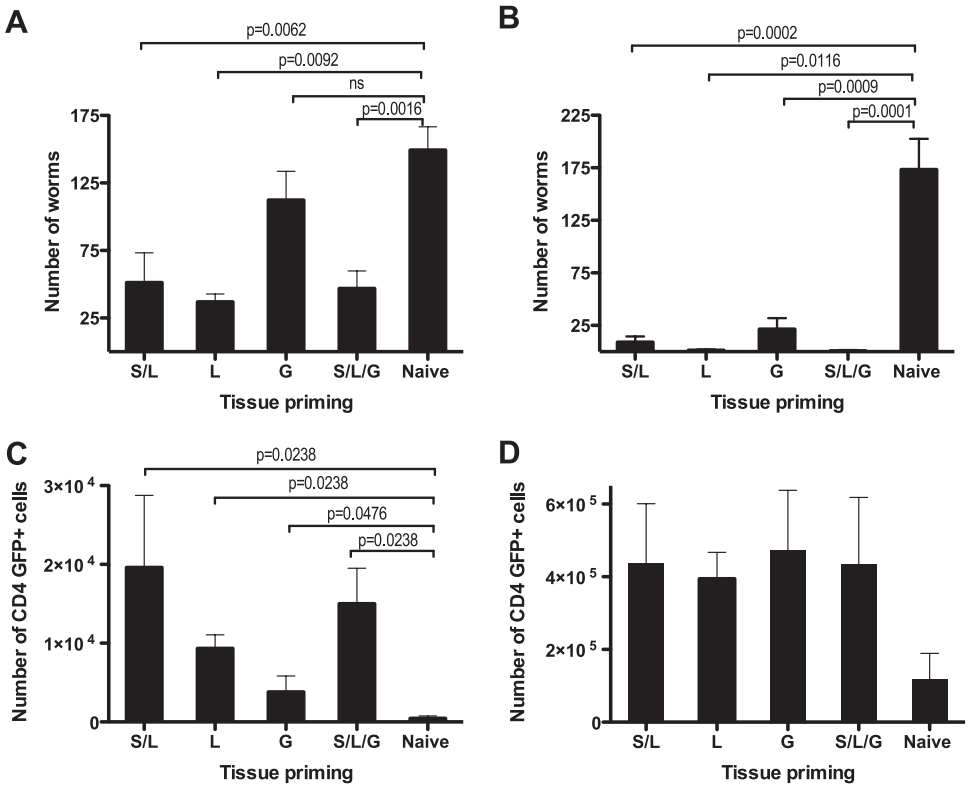

FIG. 4. Effect of truncated skin, lung, and gut primary *N. brasiliensis* infection on the development of immunity to reinfection. G4/IL-4 mice ($n = 4$) were infected via the skin and lungs, the lungs only, the gut only, or whole infection. The anthelminthic was given from day 2 to day 9 to the skin-and-lung infection and lung infection only groups. Following the 30-day rest period postinfection, mice were s.c. infected with 600 *N. brasiliensis* L3 worms and on day 2 their lungs were excised and the worms that migrated out were enumerated. The values shown represent the mean number of larvae present in the lung ± the SEM. The data shown are representative of three separate experiments. (B) Worm burdens in the gut following priming with distinct phases of the *N. brasiliensis* life cycle. G4/IL-4 mice ($n = 4$) were infected via the skin and lungs, the lungs only, the gut only, or whole infection, and an anthelminthic was given from day 2 to day 9 to the skin-and-lung and lung-only groups. Following a rest period, mice were s.c. infected with 600 *N. brasiliensis* L3 worms and on day 6, their guts were excised and the worms that migrated out were enumerated. The values shown represent the mean number of larvae present in the gut ± the SEM. The data shown are representative of three separate experiments. (C) GFP^+ CD4 T-cell responses in mediastinal lymph nodes during distinct phases of the *N. brasiliensis* life cycle. Mediastinal lymph nodes from animals ($n = 4$) infected via the skin and lungs, the lungs only, the gut only, or whole infection were harvested on day 2 and surface stained for CD3 and CD4. The values shown represent mean cell numbers ± the SEM and are representative of three independent experiments. (D) GFP^+ CD4 T-cell responses in mesenteric lymph nodes during distinct phases of the *N. brasiliensis* life cycle. Mesenteric lymph nodes from animals ($n = 4$/group) infected via the skin and lungs, the lungs only, the gut only, or whole infection were harvested on day 6 and surface stained for CD3 and CD4. The values shown represent mean cell numbers ± the SEM and are representative of three independent experiments.

N. brasiliensis to get reductions in the lung worm burden when s.c. reinfected, all of the mice that had received a skin-, lung-, or gut-only primary infection (s.c., i.n., or by gavage, respectively) significantly reduced the numbers of adult L5 worms in the gut at day 6 following reinfection with iL3 worms (Fig. 4B). This protective effect correlated with significantly higher numbers of CD4 GFP^+ T cells in the mesenteric lymph nodes (Fig. 4D) of all s.c. reinfected mice. Importantly, lung-specific priming with *N. brasiliensis* was sufficient to protect the mouse GI tract from challenge infection with adult L5 worms (see Fig. S4 in the supplemental material). The protection induced by lung infection appears to be restricted to s.c. or i.n. infection with live worms, as priming with dead L4 worms by either i.d. injection into the skin (see Fig. S5B in the supplemental material) or i.n. administration to the lung did not confer protection (see Fig. S5A in the supplemental material).

In conclusion, our finding that the s.c. reinfected mouse lung worm burden was only reduced in mice whose lungs were exposed to *N. brasiliensis* indicates that lung-only infection is sufficient to generate protective immunity against tissue-migrating parasites. The important influence of the lung as a site for the generation of protective immunological responses was emphasized in the study demonstrating that immune protection in the gut could be achieved when the primary (s.c. or i.n.) infection only occurred in the lung (see Fig. S4 in the supplemental material). The ability of priming immune responses in the gut (by gavage) to generate effective protective immunity against gut reinfection with L5 worms was consistent with previous studies.

Increased airway inflammatory responses correlate with reduced viable worm recovery in reinfected mice. We used histological analysis to follow the fate of L3 worms migrating to the airways of reinfected mice. Although the detection and reliable enumeration of nonviable worms in lung tissue proved

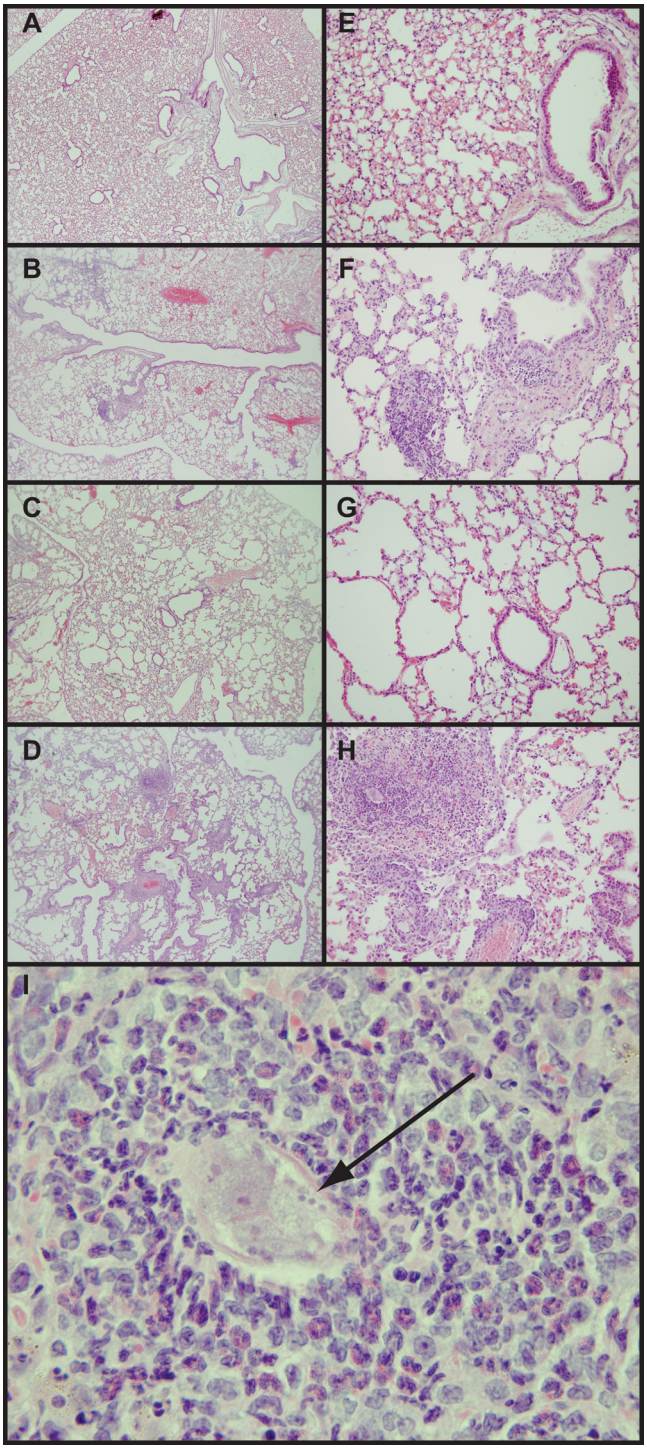

FIG. 5. Histological analysis of the lungs of *N. brasiliensis*-infected mice. BALB/c mice (n = 3/group) were infected s.c. with 600 *N. brasiliensis* worms and rested for 30 days before being reinfected s.c. with 600 iL3 *N. brasiliensis* worms. Lung tissues were harvested and fixed at the indicated time points before being separated into 4-μm sections and stained with H&E to visualize inflammatory cells. (A and E) Lung tissue from naïve mice at ×40 and ×200 magnifications, respectively. (B and F) Lung tissue from a primary s.c. *N. brasiliensis*-infected animal at day 9 postinfection at ×40 and ×200 magnifications, respectively. (C and G) Lung tissue from an *N. brasiliensis*-infected animal at day 29 after s.c. infection (prior to reinfection) at ×40 and ×200 magnifications, respectively. (D, H, and I) Lung tissue taken from mice s.c. reinfected with *N. brasiliensis* at day 6 after s.c. reinfection at ×40, ×200, and ×1,000 magnifications, respectively. The arrow in panel I indicates an *N. brasiliensis* worm.

problematic, the inflammatory response stimulated by the worms could be used as a surrogate for their presence in tissues. Lung tissue was taken from mice during the peak (day 9) of primary s.c. infection, following resolution (day 29) of the primary s.c. infection, or during the peak (day 6) of s.c. reinfection and prepared for histological analysis (Fig. 5). Examination of H&E-stained lung sections revealed marked and diffuse leukocyte infiltration of the lung tissue during primary *N. brasiliensis* infection (Fig. 5B and F). This infiltration appeared to have largely resolved by day 29 after s.c. infection (Fig. 5C and G). However, upon s.c. reinfection, extensive inflammation and leukocyte infiltration were observed as early as day 6 after s.c. reinfection, with the degree of inflammation being significantly higher than that observed on either day 9 or 29 after the primary s.c. infection (Fig. 5D and H). In addition to inflammatory markers, the alveolar structure was observed to be significantly disrupted in primary s.c. infected mice, as previously reported (19). The damage to the alveolar structure persisted even after the leukocyte infiltration had resolved (Fig. 5C and G) and was clearly evident in s.c. reinfected animals (Fig. 5D and H). Of particular note was that the airways of s.c. reinfected mice contained prominent inflammatory cell foci which surrounded identifiable worm structures. A high-power image of lung tissue from s.c. reinfected mice shows clustering of inflammatory cells around what appears to be a cross-section of an *N. brasiliensis* worm (Fig. 5I, indicated by arrow) at day 6 after s.c. reinfection, which was not observed in primary infected mice.

An assessment of mucus production within lung tissues during s.c. *N. brasiliensis* infection using PAS staining (Fig. 6A) revealed a profound increase in PAS^+ mucus-producing cells in the bronchioles of s.c. reinfected mice compared to other times during infection (Fig. 6A and E). Although significant mucus production was detected during primary *N. brasiliensis* infection (Fig. 6A and C), this resolved prior to reinfection (Fig. 6A and D). The greatly increased level of mucus production detected in the lung during reinfection correlates with the observed reduced worm recovery after reinfection with *N. brasiliensis* worms.

Taken together, these results indicate that L3 worms are able to migrate to the lungs of previously s.c. infected mice and stimulate profound airway inflammatory responses. The detection of apparently nonviable worm structures within intense inflammatory foci in the airways may be evidence of worm killing *in situ*, indicating a potential role for lung inflammatory responses in protective immunity to migrating worms.

The roles of CD4 T cells, antibodies, and cytokines in lung protective immunity against infective *N. brasiliensis* L3 worms. To further characterize the key immune mechanisms that act to reduce the worm burdens in reinfected mice, we tested mouse strains genetically deficient in cytokines, signaling pathways, or receptors in our *N. brasiliensis* model of protection (Table 1). Surprisingly, given the emphatic role established for

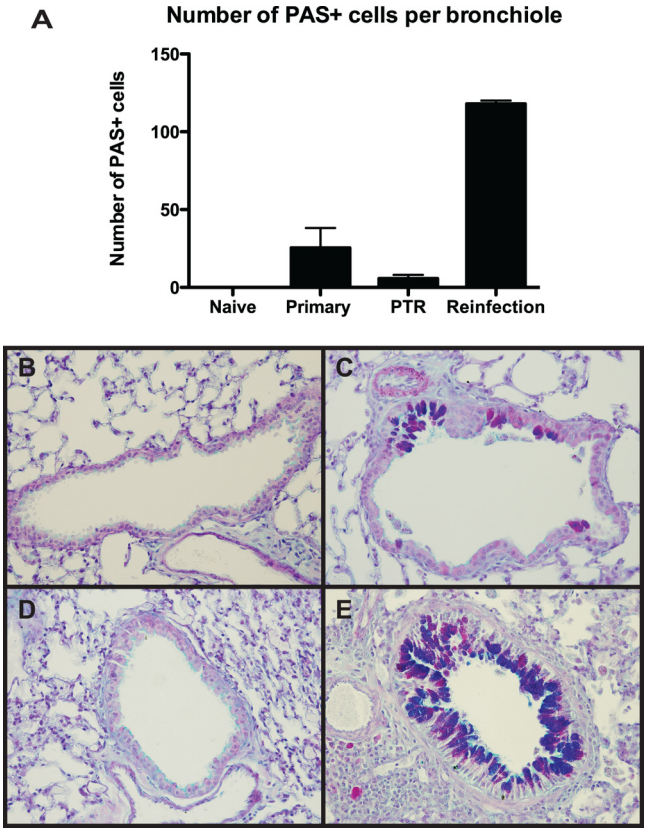

FIG. 6. Mucus production in the airways of *N. brasiliensis*-infected mice. BALB/c mice (n = 3/group) were infected s.c. with 600 worms and rested for 30 days before being reinfected s.c. with 600 iL3 *N. brasiliensis* worms. Lung tissues were harvested and fixed at the indicated time points before being separated into 4-μm sections and stained with PAS and alcian blue to visualize mucus-secreting goblet cells. (A) The number of mucus producing cells per bronchiole was determined by counting the PAS-positive cells in all bronchioles 200 to 600 μm in size per lung section. Data represent the mean number of mucus-producing cells per bronchiole ± the SEM. (B) Representative bronchiole from a naive animal, ×400 magnification. (C) Representative bronchiole from an s.c. *N. brasiliensis*-infected animal at day 9 postinfection, ×400 magnification. (D) Representative bronchiole from an *N. brasiliensis*-infected animal at day 29 after s.c. infection (prior to reinfection [PTR]), ×400 magnification. (E) Representative bronchiole from an s.c. *N. brasiliensis*-reinfected animal at day 6 after reinfection, ×400 magnification.

eosinophils in models of *N. brasiliensis* skin infection by Dent and colleagues (6, 17), we found that IL-5-deficient (−/−) mice exhibited slightly reduced protection in the lung stage from s.c. challenge infection with *N. brasiliensis*. However, MHC class II$^{-/-}$ mice completely lacking in CD4 T cells exhibited a significant loss of protection in both the lungs (Table 1) and the gut (see Fig. S1B in the supplemental material), with worm burdens in these tissues being equivalent to those found at the peak of s.c. infection of naïve mice for extended periods of time (data not shown). This finding indicates an important role for CD4 T cells in generating protective responses at these sites. The requirement for an intact STAT6 signaling pathway has previously been shown to be important for the generation and survival of Th2 memory cells in peripheral tissues such as the lung (18, 29); indeed, protection was significantly diminished in STAT6$^{-/-}$ mice. Similarly, the significantly impaired ability of IL-4$^{-/-}$ mice to reduce the numbers of viable worms in the lungs after s.c. reinfection is in contradiction to studies of gut immunity where IL-4 appears to be less important than IL-13 for the expulsion of adult parasites (28). Importantly, mu$^{-/-}$ mice, which lack B cells and cannot produce IgG and IgE antibodies specific for *N. brasiliensis*, exhibited only a minor reduction in the level of protection against reinfecting worms in the lung. CD1-restricted CD4^{+} NKT cells did not play a role in this form of protective immunity. The Toll-like receptors (TLR2 and TLR4) have previously been implicated in Th2 immunity (1, 13, 26); however, we found that deficiency in either of these receptors did not impair the ability of these mouse strains to mount a protective immune response in the lung upon s.c. reinfection with *N. brasiliensis* (data not shown). Taken together, the results in Table 1 highlight the importance in the early phase of lung immunity against migrating parasites of the CD4 T-cell-mediated and IL-4 and STAT6 signaling-dependent immune response pathways. This would most likely involve IL-4- and IL-13-mediated inflammatory processes such as increased goblet cell hyperplasia and mucus production and the development of long-lived alternatively activated macrophages that produce the appropriate reactive molecules and products toxic to helminth larvae (22, 24). In conclusion, we identify an IL-4/STAT6-dependent protective mechanism in the lung that can significantly affect the viability of migrating L3/L4 worms that is distinct from the protective immune mechanisms mediating the expulsion of adult *N. brasiliensis* worms from the gut.

DISCUSSION

Tissue-migrating helminth parasites of many species have found that a relatively easy way to use mammalian hosts for their survival is to gain access to the blood circulation through

TABLE 1. Immune requirements for protection from *N. brasiliensis* reinfection[a]

Mouse strain	Lung protection[b]
Wild type	100 ± 11
CD1$^{-/-}$	100 ± 10
IL-5$^{-/-}$	75 ± 19
Mu$^{-/-}$	77 ± 20
MHC II$^{-/-}$	47 ± 16
STAT6$^{-/-}$	39 ± 12
IL-4$^{-/-}$	25 ± 11

[a] STAT6$^{-/-}$, G4/G4 (IL-4$^{-/-}$), B6Aa0 (MHCII$^{-/-}$), IL-5$^{-/-}$, μMT (B cell$^{-/-}$), C57BL/6, and BALB/c mice (n = 3) were infected s.c. with 600 *N. brasiliensis* L3 worms, rested for 30 days, and s.c. reinfected with the same dose of *N. brasiliensis* L3. Parasite lung burdens were enumerated on day 2 after s.c. reinfection. Wild-type C57BL/6 and BALB/c mice typically reduce their lung worm burden by 90% when s.c. reinfected. Therefore, we calculated the percentage of protection for each mutant mouse strain to reflect its performance against that of a wild-type mouse. If a mutant mouse strain was able to achieve a 90% reduction of worm numbers when s.c. reinfected, we defined that mutant mouse strain as being 100% protected. If a mutant mouse strain failed to reduce its worm burden when s.c. reinfected, we defined it as 0% protected.

[b] Shown is the percent protection in the lungs compared to that seen in the lungs of wild-type animals (100% = protection maintained; 0% = no protection) ± the SEM. The variation of the wild-type strain represents the level of variation typically seen with BALB/c or C57BL/6 mice. The recovered worm burdens in naïve mice ranged from 150 to 300 worms (consistent with recoveries shown elsewhere [see Fig. S2 in the supplemental material]), and the recovered worm burdens in protected mice ranged from 25 to 60 worms.

the skin and rely on circulatory processes and the physical properties of their anatomy, movement, and size to transport themselves to the gut (often via the lung), where they mature, reproduce, and use the host excretory processes for dispersal and reinfection. Arresting worm migration in tissues is fraught with risk, as the chronic inflammatory reactions themselves can compromise the host. The gut and lung phases of helminth parasite invasion represent particular sites where the migrating larvae can be relatively easily eliminated from the host; much attention in recent years has been focused on gut "clear and sweep" mechanisms.

The data in this report clearly demonstrate that the lung has the potential to be a very important site for conferring protection against reinfection with the rodent hookworm *N. brasiliensis*. We have found not only that the lung is an important site for reducing the numbers of migrating larvae but that it is the key site for priming the protective CD4 Th2 response that occurs in the lung. Moreover, priming localized to the lung was sufficient to confer gut protective immunity to adult stages of the worm. Our results also indicate that there is a qualitative difference between priming in the lung tissue and priming in the gut tissue. Although priming in the gut tissue is sufficient to generate Th2 immune responses, protection is not as successful as the protection observed when mice are primed in the lung. The fact that only lung priming is able to achieve this level of protection indicates that the lung immune environment has a unique capacity to generate immune processes that are active at a distant site or can rapidly migrate where needed. Our conclusions are supported by other findings that the lung is a critical tissue for protection from reinfection (16). Work with schistosome parasites has also revealed that there exists an important need for pulmonary T lymphocytes in establishing protection using an attenuated cercarial vaccination (2, 5). Studies involving *Strongyloides* species have previously indicated that there is a role for parasites in modulating lung function and conferring protection from reinfection (23). Investigation into immunization against *Ascaris* migration has found i.n. administration to be most effective in protecting against the parasite infection (27).

Although we found that the skin is not a site of protection or priming for protection in our studies, as is commonly thought, this does not mean that it may not be relevant for both priming and protection in the natural environment. Although we found that restricting immunization against *N. brasiliensis* to the skin (through the injection of dead L3 worms) did not confer protection against reinfection in the lung, it could be imagined that under special circumstances of high infection rates or vaccination boosting, lung immunity could be generated (6, 10, 11, 17). One wonders why more explicit examples of lung immune response-dependent protective immunity have not been identified previously. This could in part be because it takes many years for "natural" trickle infection to build up the required levels of host lung protective immunity. The effectiveness of a "bolus" immunization strategy with the protocols used in our study may be an important factor to consider in future vaccination trials.

The immune effector mechanisms that appear to mediate protection against reinfection seem to be exquisitely dependent on lung-resident CD4 Th2 cells, STAT6 signaling, and IL-4- and/or IL-13-dependent inflammatory mechanisms. Histological analysis revealed significantly increased cellular inflammation and mucus production in the lungs of reinfected mice, which correlated with a reduced viable worm burden. Significantly, worms could be detected in the airways of reinfected mice surrounded by intense inflammatory foci, suggesting that worms can be trapped and killed within lung tissue. These novel findings raise questions about the potential mechanisms of protection; the observed increase in mucus production could be purely correlative and represent the actions of CD4 T-cell-derived IL-13 on mucus-producing goblet cells (and other cell types). Conversely, mucus production may be indicative of a novel mechanism by which large numbers of worms are trapped and cleared in a mucus bolus early during infection, before they can mature to the gut-resident stage in an acceleration of the life cycle. Further work is required to address these possibilities.

The requirement for CD4 T cells during a primary *N. brasiliensis* infection has long been established (14, 28); however, a role for CD4 T cells in protection from reinfection has only been investigated in the gut (14). A recent paper by King et al. (15) suggests that STAT6$^{-/-}$ animals have unimpaired memory Th2 responses, retaining proliferative capacity and function, as well as showing enhanced recruitment to the lung. This confirms our finding that simply mounting a Th2 response is not enough to protect from reinfection. We clearly show that STAT6$^{-/-}$ mice are not protected (Table 1), due to the fact that they cannot mount effector responses in peripheral tissues, despite seemingly intact Th2 memory responses in the lymph nodes (29). Surprisingly, we have found that B-cell-mediated antibody responses are not major forms of protection in our *N. brasiliensis* model of reinfection. Although we observed a significant reduction in the level of protection in the lung and gut, it was still minor in comparison to the contribution afforded by CD4 T cells, IL-4, and STAT6 in these strains of mice and this parasite model. It is also not clear from our results whether IL-13 is also involved in lung-mediated immune protection, as IL-4 appears sufficient. Future studies with IL-13-deficient mice are required to establish whether this key cytokine is able to contribute to lung-mediated protection. Although we find in our models that IL-5-deficient mice are almost fully protected from reinfection, this does not lessen the potential role of eosinophils as a critical mechanism of protection against parasites, with their role depending on host genetics, cytokine expression patterns, and the circumstances of infection (6, 17, 25).

In conclusion, our study raises critical issues for the design and development of formulations of vaccines against human helminths which follow the infection strategy of the rodent hookworm *N. brasiliensis*. In particular, strategies for inducing immunity in the lung need to be developed and any large-scale vaccine development needs to evaluate some appropriate markers of lung immunity for monitoring of efficacy.

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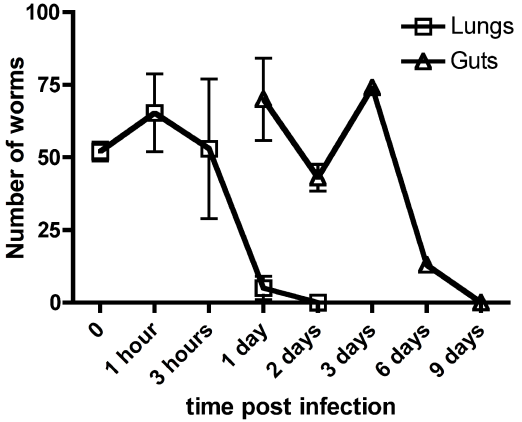
A


Lungs
Guts
Number of worms
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1 hour
3 hours
1 day
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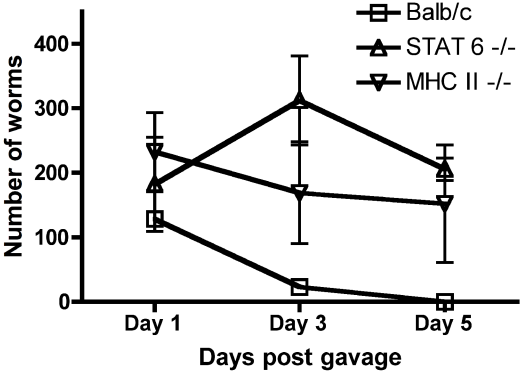
3 days
6 days
9 days
time post infection

B


Balb/c
STAT 6 -/-
MHC II -/-
Number of worms
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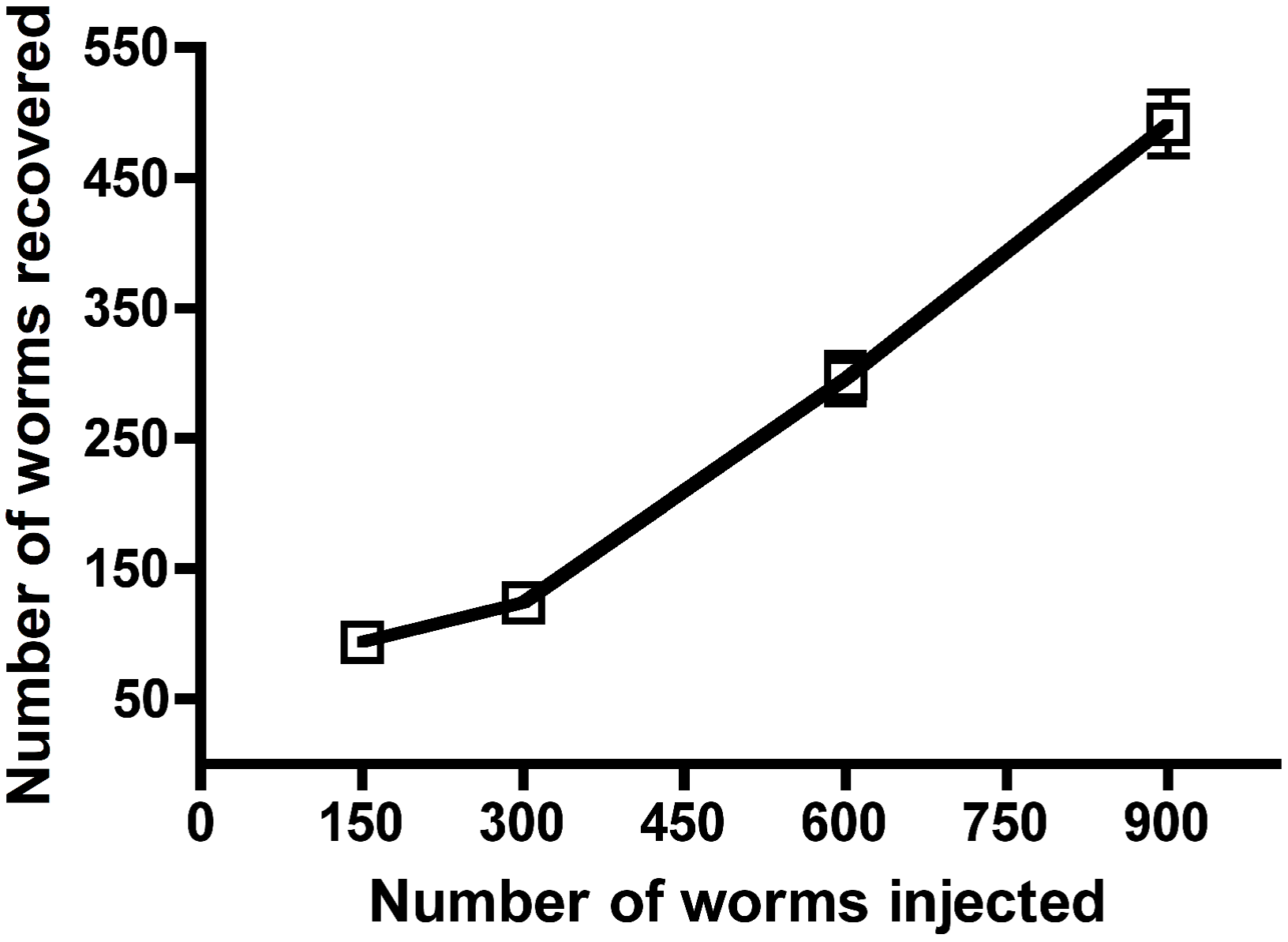
300
200
100
0
Day 1
Day 3
Day 5
Days post gavage


Number of worms recovered
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450
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250
150
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150
300
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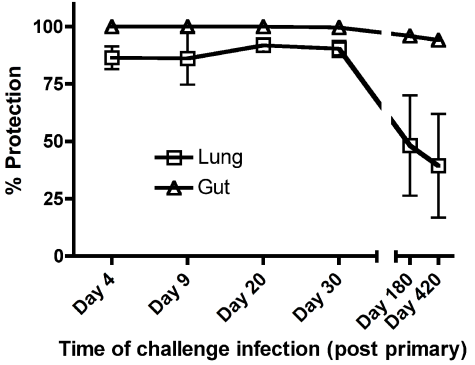
600
750
900
Number of worms injected

A


100
75
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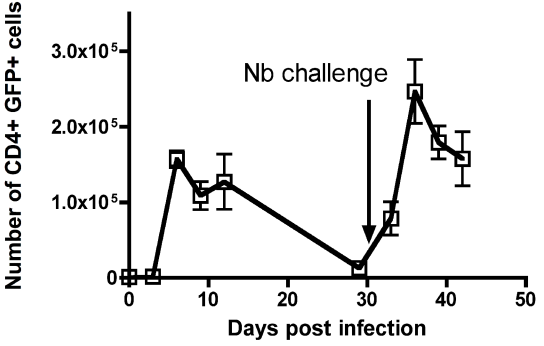
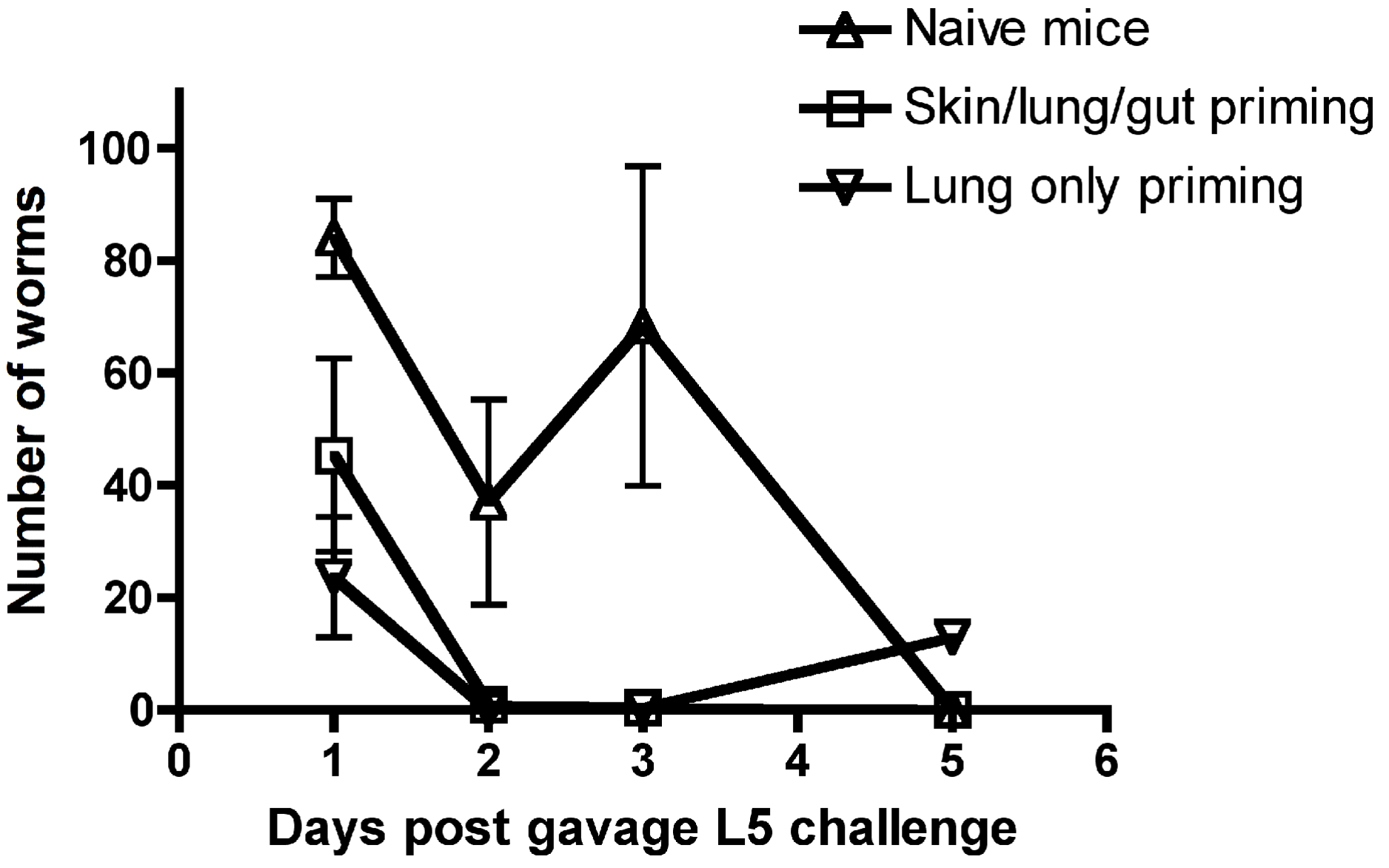
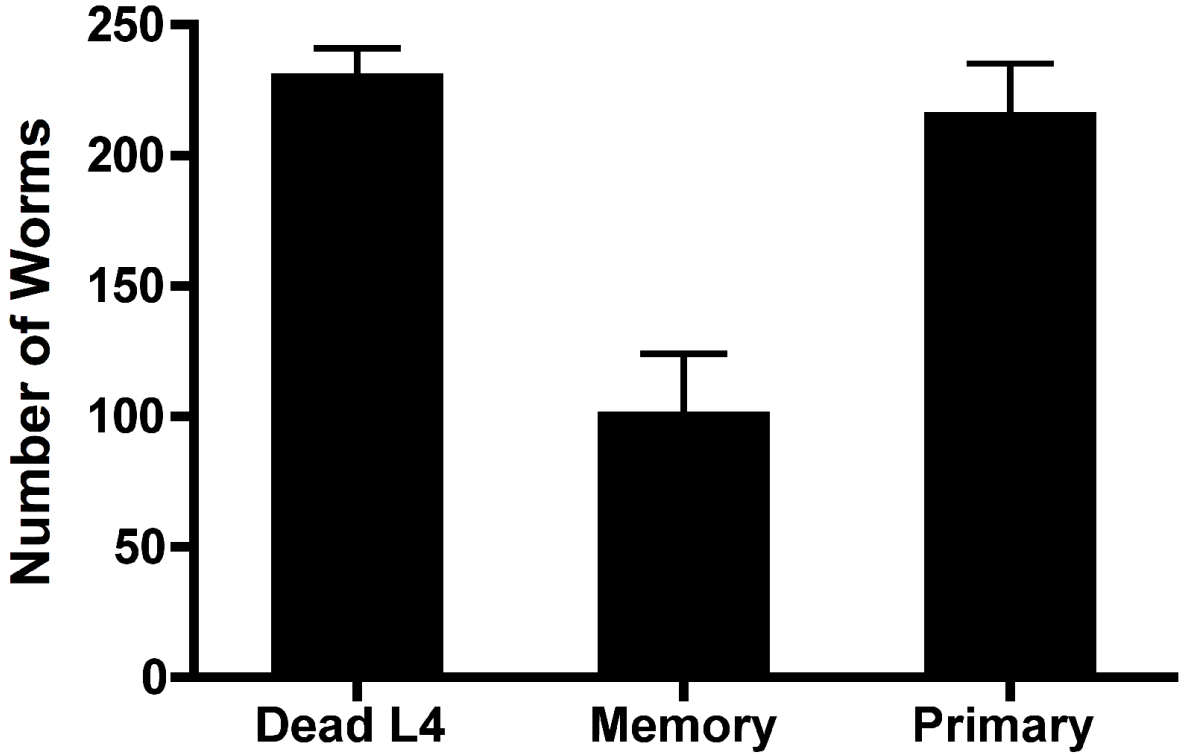
% Protection
Lung
Gut
Day 4
Day 9
Day 20
Day 30
Day 180
Day 420
Time of challenge infection (post primary)

B

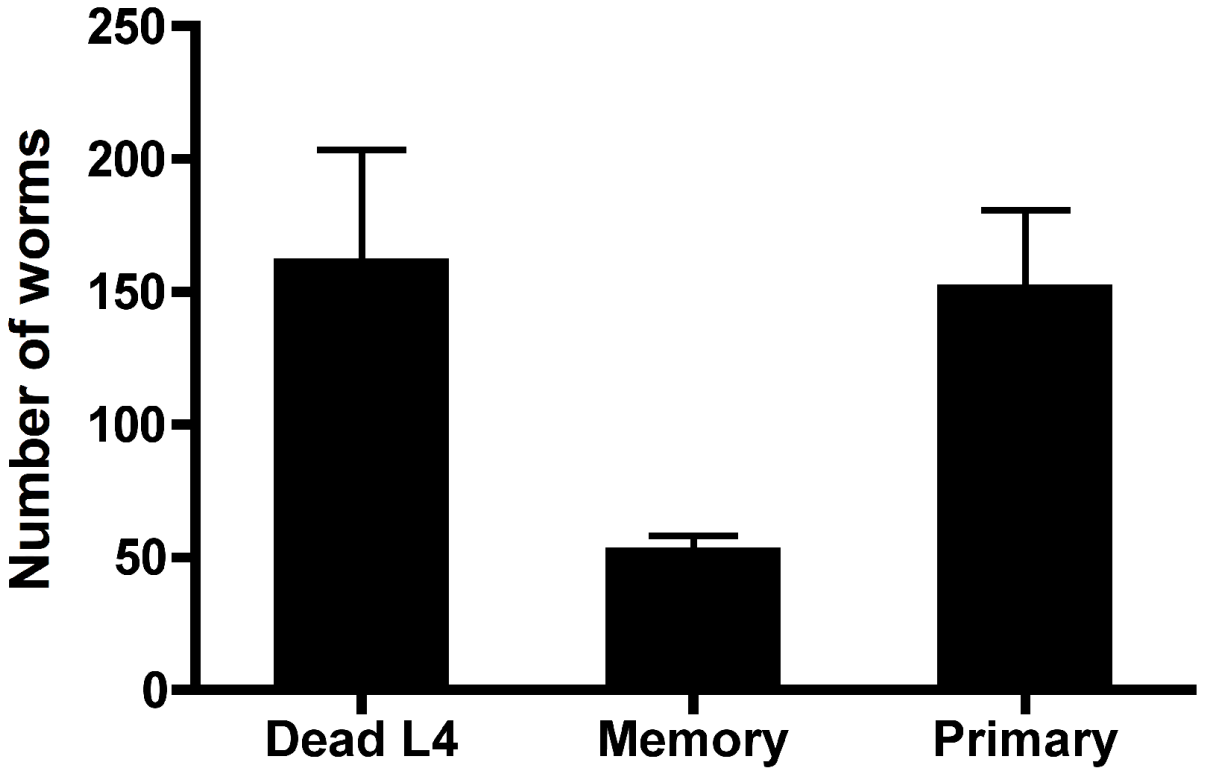

Number of CD4+ GFP+ cells
3.0x10⁵
2.0x10⁵
1.0x10⁵
0
Nb challenge
0
10
20
30
40
50
Days post infection


Naive mice
Skin/lung/gut priming
Lung only priming
Number of worms
100
80
60
40
20
0
0
1
2
3
4
5
6
Days post gavage L5 challenge

A


250
200
150
100
50
0
Number of Worms
Dead L4
Memory
Primary

B


250
200
150
100
50
0
Number of worms
Dead L4
Memory
Primary

Supplementary Figure Legends

Figure S1. (A) Intranasally inoculated L4 can complete the *N. brasiliensis* lifecycle. Worm burden in the lung (squares) & guts (triangles) following intranasal inoculation of L4. G4/IL-4 mice (n=3) were infected with 150 freshly harvested lung larvae and on indicated time points, lungs and guts were excised, worms migrated out and enumerated. Values represent mean number of larvae present ± S.E.M. **(B) Gavaged L5 can complete the *N. brasiliensis* life cycle.** Worm burden in the gut following gavage of L5 worms. Balb/c (squares), STAT 6 -/- (triangles) and MHC II -/- (inverted triangles) mice (n=2) were gavaged with 300 freshly harvested L5 worms and at indicated time points mice were sacrificed, guts excised, worms migrated out and enumerated. Values represent the mean number of larvae present in the gut ± S.E.M. and is representative of two independent experiments.

Figure S2. Dose versus recovery of Lung larvae. Dose Response of the number of larvae migrated from the lung. Balb/c mice (n=3 per time point) were infected subcutaneously in to the scruff of the neck with 900, 600, 300 or 150 iL3. Viable larvae were migrated out from excised lungs & enumerated. Values represent the mean number of larvae migrated out per lung ± S.E.M.

Figure S3. Duration of the lung protective immune response following primary infection with *N. brasiliensis*. (A) Naïve Balb/c mice (n=3 per time point) were infected subcutaneously in the scruff of the neck with 600 iL3 and then reinfected at the indicated time points with a challenge infection of 600 iL3. Following challenge infection, lungs &

guts were excised on day two & six post infection respectively and the number of viable parasites in the tissues were recovered by migration and enumerated. For each time point aged matched naïve mice were given the identical challenge infection with 600 L3 and the numbers of viable worms recovered from tissues. The degree of protection for each time point was determined from the ratio of worm burden in reinfected versus naïve mice with reinfected mice typically reducing the numbers of worms by 10 fold. If reinfected mice were able to achieve 10 fold reduction in worm numbers compared to aged match naïve mice at the specified time points following primary infection we defined that time point as maintaining 100 % protection. If reinfected mice failed to reduce worm burdens when compared to the burden in similarly challenge infected, aged matched, naïve mice we defined it as 0% protection. (B) Duration of the GFP+ CD4+ cellular response following primary and secondary infection with *N. brasiliensis*. Mice (n=4) were infected with 600 iL3, rested for 30 days and reinfected with a challenge infection of 600 iL3. At the indicated time points mice were sacrificed, mediastinal lymph nodes were harvested and the number of GFP+ CD4+ cells were assessed using flow cytometry. Values represent mean number of GFP+ CD4+ cells ± S.E.M. and are representative of at least three independent experiments

Figure S4 Mice previously infected in the lung with *N. brasiliensis* are protected against reinfection with gavaged L5. G4/IL-4 mice (n=3/timepoint) were primed with 600 iL3 or alternatively, primed with iL3 that were restricted to the lung by treatment with anti helmintic. After 30 days rest mice received a challenge infection with 300 gavaged L5 worms with a third group receiving a primary infection with gavaged L5. At

the indicated time points mice were sacrificed and gut tissues harvested. Worms were migrated out and enumerated. Values represent mean number of worms present in the gut ± S.E.M.

Figure S5. Inoculation with dead L4 does not confer protection to challenge infection with *N. brasiliensis*. (A) Effect of intranasal inoculation with dead L4 on worm burden in reinfected mice. G4/IL-4 mice (n=3) were primed with 200 dead L4 by intranasal inoculation or 600 iL3. Following a 30-day rest period, mice were reinfected with 600 iL3. On day two post infection, lungs were excised worms migrated out and enumerated. Values represent mean number of worms present in the lung ± S.E.M.
(B) Worm burden in the lung following intradermal priming in the ear with dead L4. G4/IL-4 mice (n=3) were primed intradermally in the ear with 200 dead L4 or 600 iL3. Following a 30-day rest period, mice were infected with 600 iL3. On day two post infection, lungs were excised worms migrated out and enumerated. Values represent mean number of worms present in the lung ± S.E.M.