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Pre-conception maternal helminth infection transfers via nursing long-lasting cellular immunity against helminths to offspring

Matthew G. Darby, Alisha Chetty, Dunja Mrjden, Marion Rolot, Katherine Smith, Claire Mackowiak, Delphine Sedda, Donald Nyangahu, Heather Jaspan, Kai-Michael Toellner, Ari Waisman, Valerie Quesniaux, Bernhard Ryffel, Adam F. Cunningham, Benjamin G. Dewals, Frank Brombacher, William G. C. Horsnell

Maternal immune transfer is the most significant source of protection from early-life infection, but whether maternal transfer of immunity by nursing permanently alters offspring immunity is poorly understood. Here, we identify maternal immune imprinting of offspring nursed by mothers who had a pre-conception helminth infection. Nursing of pups by helminth-exposed mothers transferred protective cellular immunity to these offspring against helminth infection. Enhanced control of infection was not dependent on maternal antibody. Protection associated with systemic development of protective type 2 immunity in T helper 2 (T\(\text{\scriptsize H}_2\)) impaired IL-4R\(^{\text{\scriptsize \alpha^{-}}\}) offspring. This maternally acquired immunity was maintained into maturity and required transfer (via nursing) to the offspring of maternally derived T\(\text{\scriptsize H}_2\)-competent CD4 T cells. Our data therefore reveal that maternal exposure to a globally prevalent source of infection before pregnancy provides long-term nursing-acquired immune benefits to offspring mediated by maternally derived pathogen-experienced lymphocytes.

INTRODUCTION
Maternal transfer of immunity both in utero and via nursing provides critical sources of early-life immune education and protection from disease. Maternally acquired protection from infection is typically associated with a passive transfer to offspring of maternal innate antibodies and antibody, which provide a transient, but critical, early-life ability to counter pathogens (1, 2).

Nursing alone provides important protection to offspring against both infectious and noninfectious diseases (3, 4). While this protection is associated primarily with transfer of maternally derived antibody (5), other immunogenic components of breast milk such as cytokines and antigen can also influence offspring immunity (6). Epidemiological and experimental evidence indicates that maternal immune transfer via nursing may also provide long-lasting pathogen-specific protection from infection, despite the short half-life of substances transferred via breast milk (7, 8). However, the mechanisms underlying any such long-term protection are not well defined, and the contribution from incorporation and maintenance of maternal components by offspring has hitherto been relatively under-addressed (9).

Helminth infections are an important cause of infection and disease and leave profound immunological footprints on a host (9, 10). These infections are extremely common and have important effects on multiple components of host immunity. Evidence supports the importance of parasite-host coevolution for optimal function and regulation of host immunity (11, 12). Helminth infections before and during pregnancy are known to have significant consequences for offspring. For example, maternal infection with the nematode *Heligmosomoides polygyrus* (Hp) provides high levels of nursing-acquired antibody that mediates protection to offspring against this infection (13). Maternal helminth infections also have bystander effects, either driving susceptibility to or protection from allergy depending on the progression of, in this case, a maternal *Schistosoma mansoni* infection (14).

Clinical studies also confirm the significant influence of maternal helminth exposure on a child’s immune system. For example, children whose mothers were infected with helminths during pregnancy display populations of lymphocytes responsive to helminth antigens (15); mothers infected with helminths during pregnancy can also confer protection to their children against eczema (16). The mechanisms underlying these maternally associated effects in offspring are, however, not well defined. As helminth exposure is a huge global coordinator of our immunological footprint, understanding this influence in the context of the maternal-offspring immunological relationship is important (9). This transgenerational immunological relationship is pivotal for our understanding of how early-life events influence lifelong ability to control disease.

In this study, we address how exposure to and resolution of a helminth infection before pregnancy influences offspring cellular immunity to this infection. To address this question, we developed mouse models of maternal helminth infection effects on offspring...
control of infection. This approach allowed us to test how pre-conception maternal infection with the nematode *Nippostrongylus brasiliensis* (*Nb*) influenced offspring ability to control an *Nb* infection both early in life and when mature. Our model allowed us to identify (i) the critical importance of nursing in transfer of protective immunity, (ii) that offspring-acquired protection was cellular and not mediated by antibody, (iii) that protection could correct germline susceptibility to infection, (iv) that protection persisted into adulthood, and (v) that this protection was maintained in an allogeneic setting and (vi) required the dissemination via nursing of maternally derived T helper 2 (Th2)–competent CD4 T cells throughout the body. Our findings therefore demonstrate that maternal transfer and offspring incorporation of nursing-derived pathogen-experienced lymphocytes can provide long-lasting immunity to infection.

**RESULTS**

Pre-conception *Nb* infection transfers protective immunity against *Nb* infection to offspring via nursing

To identify how exposure to and resolution of a helminth infection before pregnancy influenced offspring immunity, we modeled this scenario using the murine helminth parasite *Nb*. Here, female BALB/c mice were infected with *Nb* and then treated with an anti-helminth drug 7 days later. Two weeks after treatment, these female mice were mated with naïve males, and offspring immune responses were analyzed (Fig. 1A). Total cell numbers and CD4^+^ T cell numbers in the spleens of a 14-day-old offspring born to *Nb*-exposed mothers (*NbM*) and those born to naïve mothers (NvM) were similar (Fig. 1, B and C), but the numbers of activated effector CD4^+^ T cells in *NbM* offspring were raised when compared to NvM offspring (Fig. 1C).

[Image of figure 1 with data tables and graphs showing worm burdens, IgG1 levels, and IL-13 secretion under different conditions.]
Increased T cell activation suggested that NbM offspring may acquire an enhanced ability to control Nb infection. We therefore tested whether NbM transferred an enhanced interleukin-4 receptor α (IL-4Rα)–driven type 2 immunity against Nb to their offspring. Reduced intestinal worm burdens were found at day 5 post-infection (PI) in 19-day-old NbM pups in comparison to NvM pups (Fig. 1D). In addition to raised anti-Nb immunoglobulin G1 (IgG1) in NbM pups (Fig. 1E), we also found enhanced levels of the Th2 cytokine IL-13 (essential for resolving Nb infection) in the lung, mediastinal lymph nodes (MST), and spleen, demonstrating that maternal transfer of protection from infection (Fig. 1H).

We next investigated whether protection in NbM offspring was conferred in utero or via nursing. Here, we fostered 3-day-old pups born to NvM or NbM onto either NbM or NvM foster dams. Subsequent infection of fostered pups resulted in pups nursed by NbM mothers having reduced worm burdens 5 days PI and displaying early clearance of Nb by day 7 PI when compared to pups nursed on NvM (Fig. 1F). This protection was dependent on maternal Th2 competency: Wild-type (WT) offspring nursed by IL-4Rα−/− NbM did not display reduced intestinal worm burdens when compared to WT offspring nursed by WT NbM (Fig. 1G). Analysis of nursing–dependent transfer of protection in pups born to WT NvM but nursed on WT NbM also revealed both elevated Nb-specific IgG1 and systemically raised IL-13 levels associated with enhanced maternal transfer of protection from infection (Fig. 1H).

**Nursing-derived protection is dependent on maternal B cells but not antibody**

Nursing-derived protective immunity in offspring is often attributed to passive antibody transfer (18). Our findings also demonstrated associations between raised Nb-specific antibody in offspring and enhanced protection from Nb. Moreover, milk from NbM had higher Nb-specific IgG1 titers than milk from NvM (Fig. 2A). However, existing literature has shown that immunity to Nb is largely antibody independent (10, 19). Nevertheless, studies using other helminth infection models show that transfer of serum from infected mice to pups confers high levels of protection to offspring (13). In support of antibody being redundant in providing maternally derived protection in the case of Nb infection, we found that transfer of NbM serum to NvM pups did not lead to a significant reduction in worm burdens when compared to recipients of NvM serum (Fig. 2B). In addition, WT NvM pups were nursed on IgMi mice that have a functional B cell receptor but do not secrete antibody (20). Here, NvM pups nursed by IgMi NbM and WT NbM mothers showed equivalent reductions in worm burdens that were independent of Nb-specific IgG1 antibody titers in either the pups or mother (Fig. 2C). Together, these data show that transfer of maternally derived antibody is not essential to the transfer of protection against Nb to offspring.

While antibody is not directly important in immunity to Nb, B effector cell cytokine responses and antigen presentation have been shown to contribute to CD4 T cell–driven recall immunity to Nb in an IL-4Rα–dependent manner (10). We nursed WT NvM pups on...
WT and μMT NvM and NbM dams and found that no protection was transferred to pups nursed by μMT NbM dams (Fig. 2D). Associated with this reduced protection were significantly reduced CD4⁺IL-13⁺ populations in offspring nursed by μMT NbM dams when compared to protected pups nursed by WT NbM (Fig. 2D). This effect was also dependent on maternal B cells being type 2 competent. Pups nursed on NbM dams deficient in IL-4Rα specifically on B cells (MBIIIL-4Rα<sup>-/lox</sup>) had significantly higher worm burdens than pups nursed by WT NbM mothers (Fig. 2E). These data show that a maternal B cell response reflective of B effector cell–dependent activation of CD4 T cells contributes to NbM dam–derived protection in nursed pups.

**Transfer of protection from NbM is long term and associated with a systemic distribution of maternal T<sub>H2</sub> CD4 T cells in the offspring**

The systemic profile of raised T<sub>H2</sub> CD4 T cell–associated immunity in NbM nursed pups when compared to NvM nursed pups is a striking effect on pup immunity. As milk contains significant populations of immune cells, we hypothesized that these cells may be important contributors to this enhanced anti-Nb immunity in NbM-nursed offspring. This could be affected by transferred Nb-experienced maternal CD4 T cells systemically influencing offspring protection derived from NbM dams. Moreover, such a systemic effect on immune readouts of protection associated with CD4 T cells may also suggest that the effect could result in transfer of immune memory that could persist into adulthood in NbM foster pups. Direct input from passively transferred maternal antibody would be minimal in this adult setting, as transferred antibody eventually degrades after transfer (21). To identify such an effect, naïve Thy1.2 offspring were nursed on either NvM or NbM syngeneic Thy1.1 dams and infected with Nb at 6 to 8 weeks of age (Fig. 3A).

Intestinal worm burdens at day 5 PI were significantly reduced in offspring nursed by NbM dams compared to those nursed by NvM dams (Fig. 3B). This long-term maintenance of maternally acquired immunity is strongly associated with elevated numbers and proportions of both endogenous and maternally derived T<sub>H2</sub>-competent CD4 T cells in both secondary and peripheral immune sites associated with adaptive immune protection against Nb infection (10, 17) in NbM offspring (Fig. 3, C to F, and fig. S1).

Significantly higher numbers of maternally derived Thy1.1 CD4 T cells were detected in both the lungs (Fig. 3C) and MST<sup>+</sup> (Fig. S1) of NbM dam–nursed offspring than in pups nursed by NvM dams. Significantly
more maternally derived cells were activated (CD4–CD44–CD62Llo) in NbM than NvM offspring (Fig. 3D). The NbM-derived populations of Thy1.1 CD4 T cells had higher levels of T1/2 potential. Greater numbers of NbM dam-derived lung cells expressed the IL-33 receptor T1/ST2 (IL-33 signaling through this receptor can initiate type 2 immunity) when compared to offspring nursed by NvM dams (Fig. 3E). Moreover, higher numbers of NbM dam-derived CD4 T cells were also IL-13+ (Fig. 3F). This raised type 2 immune potential was also apparent in offspring’s endogenous CD4 T cells in the lung (Fig. 4, C to F) but not the MST (fig. S1). Therefore, a maternal pre-conception Nb infection enabled transfer via nursing to, and systemic integration by, offspring of populations of Nb protective CD4+ T cells. In addition, the presence of these cells is also associated with raised endogenous type 2 protective immunity in the lungs of NbM offspring against Nb. However, pups nursed on mothers exposed to a single intestinally restricted helminth Hp for 17 days before pregnancy did not demonstrate raised populations of maternally derived CD4+ T cells in the mesenteric lymph nodes or lung (fig. S2). This suggests that differences in the host-parasite relationship may influence the ability of the mother to transfer a protective CD4 T cell response.

Therefore, NbM offspring acquired long-term protective maternal immunity that associated with a systemic dissemination of maternally derived T1/2-competent CD4 T cells, which significantly enhanced both the overall and endogenous T1/2 CD4 T cell compartment of the offspring.

Long-term protection is maintained in an allogeneic setting

To test whether our findings related to transfer of maternal CD4 T cells were relevant in an outbred setting representative of typical maternal-offspring relationships, we nursed naïve C57BL/6 pups on either NvM or NbM BALB/c dams (Fig. 4A) and found that 6- to 8-week-old NbM offspring had significantly reduced Nb burdens in the intestine when compared to NbM offspring (Fig. 4B). This is associated with increased numbers of maternally derived CD4 T cells (CD3–CD4+H2Dd+) in the lung (fig. S3), albeit in reduced numbers when compared to those seen in a major histocompatibility complex (MHC)–matched setting (Fig. 3). In addition, as seen in an MHC-matched setting (Fig. 3), offspring protection in an outbred, MHC-mismatched setting is associated with a raised type 2 CD4 T cell response. Here, NbM offspring displayed significantly increased levels of CD4 T cell activation, expression of ST2, and secretion of IL-13 (Fig. 4C). This effect was also found when NvM and NbM outbred BALB/c × C57BL/6 dams were bred and BALB/c pups were subsequently fostered by them (fig. S4).

Nursing-derived protection corrects susceptibility to Nb in IL-4Rα−/− pups

We tested whether transfer of maternally derived cells via nursing to pups could support a type 2 immune environment in the lung capable of controlling the parasite (22–24) in susceptible IL-4Rα−/− pups to Nb. Transfer of WT immune cells into IL-4Rα−/− mice has been shown to reverse significant immunological pathology (i.e., graft-versus-host disease) (25). Moreover, transfer of maternal immune cells into offspring has been shown by others to correct profound immune phenotypes, e.g., in transforming growth factor–β (TGFβ−/−) offspring (26). We found that IL-4Rα−/− pups nursed on WT NbM showed significantly reduced worm burdens at day 5 PI when compared to IL-4Rα−/− pups nursed by WT NvM mothers (Fig. 5A). This protection in NbM IL-4Rα−/− pups correlated with systemically raised CD4 T cell–associated IL-13 responses when compared to NvM IL-4Rα−/− pups; specifically raised IL-13 in the lung and in CD4 T cells in the lung-draining lymph node along with increased secretion of IL-13 by anti-CD3 restimulated splenocytes (Fig. 5B).

Such a transfer of protection to IL-4Rα−/− offspring would require transfer of a range of lymphoid and myeloid cells to achieve this effect. Therefore, protection related to transfer of immune cells may not be restricted to transfer of CD4 T cells. Analysis of immune cell populations in breast milk revealed increased cell numbers of both lymphoid and myeloid cells in breast milk from NbM when compared to NvM (Fig. 5C). This suggests that nursing by NbM could

![Fig. 4. Offspring acquire, via nursing, persistent protection from Nb related to raised T1/2 CD4 T cell responses following Nb infection in an allogeneic setting.](Image)

(A) Three-day-old NvM C57BL/6 pups were fostered on BALB/c NvM or WT NbM dams before being infected at 8 weeks old with 500 × Nb L3 and euthanized at day 5 PI.(B) Intestinal worm burdens at day 5 PI in 8-week-old mice born to either NvM or NbM were established. (C) Numbers of lung CD3+CD4–, ST2–, IL-13–, and CD44–CD62Llo expressing T cell populations were quantified. All data are representative of a minimum of two experimental repeats. *P < 0.05, **P < 0.01, and ***P < 0.001.
Protection is instead associated with a systemic alteration of offspring nursing dependent but not dependent on passive antibody acquisition. Together, our data show that transfer of immunity to offspring was M mothers Nb dependent on maternally derived type 2 T cells, and these Offsprings’ nursing-derived immunity against is Nb T cells are enriched in Nb M dam milk.

DISCUSSION
This study reveals that a pre-conception maternal Nb infection transfers a strong and long-lasting protective immunity against Nb infection to her offspring and that this protection was acquired solely through offspring nursing. This maternal imprinting via nursing was profound as demonstrated by its ability to correct genetic immune susceptibility to infection. Moreover, this protection from infection persisted into adulthood and was maintained even in an allogeneic setting.

Protection was not dependent on passive antibody transfer. This is in agreement with our own and others’ findings that antibody has minimal input in immunity to Nb (10, 19). This is not the case for other helminths such as Hp, where antibody contributes significantly to host immunity against the parasite (27, 28). It also highlights that maternally derived immunological influence in offspring from helminth infection will differ depending on the species; unlike Nb, conferring protective immunity to IL-4Ra−/− offspring through the transfer of a range of not only CD4 T cells but also populations of myeloid effector cells, which, together, could coordinate an adaptive-like immunity against Nb in the IL-4Ra−/− lung.

Offsprings’ nursing-derived immunity against Nb is dependent on maternally derived type 2 T cells, and these cells are enriched in the milk of NbM mothers

Together, our data show that transfer of immunity to offspring was nursing dependent but not dependent on passive antibody acquisition. Protection is instead associated with a systemic alteration of offspring immunity related to maternal T1/2 CD4 T cells penetrating organs throughout the body. The subsequent enhanced type 2 immune footprint left on the offspring is also associated with transfer of protection in an allogeneic setting. In support of this transfer of protection coming via CD4 T cells in the milk, we identified significantly raised numbers of maternal CD3+CD4+ T cells in NbM dam milk when compared to NvM (Fig. 6A). Moreover, these NbM CD3+CD4+ T cells showed increased numbers of type 2 competent CD3+CD4+ST2+ T cells (Fig. 6A). To establish whether transfer of protection was dependent on maternal CD4 T cells being type 2 competent, we nursed naive WT offspring by NbM dams with disrupted IL-4Ra−/lox mice. Offspring nursed on NbM ilckCreIL-4Rα−lox dams failed to show enhanced control of Nb, related to an impaired systemic ability to launch a raised IL-13 response after restimulation (Fig. 6B). These data show that type 2 competent maternal CD4 T cells are required for transfer of immunity to offspring and that these protective CD4 T cells are enriched in NbM dam milk.

Fig. 5. IL-4Rα−/− offspring nursed on NbM dams have enhanced lymphocyte-mediated immunity against Nb infection. (A) Three-day-old IL-4Rα−/− pups were fostered on either WT NvM or NbM dams. Offspring were infected when 14 days old with 250× Nb L3 and euthanized at day 5 PI, and intestinal worm burdens were quantified. (B) Nb-specific serum IgG1, anti-CD3 restimulated splenocyte IL-13, lung homogenate levels of IL-13, and CD3+CD4+IL-13+ T cells/MST were quantified in IL-4Rα−/− pups. (C) Numbers of immune cells in breast milk pellets from the stomachs of 10-day-old pups nursed by WT NvM or NbM dams. All data are representative of a minimum of two experimental repeats. *P < 0.05, **P < 0.01, and ***P < 0.001.

Fig. 6. Maternally derived type 2 CD4+ T cells are enriched in NbM dam milk and are required for transfer of protection. (A) Numbers of Thy1.1+CD4+ and Thy1.2+CD4+ST2+ T cells in milk pellet from the stomach of Thy1.2 pups at day 10 after birth. (B) Three-day-old NvM pups were fostered on WT NvM/NvM or ilckCreIL-4Rα−/lox dams. Pups were infected when 14 days old with 250× Nb L3 and euthanized at day 5 PI. Offspring intestinal worm burden was analyzed, and IL-13 secretion by anti-CD3–restimulated splenocytes was quantified at day 5 PI. All data are representative of a minimum of two experimental repeats. *P < 0.05 and **P < 0.01.
nursing-derived protection against *Hp* is dependent on antibody transfer (13). While our findings showed that antibody was not essential, we still found a requirement for maternal B cells in transfer of protection that related to offspring acquiring a type 2 CD4 T cell response. These findings show a maternally driven B effector–type response, which may be mediated via B cell–secreted cytokines and/or antigen presentation (10), driving the subsequent transfer of CD4 T cell protection.

Transfer of protection to NbM offspring was dependent on a systemic increase in offspring of CD4 T cell populations with raised levels of canonical anti-helminth T12 competency and thereby an enhanced immunological ability to resolve an Nb infection (10). The biological potential of this effect to transfer protection was highlighted by our demonstration that nursing by WT NbM transferred protection against Nb to IL−4Ra−/− offspring, which has a genetically impaired ability to launch type 2 immune responses. This protection was coincident with IL−4Ra−/− offspring having a heightened systemic T12 competency. Typically, IL−4Ra−/− mice display an abrogated ability to secrete T12 cytokines such as IL−13 (29); however, here, we found that IL−4Ra−/− offspring nursed by NbM are able to secrete IL−13 at levels up to fourfold higher than IL−4Ra−/− offspring nursed by NvM. Moreover, we found that populations of maternally derived myeloid cells known to affect pulmonary anti-Nb immunity were also present in high numbers in the breast milk. Therefore, the required complement of type 2 immune cells for driving pulmonary immunity to Nb could be acquired via nursing.

Others have also shown that maternal cell transfer occurs and can dramatically influence early-life immunity in different contexts, e.g., by reversing the immune pathology resulting from lack of TGFβ (26). However, studies addressing immune cell transfer via nursing have not demonstrated whether this effect confers long-term influence on offspring ability to control infection. Our data show that pathogen-experienced maternal T cells can systemically train offspring immunity. We show that the influence and penetration of these cells in offspring are not restricted to the digestive tract (the site of cell uptake) (30); these cells can also be found in (for example) the lung and spleen. As this T12 CD4 T cell–associated enhanced control of infection was maintained in an allogeneic setting, our observations are applicable in an immune setting reflective of normal MHC diversity in humans. In this allogeneic setting, antigen presentation would be impaired between maternally derived cells and foster pup cells. Moreover, transferred cells are likely to be exposed to high levels of cytotoxic killing by foster pup cells, although, as outlined below, the tolerogenic environment promoted during gestation and nursing may abrogate the cytotoxicity. Together, these data suggest that transfer of protection may be affected by secreted factors (such as cytokines) derived from maternally transferred cells as well as interactions dependent on direct contact between maternal and offspring cells.

Understanding the maternal–offspring interface in the context of type 2 immunity is also important. In early life, the immune system shows an inherent type 2 immune bias (31) and an ability to suppress T111 immunity (32). This elevated prenatal type 2 immunity appears to contribute to fetal allograft survival by impeding potential cytotoxic events against it (33). This tolerogenic effect may also provide a permissive window that promotes an extended survival of maternal cells that is sufficient to allow immunological imprinting in the offspring to occur. In the neonate, the maintained T112 bias can also contribute to the coordination of early-life pulmonary development (34), possibly at a cost of enhanced risk of neonatal allergic disease developing (35). Our findings add an important further dimension to this understanding of early-life T112 bias: It may provide protection against helminth infection in early life.

Therefore, in this study, we demonstrate that maternal helminth infection before pregnancy can provide offspring with the ability throughout life to launch a heightened protective cellular immune response against the parasite. This protection is not dependent on antibody but is instead dependent on transfer of maternal type 2 competent cells. We show that maternal T112-competent CD4 T cells were enriched in the milk of NbM dams, dispersed systemically throughout the offspring, and provided an environment conducive to initiating an enhanced and long-term ability in the offspring to launch a protective immune response against subsequent helminth infection. Therefore, transfer to offspring of maternally derived pathogen-conditioned lymphocytes via nursing represents a novel transgenerational mechanism by which offspring may acquire early-life immunity that persists into adulthood, but how this maternal cellular transfer of immunity manifests itself in clinically relevant human and veterinary settings is not yet defined and represents a potentially significant new paradigm in our understanding of maternal transfer of immunity to offspring.

**MATERIALS AND METHODS**

**Animal work**

**Mice used**

The following BALB/c background mouse strains were used in the study: BALB/c (CD90.2), IL−4Ra−/− (described as IL4ra tm1Fbb / IL4ra tm1Fbb), μMT (B cell deficient), Thy1.1 (CD90.1), MBI−/−IL−4Ra−/− (B cell–specific disruption of IL−4Ra expression (36)), and Ick−/−IL−4Ra−/− [CD4 T cell–specific disruption of IL−4Ra expression (37)]. C57BL/6 background mouse strains used were C57BL/6 and IgMi (20). Mice were bred and housed in specific pathogen–free conditions at the Animal Unit of the University of Cape Town, South Africa. All experimental mice were used between 1 and 15 weeks of age with appropriate littermate controls of the same generation. Mice were euthanized by lethal halothane inhalation.

**Ethics statement**

All studies were carried out in accordance with protocol 012/054 or 014/021 approved by the Faculty of Health Sciences Animal Ethics Committee from the University of Cape Town.

**Mating and litter swaps**

Female mice aged between 7 and 8 weeks were mated with male mice at one male per cage of two females over 2 weeks, after which the male was removed. Females gave birth approximately 21 days after fertilization, and the birth of pups was monitored daily to ensure age matching. A maximum of two mothers and eight pups per mother were housed per cage. In the long-term experiments, pups were weaned at 3 weeks of age, separated from their mothers, and housed by gender.

**Infection with Nb**

Adult mice (older than 6 weeks) were injected subcutaneously with 500 × Nb L3 larvae. Pups were infected with a half-dose of 250 × Nb L3 larvae. To enumerate adult worms, mice were euthanized at various times PI, intestines were opened longitudinally and incubated in 10 ml of saline for 3 hours at 37°C, and parasites were counted under a dissecting microscope.

The mice used (except IL−4Ra−/−) for infection experiments would naturally clear Nb at approximately 9 days PI, but to ensure
that all worms were expelled, mothers were treated with ivermectin (10 mg/ml) in their drinking water from days 7 to 14 after primary infection to clear the pathogen before mating.

**Flow cytometry**

Single-cell suspensions were prepared, and $1 \times 10^6$ cells were incubated in phosphate-buffered saline + 0.5% bovine serum albumin, 1% normal rat serum, and appropriate antibody cocktails. Cell populations were acquired and determined on a BD FACS Fortessa (Becton Dickinson). Cell populations were identified by the following antibody staining strategies: CD4 T cells; CD3+CD4+. CD4 T cells populations were additionally stratified into activated effector (CD4+CD62Llo), T1/ST2+ and Thy1.1+Thy1.2−, or Thy1.2+Thy1.1− T cell populations.

Intracellular cytokine staining was also carried out on T cells to determine IL-13 expression. Cytokines were resuspended in complete medium [Iscove's modified Dulbecco's medium (IMDM; Gibco/Invitrogen, Carlsbad, CA)] + 10% fetal calf serum (FCS), penicillin/streptomycin] at 2.5 $\times$ 10$^7$/ml and stimulated with phorbol 12-myristate 13-acetate/ionomycin (10 $\mu$g/ml) and GolgiStop (as per the manufacturer's protocol; BD Pharmingen) at 37°C for 4 hours. After restimulation, cells were surface-stained for CD3 and CD4 and then fixed and permeabilized with Cytofix/Cytoperm Plus (as per the manufacturer's instructions; BD Pharmingen). Intracellular staining was performed by staining cells with IL-13 or appropriate control. All analyses were performed with FlowJo software.

**Antibody ELISA**

Relative antigen-specific serum antibody levels were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, plates were coated with Nb somatic antigen (10 $\mu$g/ml). Blood serum was added and incubated overnight before the commercially available alkaline phosphatase–conjugated secondary antibody for IgG1 (BD Pharmingen) was added. 4-p-Nitrophenol-phosphate was used to develop the reaction. Absorbance was read at $\lambda = 405$ nm against a reference measurement of $A = 490$ nm using the SoftMax Pro software on a VersaMax microplate reader. Relative antigen-specific antibody levels were plotted as dilution graphs. Nb somatic antigen was generated by isolating the soluble fraction from homogenized Nb L3 larvae.

**Milk preparation**

A modified method of the one described by Cabin et al. (30) was used to prepare milk for analysis of cell populations. The milk bolus was extracted from the stomachs of 10-day-old pups and conserved in complete medium (IMDM + 10% FCS, penicillin/streptomycin, Hepes). The bolus was passed through a 100-μm cell strainer to generate a single-cell suspension, and the washer was washed with 0.17 M tris (pH 7.6) and 0.83% NH4Cl buffer. The cell suspension was centrifuged at 400g for 15 min. Pellets containing cells were washed a further three times in complete medium before further analysis.

**Cytokine ELISA**

Cytokine ELISAs were performed as previously described (38) using coating and biotinylated detection antibodies from R&D Systems. Streptavidin–conjugated horseradish peroxidase was used for detection with a commercially available substrate solution. Spleen cells were plated at $1 \times 10^6$ cells per well in 48-well plates precoated with anti-CD3 (20 $\mu$g/ml) and restimulated for 72 hours before the supernatant was analyzed for cytokines. Homogenates of lung sections were prepared using a Polytron homogenizer, and the protein concentration of the soluble fraction of the lung samples was equalized before ELISA.

**Statistics**

The results are expressed as either individual mice/data points or group mean ± SD. P values and significances were determined using either the one-tailed Mann-Whitney U test or nonparametric one-way analysis of variance (ANOVA) (GraphPad Prism Software). Groups were judged to be significantly different if the P value was less than 0.05 (*P < 0.05, **P < 0.01, and ***P < 0.001).

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/5/eaav3058/DC1.

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