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## IMMUNOLOGY

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

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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<sub>H</sub>2) impaired IL-4R $\alpha^{-/-}$  offspring. This maternally acquired immunity was maintained into maturity and required transfer (via nursing) to the offspring of maternally derived T<sub>H</sub>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 opsonins 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 unaddressed (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

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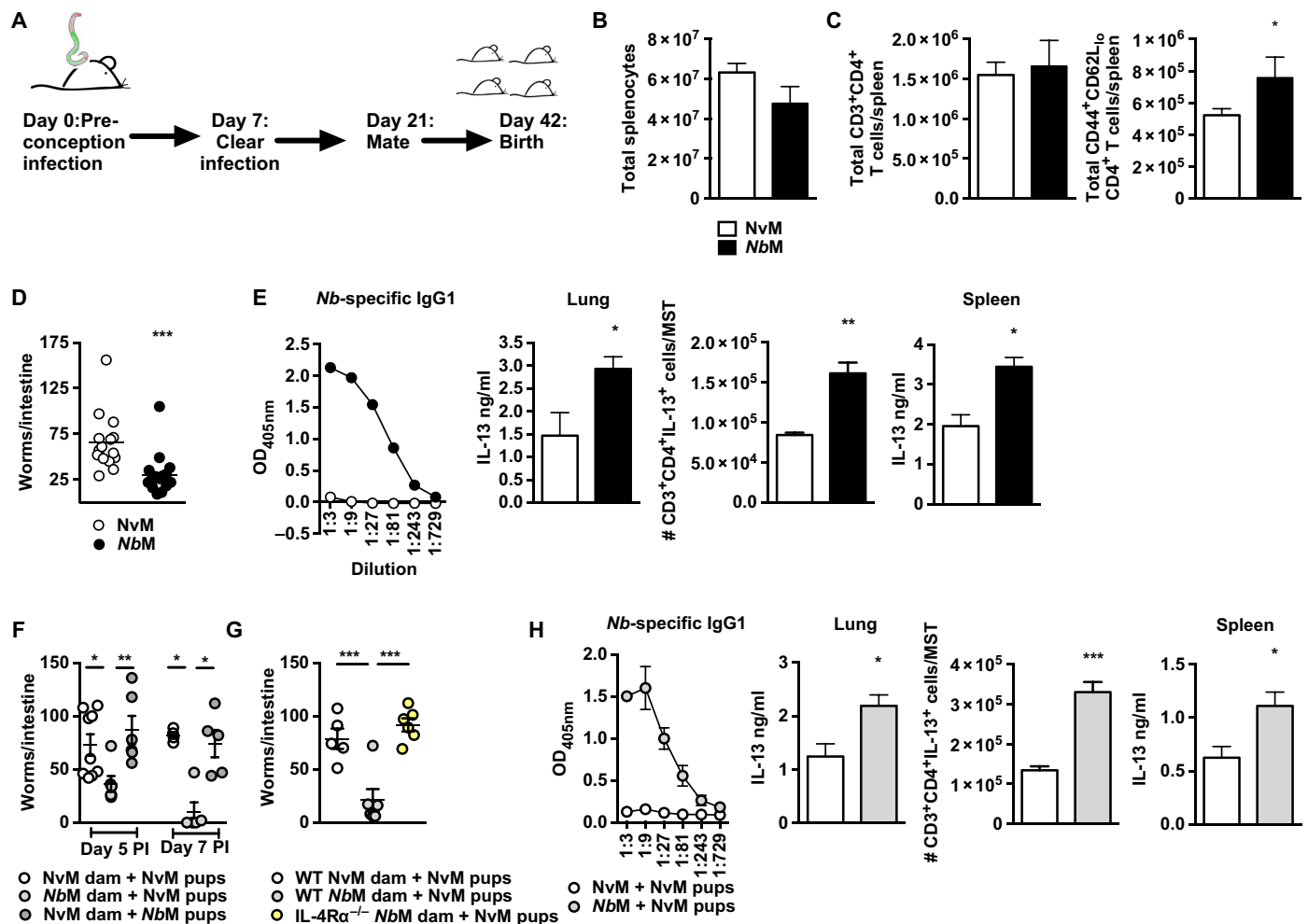
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control of infection. This approach allowed us to test how pre-conception maternal infection with the nematode *Nippostrongylus brasiliensis* (*Nb*) influenced offspring ability to control an *Nb* infection both early in life and when mature. Our model 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 (T<sub>H</sub>2)-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<sup>+</sup> 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<sup>+</sup> T cells in *NbM* offspring were raised when compared to *NvM* offspring (Fig. 1C).



**Fig. 1. Maternal helminth infection confers systemic protective immunity against *Nb* to pups via nursing.** (A) Female BALB/c mice were infected with 500 × *Nb* L3 and, 7 days PI, were cleared of *Nb* by a 5-day oral treatment with ivermectin; at day 21 PI, mice were mated and immunity in subsequent pups was established at various times after birth and/or *Nb* infection. (B) Total splenocyte cellularity in 14-day-old offspring. (C) Total numbers of CD3<sup>+</sup>CD4<sup>+</sup> T cells and activated T effector CD4 T cell populations (CD3<sup>+</sup>CD4<sup>+</sup>CD44<sup>+</sup>CD62L<sub>lo</sub>) in 14-day-old offspring. (D) Intestinal worm burdens at day 5 PI of pups born to *NvM* or *NbM* mothers and infected with 250 × *Nb* L3 when 14 days old. (E) *NvM* and *NbM* offspring anti-CD3 restimulated splenocyte IL-13 secretion, lung tissue homogenate levels of IL-13 and IL-13<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> T cells/MST, and *Nb*-specific serum immunoglobulin G1 (IgG1) at day 5 after *Nb* infection. OD<sub>405nm</sub>, optical density at 405 nm. (F) Worm burdens of *NvM* and *NbM* offspring infected when 14 days old and euthanized at day 5 PI following fostering on *NvM* or *NbM* dams at 3 days old. (G) Worm burdens of *NvM* and *NbM* offspring infected when 14 days old and euthanized at day 5 PI following fostering on WT or IL-4Rα<sup>-/-</sup> *NvM* and *NbM* dams. (H) *NvM* pups fostered by either *NvM* or *NbM* anti-CD3 restimulated splenocyte IL-13 secretion, lung tissue homogenate levels of IL-13 and IL-13<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> T cells/MST, and *Nb*-specific serum IgG1 at day 5 after *Nb* infection. All data are representative of a minimum of two experimental repeats. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001.

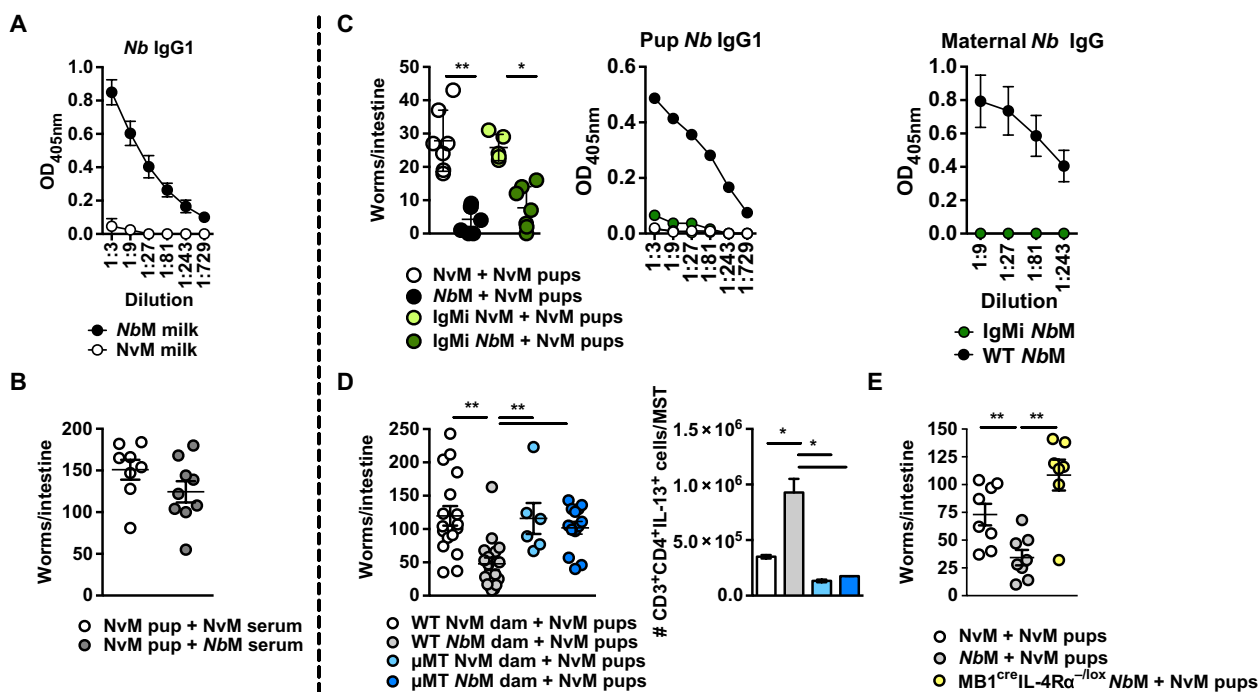
Increased T cell activation suggested that *NbM* offspring may acquire an enhanced ability to control *Nb* infection. We therefore tested whether *NbM* transferred an enhanced interleukin-4 receptor  $\alpha$  (IL-4R $\alpha$ )-driven type 2 immunity against *Nb* to their offspring. Reduced intestinal 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 T<sub>H</sub>2 cytokine IL-13 (essential for resolving *Nb* infection) in the lung, mediastinal lymph nodes (MST), and spleen, demonstrating that maternal transfer of immunity resulted in a systemic T<sub>H</sub>2 priming in *NbM* offspring reflective of acquired host-protective pulmonary T<sub>H</sub>2 immunity to *Nb* (17).

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 T<sub>H</sub>2 competency: Wild-type (WT) offspring nursed by IL-4R $\alpha$ <sup>-/-</sup> *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-4R $\alpha$ -dependent manner (10). We nursed WT *NvM* pups on



**Fig. 2. Protection in *NbM* pups is maternal B cell dependent but not antibody dependent.** (A) *Nb*-specific IgG1 levels in breast milk taken from the stomach of pups nursed by *NvM* or *NbM*. (B) Intestinal worm burdens at day 5 PI of *NvM* offspring infected at 14 days old with  $250 \times Nb$  L3 following intravenous administration of  $150 \mu\text{l}$  of heat-inactivated serum from either *NvM* or *NbM*. (C) Intestinal worm burdens and *Nb*-specific serum IgG1 levels at day 5 PI of *NvM* offspring infected at 14 days old with  $250 \times Nb$  L3 after being fostered on either WT and IgMi *NbM* mice or WT and IgMi maternal *Nb*-specific serum IgG1 levels. (D) Three-day-old *NvM* pups were fostered on WT *NvM/NbM* or  $\mu\text{MT}$  *NvM/NbM* dams. Pups were infected when 14 days old with  $250 \times Nb$  L3 and euthanized at day 5 PI. Offspring intestinal worm burden was analyzed, and MST IL-13<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup>IL-13<sup>+</sup> T cells were quantified at day 5 PI. (E) Intestinal worm burdens at day 5 PI of *NvM* offspring infected at 14 days old with  $250 \times Nb$  L3 after being fostered on either WT or B cell-IL-4R $\alpha$ -deficient (MB1<sup>cre</sup>IL-4R $\alpha$ <sup>-/-</sup>) dams. All data are representative of a minimum of two experimental repeats. \* $P < 0.05$  and \*\* $P < 0.01$ .

WT and  $\mu$ MT NvM and *NbM* dams and found that no protection was transferred to pups nursed by  $\mu$ MT *NbM* dams (Fig. 2D). Associated with this reduced protection were significantly reduced CD4<sup>+</sup>IL-13<sup>+</sup> populations in offspring nursed by  $\mu$ MT *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-4R $\alpha$  specifically on B cells (MB1<sup>cre</sup>IL-4R $\alpha$ <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>H</sub>2 CD4 T cells in the offspring**

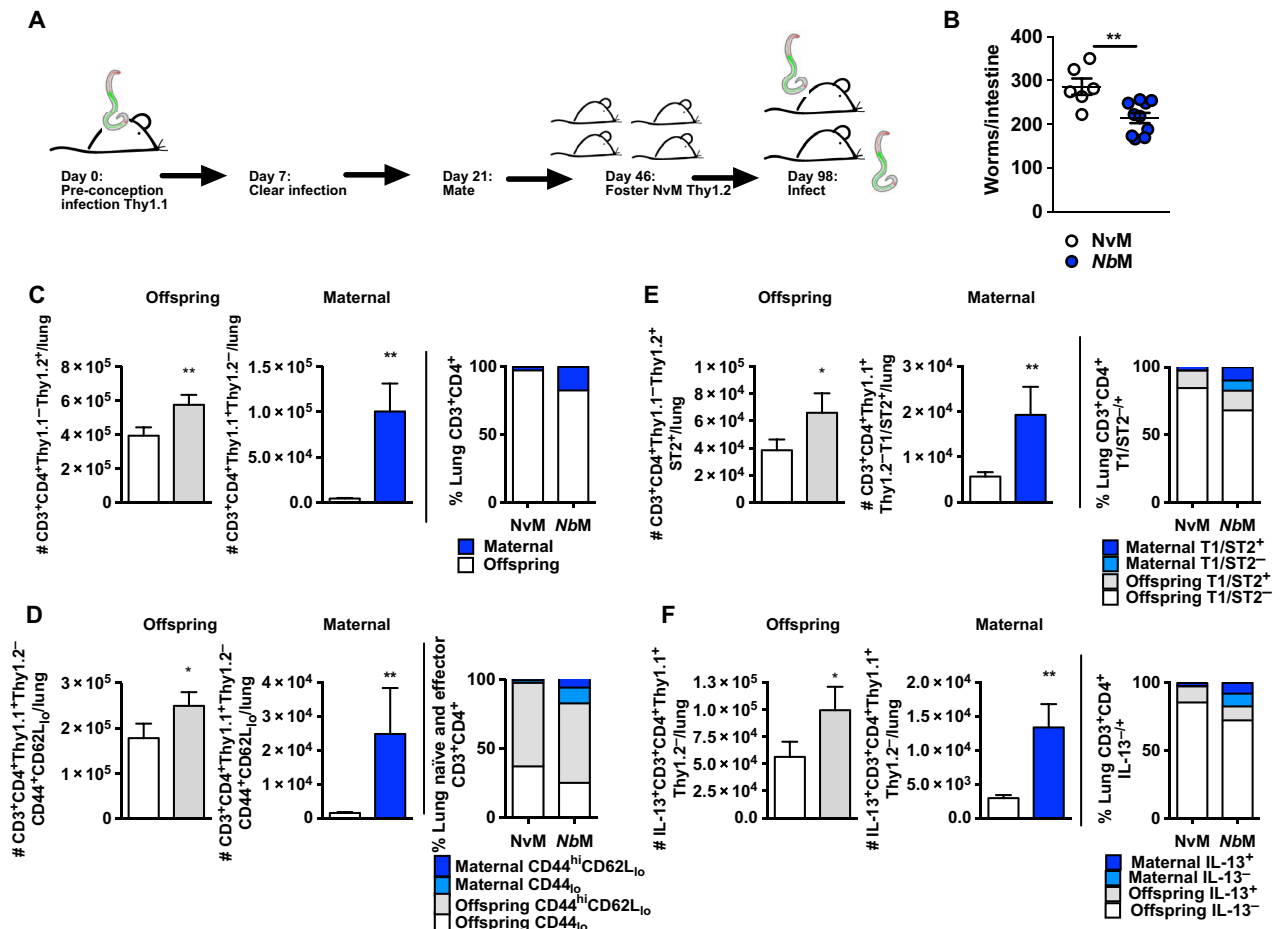
The systemic profile of raised T<sub>H</sub>2 CD4 T cell-associated immunity in *NbM* nursed pups when compared to NvM nursed pups is a striking effect on pup immunity. As milk contains significant populations of immune cells, we hypothesized that these cells may be important contributors to this enhanced 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, naive 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>H</sub>2-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 (fig. S1) of *NbM* dam-nursed offspring than in pups nursed by NvM dams. Significantly

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**Fig. 3. Offspring acquire, via nursing, persistent protection from *Nb* related to acquisition of a population of maternally derived CD4 T cells with a heightened T<sub>H</sub>2 responsiveness following *Nb* infection.** (A) Three-day-old NvM Thy1.2<sup>+</sup> pups were fostered on Thy1.1<sup>+</sup> WT NvM/*NbM* dams. Eight-week-old NvM Thy1.2<sup>+</sup> mice that had been fostered on either NvM or *NbM* Thy1.1<sup>+</sup> dams were infected with 500 × *Nb* L3 and euthanized at day 5 PI. (B) Intestinal worm burdens in offspring at day 5 PI in 8-week-old mice born to either NvM or *NbM* were established. (C to F) Numbers and proportions of offspring endogenous (Thy1.2<sup>+</sup>Thy1.1<sup>-</sup>) and maternally derived (Thy1.1<sup>+</sup>Thy1.2<sup>-</sup>) lung (C) Thy1<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup>, (D) CD44<sup>hi</sup>CD62L<sub>lo</sub>, (E) T1/ST2<sup>+</sup>, and (F) IL-13<sup>+</sup> expressing T cell populations. All data are representative of two experimental repeats. \*P < 0.05 and \*\*\*P < 0.01.

more maternally derived cells were activated ( $CD4^+CD44^+CD62L_{lo}$ ) in *NbM* than *NvM* offspring (Fig. 3D). The *NbM*-derived populations of *Thy1.1*  $CD4^+$  T cells had higher levels of  $T_H2$  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  $T_H2$ -competent  $CD4^+$  T cells, which significantly enhanced both the overall and endogenous  $T_H2$   $CD4^+$  T cell compartment of the offspring.

### Long-term protection is maintained in an allogeneic setting

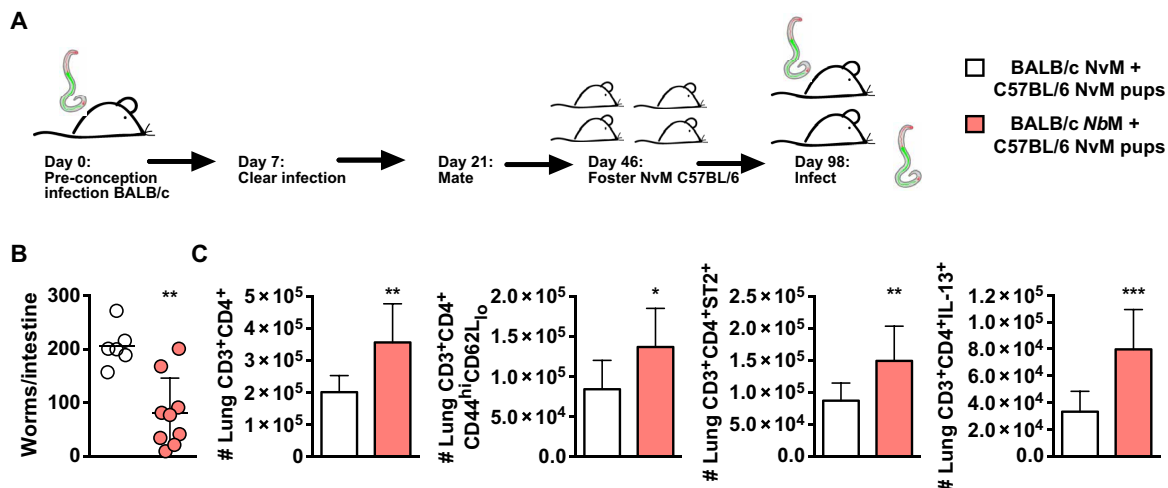
To test whether our findings related to transfer of maternal  $CD4^+$  T cells were relevant in an outbred setting representative of typical maternal-offspring relationships, we nursed naïve *C57BL/6* pups on either *NvM* or *NbM* *BALB/c* dams (Fig. 4A) and found that 6- to 8-week-old *NbM* offspring had significantly reduced *Nb* burdens in the intestine when compared to *NvM* 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).

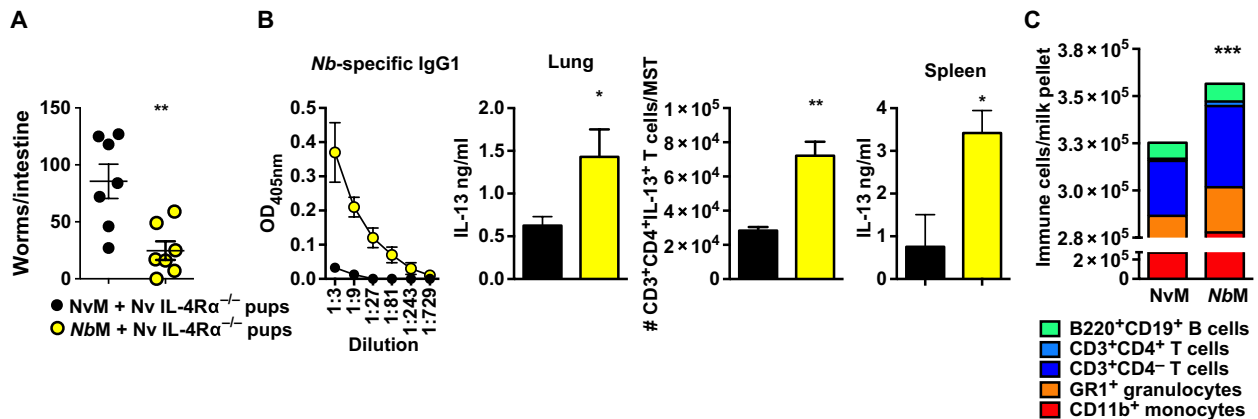
### Nursing-derived protection corrects susceptibility to *Nb* in $IL-4R\alpha^{-/-}$ pups

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  $IL-4R\alpha^{-/-}$  pups to *Nb*. Transfer of WT immune cells into  $IL-4R\alpha^{-/-}$  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- $\beta$  ( $TGF\beta^{-/-}$ ) offspring (26). We found that  $IL-4R\alpha^{-/-}$  pups nursed on WT *NbM* showed significantly reduced worm burdens at day 5 PI when compared to  $IL-4R\alpha^{-/-}$  pups nursed by WT *NvM* mothers (Fig. 5A). This protection in *NbM*  $IL-4R\alpha^{-/-}$  pups correlated with systemically raised  $CD4^+$  T cell-associated  $IL-13$  responses when compared to *NvM*  $IL-4R\alpha^{-/-}$  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  $IL-4R\alpha^{-/-}$  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  $T_H2$   $CD4^+$  T cell responses following *Nb* infection in an allogeneic setting.** (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  $500 \times Nb$  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  $CD44^+CD62L_{lo}$  expressing T cell populations were quantified. All data are representative of a minimum of two experimental repeats. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .



**Fig. 5. IL-4Rα<sup>-/-</sup> offspring nursed on NbM dams have enhanced lymphocyte-mediated immunity against Nb infection.** (A) Three-day-old IL-4Rα<sup>-/-</sup> 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<sup>+</sup>CD4<sup>+</sup>IL-13<sup>+</sup> T cells/MST were quantified in IL-4Rα<sup>-/-</sup> 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.

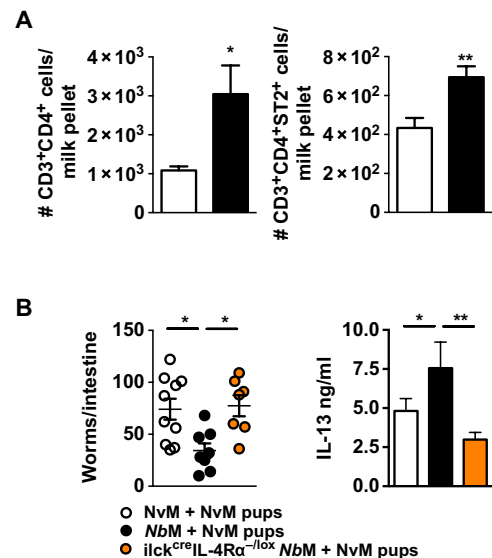
confer protective immunity to IL-4Rα<sup>-/-</sup> offspring through the transfer of a range of not only CD4 T cells but also populations of myeloid effector cells, which, together, could coordinate an adaptive-like immunity against Nb in the IL-4Rα<sup>-/-</sup> lung.

**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 TH2 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<sup>+</sup>CD4<sup>+</sup> T cells in NbM dam milk when compared to NvM (Fig. 6A). Moreover, these NbM CD3<sup>+</sup>CD4<sup>+</sup> T cells showed increased numbers of type 2 competent CD3<sup>+</sup>CD4<sup>+</sup>ST2<sup>+</sup> 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-4Rα expression specifically in their T cell compartment (ilck<sup>Cre</sup>IL-4Rα<sup>-/lox</sup> mice). Offspring nursed on NbM ilck<sup>Cre</sup>IL-4Rα<sup>-/lox</sup> 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.

**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



**Fig. 6. Maternally derived type 2 CD4<sup>+</sup>T cells are enriched in NbM dam milk and are required for transfer of protection.** (A) Numbers of Thy1.1<sup>+</sup>CD4<sup>+</sup> and Thy1.1<sup>+</sup>CD4<sup>+</sup>ST2<sup>+</sup> 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/NbM or ilck<sup>Cre</sup>IL-4Rα<sup>-/lox</sup> 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.

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,

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 T<sub>H</sub>2 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-4R $\alpha^{-/-}$  offspring, which have a genetically impaired ability to launch type 2 immune responses. This protection was coincident with IL-4R $\alpha^{-/-}$  offspring having a heightened systemic T<sub>H</sub>2 competency. Typically, IL-4R $\alpha^{-/-}$  mice display an abrogated ability to secrete T<sub>H</sub>2 cytokines such as IL-13 (29); however, here, we found that IL-4R $\alpha^{-/-}$  offspring nursed by *NbM* are able to secrete IL-13 at levels up to fourfold higher than IL-4R $\alpha^{-/-}$  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 TGF $\beta$  (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 T<sub>H</sub>2 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 T<sub>H</sub>1 immunity (32). This elevated prenatal type 2 immunity appears to contribute to fetal allograft survival by impairing 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 T<sub>H</sub>2 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 T<sub>H</sub>2 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 T<sub>H</sub>2-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-4R $\alpha^{-/-}$  (described as *IL4ra<sup>tm1Fbb</sup>/IL4ra<sup>tm1Fbb</sup>*),  $\mu$ MT (B cell deficient), Thy1.1 (CD90.1), MB1<sup>cre</sup>IL-4R $\alpha^{-/lox}$  [B cell-specific disruption of IL-4R $\alpha$  expression (36)], and *ilck<sup>cre</sup>IL-4R $\alpha^{-/lox}$*  [CD4 T cell-specific disruption of IL-4R $\alpha$  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  $\times$  *Nb* L3 larvae. Pups were infected with a half-dose of 250  $\times$  *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-4R $\alpha^{-/-}$ ) 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<sup>+</sup>CD4<sup>+</sup>. CD4 T cells populations were additionally stratified into activated effector (CD44<sup>+</sup>CD62L<sub>lo</sub>), T1/ST2<sup>+</sup> and Thy1.1<sup>+</sup>Thy1.2<sup>-</sup>, or Thy1.2<sup>+</sup>Thy1.1<sup>-</sup> T cell populations.

Intracellular cytokine staining was also carried out on T cells to determine IL-13 expression. Cells 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 µg/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 µg/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  $\lambda = 490$  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 Cabinian *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 strainer was washed with 0.17 M tris (pH 7.6) and 0.83% NH<sub>4</sub>Cl 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 µg/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  $\pm$  SD. *P* values and significances were determined using either the one-tailed Mann-Whitney *t* 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>

- Fig. S1. Offspring acquire, via nursing, a population of maternally derived CD4 T cells with a heightened Th<sub>2</sub> responsiveness following *Nb* infection.  
 Fig. S2. Offspring nursed on *Hp*-infected mothers do not demonstrate maternal cell transfer.  
 Fig. S3. Allogeneic *NbM* offspring have raised numbers of maternally derived lung CD4<sup>+</sup> T cells following *Nb* infection.  
 Fig. S4. Offspring acquire, via nursing, persistent protection from *Nb* related to raised Th<sub>2</sub> CD4<sup>+</sup> T cell responses following *Nb* infection in an allogeneic setting.

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

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### ARTICLE TOOLS

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