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Impaired Basophil Induction Leads to an Age-Dependent Innate Defect in Type 2 Immunity during Helminth Infection in Mice

Hendrik J. Nel,* Emily Hams,* Sean P. Saunders,* Niamh E. Mangan,* Philip Smith,* Ann Atzberger,* Richard A. Flavell,† Shizuo Akira,‡ Andrew N. McKenzie,§ and Padraic G. Fallon*,*

Parasitic-infection studies on rhesus macaque monkeys have shown juvenile animals to be more susceptible to infection than adults, but the immunological mechanism for this is not known. In this study, we investigated the age-dependent genesis of helminth-induced type 2 immune responses using adult (6–8-wk-old) and juvenile (21–28-d-old) mice. Following infection with the parasitic nematode *Nippostrongylus brasiliensis*, juvenile mice had increased susceptibility to infection relative to adult mice. Juvenile mice developed a delayed type 2 immune response with decreased Th2 cytokine production, IgE Ab responses, mouse mast cell protease 1 levels, and intestinal goblet cell induction. This innate immune defect in juvenile mice was independent of juvenile mice developed a delayed type 2 immune response with decreased Th2 cytokine production, IgE Ab responses, mouse mast cell protease 1 levels, and intestinal goblet cell induction. This innate immune defect in juvenile mice was independent of TLR signaling, dendritic cells, or CD4+ cell function. Using IL-4-eGFP mice, it was demonstrated that the numbers of IL-4-producing basophil and eosinophils were comparable in young and adult naive mice; however, following helminth infection, the early induction of these cells was impaired in juvenile mice relative to older animals. In nonhelminth models, there was an innate in vivo defect in activation of basophils, but not eosinophils, in juvenile mice compared with adult animals. The specific role for basophils in this innate defect in helminth-induced type 2 immunity was confirmed by the capacity of adoptively transferred adult-derived basophils, but not eosinophils, to restore the ability of juvenile mice to expel *N. brasiliensis*. The defect in juvenile mice with regard to helminth-induced innate basophil-mediated type 2 response is relevant to allergic conditions. *The Journal of Immunology*, 2011, 186: 4631–4639.

A pproximately one third of the world’s population, or ∼2 billion people, is infected with various helminths (1). This high prevalence of helminth infections in humans, and more relevantly in our primate ancestors, will have exerted considerable evolutionary pressure on selection of genes that function in the immune system of humans. The consequence of such evolutionary pressure was elegantly exemplified by a recent study showing that helminth infections exerted the dominant pressure, compared with viruses, fungi, or bacteria, on the selection of the IL genes, including genes associated with prevailing autoimmune diseases (2). This selection-pressure concept supports the hypothesis that type 2 immunity evolved specifically to control and expel parasitic helminths and that the escalation in allergic disease seen today developed as a consequence of the absence of helminth immune regulation (3). Indeed, experimental models using helminths as natural inducers of type 2 immunity have been instrumental in dissecting the innate and adaptive components of the type 2 response. This has resulted in recognition of the importance of non-Th2 (CD4+ T cell) responses in the genesis of innate type 2 immunity, in particular the crucial roles of basophils and eosinophils (4–10). More recently, the use of helminth models of type 2 immunity has been instrumental in the identification of additional novel innate type 2 effector cell types, which can be characterized by high expression of IL-4, IL-5, and IL-13 (11–14). Discoveries such as these emphasize the benefits and importance of mouse helminth models in dissecting immune mechanisms to enhance our understanding of the genesis of Th2 responses (15).

Host age is an important factor in the incidence and burden of certain helminth diseases [reviewed in Hotez et al. (16)], with young animals being more susceptible to infection than are adults with increased worm burden. However, this phenomenon is less clear in the case of human helminth infection. Thus, although children are more susceptible to certain helminths, infection with other helminth species increases into adulthood (17, 18). With respect to the infection of man with the nematode parasite *Schistosoma mansoni*, contradictory results on age prevalence exist, with some studies suggesting that juveniles are more susceptible and others suggesting that prevalence increases in adulthood; however, this may be due, in part, to varying levels of exposure to the helminth in daily activities (19, 20).

It remains to be elucidated whether the increased susceptibility of juveniles to certain helminth infections is due to a defect in the...
genesis of the characteristic helminth-induced type 2 immunity in younger animals or whether adults have fewer worms as a result of their ability to evoke adaptive type 2 antihelminth immunity. This age-dependent phenomenon was addressed experimentally using S. mansoni infection of rhesus macaque monkeys (21). Following a primary S. mansoni infection, juvenile monkeys had greater numbers of worms present then did comparable infected adult animals (21, 22). Additionally, despite having more worms present, the juvenile monkeys had a strikingly lower type 2 immune response compared with the adult animals (21). However, the specific immunological mechanisms that control these age-dependent differences in the helminth-induced type 2 response are unknown.

In this study, we used mouse helminth models to compare the generation of type 2 responses in adult and juvenile mice and, subsequently, to address the mechanisms underlying the apparent age-associated defect. The data presented demonstrate that juvenile mice have an innate defect in the generation of basophils that impairs adaptive type 2 immunity.

Materials and Methods

Animals
BALB/c and C57BL/6j mice were purchased from Harlan (Bicester, U.K.). IL-4-enhanced GFP (eGFP) reporter (4get), RAG-1−/−, and DO.11.10 OVA-TCR mice, all on a BALB/c background, were obtained from Jackson Laboratories (Bar Harbor, ME) and subsequently bred in-house. 4get mice were backcrossed to a C57BL/6 strain in-house. IL-13–eGFP reporter mice on a BALB/c background were as described (11). Foxp3–monomeric red fluorescent protein (mRFP) reporter mice (23) were bred in-house. TLR2−/−, TIRAP−/−, and TRIF +/+ wild-type mice were originally from S. Akira. All TLR knockout mice were on a C57BL/6 strain in-house. TLR2−/− mice have an innate defect in the generation of basophils that is associated with a defect. The data presented demonstrate that juvenile mice have an innate defect in the generation of basophils that impairs adaptive type 2 immunity. 

Cytokine and Ab ELISAs

Supernatants from in vitro cultures and serum collected from mouse blood were analyzed by sandwich ELISAs to quantify levels of specific cytokines and other blood-borne proteins. Total TGF-β (acidified samples) was measured using a commercial kit (Promega), according to the manufacturer’s instructions. Abs and reagents for detection of IL-4 and IFN-γ were obtained from BD Pharmingen, whereas all other cytokines (IL-5, IL-13, IL-17, and IL-10) were measured with the DuoSet ELISA development system from R&D Systems. Serum mouse mast cell protease 1 (mMCP-1) was quantified using a kit purchased from Moredun Scientific. Total IgE and IgG were detected with anti-mouse IgE (R35-72; BD Pharmingen) and anti-mouse IgG (Zymed).

Lung tissue processing

For flow cytometric analysis, lung tissue was harvested and homogenized as previously described (26).

In vitro Th cell differentiation and analysis

CD4+ cells were isolated from spleens of adult and juvenile mice using Mouse CD4+ T Cell Enrichment Columns (R&D Systems). A total of 1 × 10^6 CD4+ cells were cultured for 4 d under the following conditions: Th1 differentiation, 10 ng/ml IL-2, 1 ng/ml IL-12, and 10 ng/ml anti–IL-4; Th2 differentiation, 10 ng/ml IL-2, 10 ng/ml IL-4, and anti–IFN-γ; and Th17 differentiation, 100 ng/ml IL-6, 1 ng/ml TGF-β, 1 ng/ml anti–IL-4, and 1 ng/ml anti–IFN-γ. Cells were washed and restimulated in the presence of 20 ng/ml PMA and 1 μg/ml ionomycin (both from Sigma). After 12 h, supernatants and cells were harvested for cytokine ELISA analysis or RNA isolation for gene-expression analysis, respectively.

Adaptive CD4+ cell-transfer assay

Spleen CD4+ cells were isolated from juvenile and adult BALB/c mice, and 8 × 10^6 cells were injected i.v. into RAG-1−/− recipient mice. Recipient animals were subsequently infected with N. brasiliensis larvae before being sacrificed 8 d later for intestinal worm counts.

Real-time PCR

Total RNA was isolated using the RNeasy Mini kit and reverse transcribed with the Quantitect Reverse Transcription kit (both from Qiagen) to obtain cDNA. TaqMan-labeled probes for T-bet (Th1 transcription factor), GATA3 (Th2 transcription factor), and RORγT (Th17 transcription factor), using Il18 as a reference gene, were analyzed on an ABI 7900 Real-Time PCR cycler (all from Applied Biosystems), according to the manufacturer’s protocols. Levels of Th1-, Th2-, and Th17-specific transcription factors were quantified using the ∆∆CT method of relative quantification after real-time PCR.

Dendritic cell analysis

For dendritic cell (DC) functional analysis, CD11c-expressing DCs were isolated from collagenase-digested cell suspensions from spleen from juvenile and adult BALB/c mice. Spleen DCs were isolated using autoMACS CD11c columns (Miltenyi Biotec), and the purity was >95% CD11c+. DCs were cultured in vitro with OVA (323-339) peptide (Cambridge Research Biochemicals, Cleveland, U.K.; <0.01 endotoxin units/mg protein) or PBS for 6 h. DCs were harvested, washed, and injected i.p. into recipient BALB/c strain mice that had been injected the previous day with 3 × 10^6 CD4+ cells from DO.11.10 OVA-TCR mice. Purified CD4+ cells were isolated from DO.11.10 mice using Mouse CD4+ T Cell Enrichment Columns (R&D Systems). On day 4 after DC transfer, the footpad-draining popliteal lymph nodes (PLNs) were removed. Flow cytometric analysis was performed to identify the presence of OVA (KJ1-26)-specific CD4+ cells, as described (26), in the PLNs.

PLN cells were cultured under increasing OVA concentrations for 72 h and pulsed with 1 μCi [3H]thymidine for the last 16 h of culture. Thymidine incorporation was measured (cpm) with the Microbeta liquid scintillation system (1450 Microbeta plus liquid scintillation counter, Wallac). Cytokine ELISA measurements of supernatants from OVA-stimulated PLN cell cultures from recipients of juvenile or adult DCs were performed to detect IFN-γ, interleukin (IL)-10, and IL-17. Abs and reagents for detection of IL-4 and IFN-γ were obtained from BD Pharmingen, whereas IL-10 and IL-17 were measured with the DuoSet ELISA development system from R&D Systems.
**Cell culture and immunological assays**

Single-cell suspensions were prepared from blood, lung, spleen, mesenteric lymph nodes (MLNs), or PLNs and were depleted of erythrocytes with ammonium chloride solution (if required), as described (27). Purified CD4+ cells were isolated using the CD4+ cell enrichment columns (R&D Systems) or the EasySep Mouse CD4 T Cell Enrichment Kit (StemCell Technologies). For in vitro cultures, cells were suspended in RPMI 1640 (Invitrogen Life Technologies), supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. For nonspecific stimulation, cells were cultured with plate-bound anti-CD3 (145-2C11) and soluble anti-CD28 mAb (37.51; both from BD Biosciences). Cultures were incubated at 37°C and 5% CO2, and supernatants for cytokine analysis by ELISA were harvested after 72 h.

**Isolation of basophils**

Spleens, bone marrow, and blood were harvested from naive adult or juvenile 4Get mice, and single-cell suspensions were prepared. Flow buffers used contained 2 mM EDTA to exclude doublets. CD49b+ (DX5) cells were enriched by positive selection using magnetic microbeads, according to the manufacturer’s instructions (Miltenyi Biotec). The cells were further incubated with anti-mouse c-Kit– and CCR3-fluorochrome–conjugated mAbs before basophils were purified using a MoFlow cytometer (IL-4-eGFP+NBNT SiglecF-). The purity of sorted basophils was confirmed by flow cytometry to be >96% (Supplemental Fig. 1), with <0.5% contamination with DCs or monocytes (data not shown). Sorted cells were also visually validated by Wright-Giemsa–stained cytopsin.

**Adoptive basophil cell-transfer assay**

Basophils were isolated from naive adult and juvenile 4Get mice, as illustrated in Supplemental Fig. 1. Between 0.5 and 1 × 10^6 unactivated basophils were adoptively transferred i.v. into recipient N. brasiliensis–infected BALB/c juvenile mice every 48 h on days 0, 2, and 4 postinfection. The period between transfer was based on the predicted 60-h in vivo life expectancy of basophils (28).

**Isolation of eosinophils and adoptive cell transfer**

Spleens, bone marrow, and blood were harvested from naive adult or juvenile 4Get mice, and single-cell suspensions were prepared. Flow buffers used contained 2 mM EDTA to exclude doublets. The cells were incubated with anti-mouse CD4+, CD8+, CD19-, and SiglecF-conjugated mAbs before eosinophils were purified using a MoFlow cytometer (IL-4-eGFP+c-Kit CCR3+). The purity of sorted eosinophils was confirmed to be >96% by flow cytometry and was visually validated with cytopsins. Adult and juvenile BALB/c mice were infected with N. brasiliensis before subsequent i.v. adoptive transfer of 0.2–0.5 × 10^6 sorted eosinophils on days 0, 2, and 4 postinfection.

**Air pouch model of eosinophil recruitment**

Subcutaneous air pouches were induced in adult and juvenile 4Get mice, as previously described (29, 30). Briefly, mice were maintained under anesthesia while air pouches were raised by two s.c. injections, 3 d apart, of sterile air (5 ml) into the dorsal area. Two days after the second injection, mice were injected i.v. with 300 ng recombinant mouse IL-5, followed 30 min later by injection of 2 µg recombinant mouse eotaxin-1 (both from R&D Systems) into the air pouch lumen. The air pouches were lavaged 4 h later with 5 ml cold PBS, and the recovered cells were quantified by flow cytometry, as above, and differential cell counts.

**Differential cell counting**

Cytospins (50,000 cells/slide) were prepared from air pouch lavage fluid or cell sorting populations. All slides were Wright-Giemsa stained for differential counting of 400 leukocytes per slide.

**Identification and analysis of the nuocyte population**

Juvenile and adult IL-13–eGFP mice were injected i.p. with 0.4 µg recombinant mouse IL-25 (R&D Systems) daily for 3 d to induce nuocyte activation (11, 27). The nuocyte population was identified in the MLNs by flow cytometry. These cells were determined to be Lin− IL-13–eGFP+c-Kit− FcRn− (11).

**Statistics**

Statistical analysis was performed using GraphPad InStat. Results are presented as mean and SEM or SD where indicated. Differences, indicated as two-tailed p values, were considered significant when p > 0.05, as assessed by unpaired Student t test, with the Welch correction applied as necessary.

**Results**

Juvenile mice are more susceptible than adult mice to primary *N. brasiliensis* infection

Previous studies in juvenile and adult rhesus monkeys demonstrated an increased susceptibility to primary *S. mansoni* infection in younger animals (21, 22). To explore the immunological basis of this, we initially sought to use murine *S. mansoni* infections, a widely used animal model of many aspects of human schistosome infection (31). However, because *S. mansoni* infection takes >5 wk to establish in vivo (32), by this stage juvenile mice would have matured and could no longer be considered juvenile. Therefore, we used *N. brasiliensis*, a well-characterized type 2-inducing mouse model of a primary helminth infection (33), which is rapidly rejected in 5–7 d postinfection of adult mice. Adult mice infected with *N. brasiliensis* have worms present in the intestines by day 5, but these are expelled by day 7 (Fig. 1A), with no parasite eggs detected by day 8 (Fig. 1B). In contrast, juvenile mice demonstrated greater susceptibility than did adult mice, with more worms (p < 0.001) and eggs (p < 0.05) at day 5 postinfection, as well as a significant delay in the expulsion of the worms (Fig. 1A, 1B). When mice first infected with *N. brasiliensis* as juveniles were rechallenged, they developed acquired resistance to secondary infection (Fig. 1C, 1D). Therefore, juvenile mice are innately more susceptible to a primary helminth infection with delayed rejection.

![FIGURE 1.](http://www.jimmunol.org/...)

Juvenile mice are more susceptible than adult mice to primary *N. brasiliensis* infection than are adults with delayed type 2 immunity. *A* and *B*, Intestinal worm and eggs/g feces (EPG) counts during primary *N. brasiliensis* infection of juvenile (21–23-d-old) and adult (6–8-wk-old) BALB/c mice. *C* and *D*, Intestinal worm and egg counts 5 d after a secondary *N. brasiliensis* reinfection. Reinfections were administered 20 d after primary infection. *E*, Intestinal worm counts in juvenile and adult C57BL/6J mice following infection with 500 *N. brasiliensis* larvae. Data are from at least three separate experiments with 7–14 mice/group. *p < 0.05, **p < 0.001, two-tailed Student t test. ND, not detected.
worm expulsion, but they do develop resistance to reinfection. This effect is not specific to the BALB/c mice; it was also apparent in juvenile C57BL/6J mice in response to *N. brasiliensis* infection (Fig. 1E).

**Juvenile mice have a defect in helminth infection-induced type 2 immunity**

The expulsion of *N. brasiliensis* is associated with the generation of robust type 2 immunity characterized by elevated type 2 cytokines (IL-4, IL-5, IL-13), increased IgE, the development of mastocytosis, and increased intestinal mucus production by goblet cells (33, 34). In adult mice following *N. brasiliensis* infection, cells isolated from the MLNs demonstrated a progressive increase in type 2 cytokine expression upon in vitro restimulation, peaking at ~day 9 (Fig. 2A). In contrast, infected juvenile mice had significantly (p < 0.001–0.0001) reduced production of type 2 cytokines in early stages of infection (days 5 and 9), with Th2 cytokine production comparable to that seen in adult mice after worm expulsion on day 12 (Fig. 1A–C). There were no significant differences in the levels of cytokines associated with a type 1 (IFN-γ), Th17 (IL-17), or regulatory cell phenotype (TGF-β and IL-10) between juvenile and adult animals (Supplemental Fig. 2A–D).

Consistent with a defect in initial type 2 cytokine responses in juvenile mice relative to adult mice following primary infection, there was delayed generation of characteristic type 2 phenotypes, including levels of mMCP-1 (Fig. 2B), serum IgE (Fig. 2C), and numbers of intestinal goblet cells (Fig. 2D). However, the intact resistance of juvenile mice to secondary infection (Fig. 1C, 1D) was associated with generation of a functional adaptive type 2 response, with mMCP-1 (Fig. 2B), serum IgE (Fig. 2C), and goblet cells (Fig. 2D) increased to levels comparable to those in reinfected adult mice. These data suggested that juvenile mice are more susceptible to primary *N. brasiliensis* infection, with a delay in the expulsion kinetics that is associated with diminished capacity to generate early type 2 immunity.

**Juvenile mice have normal CD4+ cell and DC functions with no role evident for TLRs**

Because rejection of *N. brasiliensis* infection is mediated by CD4+ T cells (35), the delay in type 2 immune responses in juvenile mice during primary helminth infection raises questions regarding the development and functionality of CD4+ Th2 cells in juvenile animals. CD4+ T cells isolated from juvenile mice had the capacity to differentiate in vitro into Th1, Th2, and Th17 cells, as did cells from adult animals, based on relevant cytokine protein production (Fig. 3A–C) or transcription factor expression (Fig. 3D). Furthermore, the adoptive transfer of CD4+ cells isolated from juvenile mice was also able to transfer resistance to *N. brasiliensis* infection in RAG-1−/− recipient mice (Fig. 3E). Additionally, we found comparable frequencies of FoxP3+ regulatory T cells in juvenile and adult mice (data not shown).

The temporary defect in innate priming of the immune response in helminth-infected juvenile mice implies possible involvement of TLR signaling or DC functionality. To investigate the role of TLRs in helminth infection, we infected adult and juvenile TLR2−/− or TLR4−/− mice, as well as mice deficient in the adaptor proteins MyD88, Toll/IL-1R–domain containing adaptor protein, and TRIF. Importantly, worms were still detectable in TLR2−/−, TLR4−/−, MyD88−/−, TIRAP−/−, and TRIF−/− juvenile mice, but not in adults, after *N. brasiliensis* infection, indicating that the juvenile defect is TLR independent (Fig. 4). Extensive studies revealed that juvenile mouse DCs are not defective in vivo and are capable of rejection of *N. brasiliensis* infection.

**FIGURE 2.** Juvenile mice have a delayed type 2 cytokine immune response to *N. brasiliensis* infection compared with adults. A, MLN cells removed at indicated days from juvenile and adult *N. brasiliensis*-infected mice were restimulated with anti-CD3 mAb, and supernatants were analyzed by ELISA for the type 2 cytokines IL-4, IL-5, and IL-13 (mean and SD). Serum levels of mMCP-1 (mean + SEM) (B), total IgE (mean + SD) (C), and intestinal goblet cell numbers per villous crypt units (VCU; mean + SEM) (D) were measured on the indicated days. Data are from at least three separate experiments, with 7–14 mice/group. *p < 0.05, **p < 0.001, two-tailed Student t test.

**FIGURE 3.** Intact Th cell differentiation of CD4+ cells from juvenile mice in vitro. A, Production of IL-4, IL-5, and IL-13 in supernatant of splenic CD4+ cells from juvenile and adult mice cultured in vitro for 4 d under Th2 cell-differentiation conditions. Cells were washed and restimulated with PMA/ionomycin, and supernatants were analyzed by ELISA. IFN-γ (B) and IL-17 (C) production in cells treated as in A, but cultured in Th1- and Th17-differentiation conditions, respectively. D, RT-PCR detection of transcription factors in Th1 (Tbet)-, Th2 (GATA3)-, and Th17 (RORγT)-differentiated cells from juvenile and adult mice. E, Intestinal worm burden in RAG-1−/− mice 8 d postinfection with *N. brasiliensis*. RAG-1−/− mice were not treated (−) or received CD4+ splenocytes isolated from juvenile or adult mice. Data are from at least two separate experiments. Two-tailed Student t test.
of eliciting functional Ag-specific CD4+ cell responses comparable to DCs from adult animals (Supplemental Fig. 3A–C). Collectively, these data suggested that adaptive CD4+ T cell responses are intact in juvenile mice in response to helminth infection, suggesting that the delayed type 2 response evoked in juvenile mice may be due to a defective non-T cell-related innate priming event that is TLR and DC independent.

**Impaired basophil and eosinophil induction following helminth infection of juvenile mice**

To dissect the generation of type 2 cell populations following *N. brasiliensis* infection, mice coexpressing an eGFP with IL-4 mRNA, designated 4Get mice (36), were used. Following *N. brasiliensis* infection of adult mice, there was an early expansion of the innate type 2 CD4+IL-4+ Th2 cells (Fig. 5A). In contrast, in juvenile mice, there was an initial absence of CD4+IL-4+–producing cell populations up to day 9 postinfection, with a secondary delayed generation of Th2 cells (Fig. 5B). The data indicated that, in juvenile mice, an early deficiency in the generation of CD4+IL-4+ cell population(s) precedes the delayed expansion of Th2 cells and the associated type 2 immunity that causes worm expulsion following helminth infection.

Flow cytometry was used to further characterize the specific cells within the CD4+IL-4+ population, in particular basophils and eosinophils. FACs profiles used for detection of basophils [non-lymphocytic cells, designated CD45+CD8+/CD19− (non-B non-T cell [NBNT]), IL-4+, FcεRI+, CD49b (DX5)+, FcγRIIIB/IIA (CD16/CD32)+, c-Kitε and chemokine receptor 3 (CCR3)+] and eosinophils (NBNT, IL-4+, CCR3+, SiglecF+, c-Kitε and FcεRIε) included a gated cell population confirmed to be side scatter (SSC)low, contrasting with gated eosinophils being SSChi (Supplemental Fig. 4A, 4B). Using these criteria, the levels of basophils were quantified in the lungs as the initial site of worm migration and innate induction of type 2 responses following *N. brasiliensis* infection (7). There was a marked increase in basophils in adult mice, with levels peaking at day 7 postinfection (Fig. 5C). Importantly, although basophils were induced in juveniles on day 3, there was a significant reduction in basophil levels in juveniles relative to adults at this point in the infection. We also assessed baseline levels of basophils and found that uninfected adult mice had significantly higher baseline levels of basophils in bone marrow compared with juvenile mice. However, in the spleen, MLN, and the periphery, adult and juvenile mice had similar basal levels of basophils (data not shown).

Recently, Sokol et al. (9) identified basophils as major cells in the initiation of type 2 immune responses using the cysteine protease papain as a model allergen. It is also known that certain helminth species require secreted proteases to establish infection (37). Using the model of Sokol et al. (9), proteolytically active NbES (protease activity 0.64 μg/ml) or heat- or E-64–inactivated NbES (both with undetectable levels of protease activity) were generated and injected into footpads of adult and juvenile 4Get mice. NbES evoked a significantly (p < 0.05) greater recruitment of basophils to the PLN in adult mice relative to juvenile animals (Fig. 5D). Consistent with a role for protease activity in the activation of basophils, protease-inactive NbES (heat inactivation or E-64 inactivation) did not cause basophil recruitment in any mice (Fig. 5D). These data indicated that the induction and recruitment of basophils by helminth-ES proteins act via a protease-specific pathway and validated the presence of a defective basophil response in juvenile mice.

In addition to basophils, *N. brasiliensis* infection is known to induce the expansion of eosinophils in mouse lungs (4). Indeed, there was a marked expansion of pulmonary eosinophils following *N. brasiliensis* infection in adult mice (Fig. 6A). However, there was a significant delay in eosinophil expansion in juvenile mice, but this delayed expansion was subsequent to the initial impaired basophil response (Fig. 6A). Baseline eosinophil levels in the bone marrow, spleen, and MLNs were not significantly different between adult and juvenile mice, although naive juveniles presented with slightly higher levels in the periphery (data not shown). To further address whether the delayed recruitment of eosinophils into the lungs of helminth-infected juvenile mice was due to an innate eosinophil defect, we assessed eosinophil expansion and recruitment in response to IL-5 and eotaxin-1 in a mouse air pouch model (Fig. 6B). There was no difference in the recruitment of eosinophil (NBNT, IL-4–eGFPε SiglecFε) cells into the air pouches of juvenile mice relative to adult animals (Fig. 6C). These data implied that the delay in the generation of the type 2 response in juvenile mice involves an initial defect in basophil induction, with secondary delays in eosinophil expansion.

**Intact nuocyte generation in juvenile mice**

In addition to basophils, a previously identified IL-25–dependent cell population (Lin-ε, IL-13ε, c-Kitε, FcεRIε), recently termed the nuocyte, was shown to be an early source of IL-13 during *N. brasiliensis* infection (11). To address whether juvenile animals have a defect in the induction of nuocytes, we administered rIL-25 (0.4 μg) or PBS as control to IL-13–eGFPε mice daily for three consecutive days, as described (11). Nuocytes were induced in the MLNs following rIL-25 treatment at comparable levels in adult and juvenile mice (Fig. 7A). We also examined levels of nuocyte induction in MLNs of juvenile and adult mice following *N. brasiliensis* infection. Nuocyte cell numbers increased markedly as
Impaired basophil responses in juvenile mice after helminth infection and injection with helminth-derived Ags. A, Total numbers of CD4+IL-4+ (Th2) and CD4+IL-4- cells in MLNs of adult 4Get mice during N. brasiliensis infection. B, Total numbers of CD4+IL-4- (Th2) and CD4+IL-4+ cells in MLNs of juvenile 4Get mice during N. brasiliensis infection. C, Flow cytometry detection of the total numbers of basophils (SSClow, IL-4+, NBNT, CCR3+, c-Kit+, CD49b+, FcεRI+) in lungs of juvenile and adult 4Get mice during N. brasiliensis infection. D, Numbers of basophils in PLNs of adult and juvenile 4Get mice 3 d after footpad injection with NbES Ags or inactivated NbES (E-64). Control animals were injected with PBS. Data are mean ± SD (A, B) or mean ± SEM (C, D) from at least three separate experiments with five mice/group. *p < 0.05, **p < 0.001, two-tailed Student t test.

Early as 4 d postinfection, showing a steady decline on days 6 and 10, with no differences between adult and juvenile mice (Fig. 7B). These data demonstrated that juvenile mice have no defect in the generation of nuocytes induced by exogenous rIL-25 or by helminth infection.

Adoptive transfer of basophils, but not eosinophils, from adult mice restores resistance to primary helminth infection in juvenile mice

To formally validate that the defect in type 2 immunity in juvenile mice following helminth infection was specifically due to defective basophils, we performed an adoptive-transfer experiment with basophils isolated from adult or juvenile mice. Using unprimed adult or juvenile 4Get mice, we isolated nonactivated basophils, as illustrated in Supplemental Fig. 1, and adoptively transferred them into recipient BALB/c juvenile mice every 48 h during N. brasiliensis infection. Although adult animals had mostly expelled the parasites by day 7, juvenile mice had significantly more worms (p < 0.001) than did the older mice (Fig. 8A). Juvenile mice that received basophils isolated from adult mice had a worm burden comparable to adult mice, with significantly reduced numbers of worms compared with juvenile mice that did not receive cells (p < 0.01) (Fig. 8A). In contrast, transfer of basophils from juvenile mice did not reduce the number of worms present in juvenile animals (Fig. 8A). We examined the blood of recipient mice and could not find any donor 4Get mouse IL-4-eGFP+ basophils, possibly because of the short life expectancy of such cells in vivo (28). However, nondonor basophil levels in the blood of juvenile mice that had received adult-derived basophils were elevated significantly (p < 0.05) above the levels in control juvenile mice and were at levels similar to those in adult mice (Fig. 8B).

To formally exclude a role for eosinophils in the helminth-specific defect in the juvenile type 2 innate immune response, we again performed an adoptive-transfer experiment, with juvenile mice receiving eosinophils isolated from naive adult mice before and during N. brasiliensis infection. Seven days postinfection, adult mice had expelled worms, whereas juvenile mice and juvenile mice that received adult-derived eosinophils had fecund worms that were excreting parasite eggs (Supplemental Fig. 5).

Discussion

Our original objective for this study was to experimentally address in mouse models the immunological mechanism to explain why the prevalence of certain parasitic helminth infections seems to be associated with age (16). Previously, rhesus monkeys were used to show that juveniles are more susceptible to primary infection with S. mansoni relative to comparably infected adult monkeys, which is associated with a marked defect in the generation of a range of helminth-specific type 2 responses in younger monkeys (21). The defective ability of juvenile animal hosts to initiate such an im-
mune response to helminths is not restricted to rhesus monkeys; it is also seen in rats, with a delayed worm expulsion in juvenile rats following primary infection with *N. brasiliensis* or *S. mansoni* (data not shown). This raised the question, “Are juvenile animals inherently more susceptible to certain helminth infections because of an age-dependent defect in the genesis of type 2 immunity?” In juvenile 21–28-d-old mice, a range of immunological processes associated with type 2 immunity were shown to be functional, including priming of DCs, TLR-mediated activation and signaling, nuocyte generation, Th1/2/17 cell differentiation, and CD4+ T cell function in vivo. We identified that BALB/c and C57BL/6J strains of juvenile mice have a defect in basophil induction following helminth challenge. We validated that this basophil defect is responsible for the impaired type 2 immunity in juvenile mice using adoptive-transfer studies, with basophils from adult mice restoring the capacity of young mice to mount a type 2 response and reject helminths following infection. Although there is also a delay in eosinophil recruitment to lungs of helminth-infected juvenile mice, we demonstrated that juvenile mice have an intact eosinophil recruitment response in an air pouch model. Furthermore, adoptive transfer of adult eosinophils during infection did not restore the defective type 2 immune worm-expulsion response in juvenile mice. Interestingly, although transfer of eosinophils from adult mice to juvenile mice during infection restored the levels of blood eosinophilia to that detected in infected adult mice, it did not lead to expulsion of the worms. These data are consistent with the fact that following infection, there is an initial impairment in basophil induction in juvenile mice, with a secondary defect in eosinophils, Th2 cells, and type 2 responses.

There is a growing recognition that basophils play a central role in innate induction of type 2 responses and, thereby, facilitate the generation of adaptive immunity (38–40). The dominant role that helminths have in driving basophil functions is well described (41). Indeed, consistent with a major role for basophils in helminth infections, a recent clinical trial involving deliberate infection of IgE-seronegative humans with the hookworm *Necator americanus* showed that sensitization of basophils to parasite Ag already occurs at an early stage following infection, even before parasite-specific IgE is measurable in the serum (42). The innate role for basophils in the generation of type 2 responses is illustrated by the fact that expansion of eosinophils after *N. brasiliensis* infection is dependent on an initial basophil response (28). Indeed, in support of this, the defect in basophil generation in juvenile mice reported in this article precedes the delayed expansion of eosinophils and Th2 cells in younger animals.

Using helminth infection as a model, we identified an apparently previously unrecognized age-dependent deficiency in the innate induction of basophils. Further definition of this phenotype was achieved using helminth excretory/secretory proteases, which again resulted in the induction of basophils in adult mice, but not juvenile animals. Our data are consistent with a mechanism involving helminth proteases evoking type 2 immune responses through activation of basophils (9), because inactivation of such helminth proteases ablated the observed basophil recruitment to PLNs in adult mice. In separate experiments, we confirmed that adult and juvenile basophils expanded equally in response to external IL-3 administration in vivo and in vitro (43) (data not shown). A number of recent publications also identified new innate type 2 cells that play an important role in innate immunity (11–14). Following helminth infection using novel IL-13–eGFP mice, we found that nuocyte generation was not compromised in juvenile mice, supporting our findings that the impaired type 2 response in young animals is a basophil-specific defect.

The emerging paradigms on the mechanisms and functions of basophils are derived from studies on cells from adult experimental animals or humans (39, 41, 44, 45). Data presented in this article outline a clear defect in the innate induction of basophils in juvenile animals, thus suggesting the need to assess basophil levels in infants and children. Given that we focused on juvenile 21–28-d-old mice, it is prudent to mention that it is known that neonate mice have an impaired development of Th function (46–48). For example, CD4+ cells isolated from neonatal murine lymph nodes have a Th2-skewed primary immune response that induces a Th2-biased memory response toward secondary insults (49). In contrast, 3-wk-old juvenile mice were used in this study in models of helminth infection, demonstrating decreased or delayed type 2 immune responses compared with adult animals. It was recently identified that basophils play a role in enhancing humoral memory responses through their ability to bind Ag on their surface (50). In these studies, depletion of basophils in adult mice led to impaired generation of allergen-specific Ab responses, with...
B cell function enhanced by activated basophils (50). Thus, it is interesting that juvenile mice in this study had a defective capacity, relative to adult mice, in the generation of Ag-specific IgE following helminth infection. Therefore, the humoral defect in juvenile mice is consistent with impaired basophil function.

A number of groups recently demonstrated that basophils in adult mice can present Ag to Th2 cells under specific experimental conditions (51–53), a phenomenon with potential relevance to this study. However, recent publications highlighted some discrepancies in the suggestion that basophils may function as APCs, with demonstrations of a redundant role for basophils in Th2 induction (54–57) or the possibility for a cooperative role for DCs and basophils in Th2 induction under certain conditions (58). Once there is a consensus on the mechanisms that basophils use in the generation of type 2 immunity, such as Ag presentation or processing, it would be pertinent to further examine the functions of these cells in juvenile and adult animal models.

The ability of basophils to capture Ags indirectly though Ag-specific FcεRI-bound IgE could be another important mechanism for developing long-term resistance to helminth infections. It might be possible that the defective type 2 immune responses seen in juvenile mice could be due to altered surface expression of IgE (FceRI) or IgG (FcγRII/III) receptors on basophils. It is also possible that juvenile mouse basophils are in some other way immature or defective in their ability to bind IgE or IgG Abs accompanied by an insufficient capacity or an inability to induce intracellular signal. We have not fully investigated the underlying mechanism behind the defective basophil response in juvenile mice, and future work will need to address the possibility of a humoral defect in juvenile animals with respect to immune function, in particular with regard to basophil functions.

Helminth species have coexisted with humans and human ancestors by evolving to establish and maintain chronic infections (2, 59). Indeed, it is widely postulated that the escalation in allergic disease in Westernized societies is linked to the absence of helminth immune modulation (3, 60). Because basophils are induced early following helminth infection in experimental animals (5, 7) it is indicative that basophils were selected as the innate cellular response to initial helminth infection. In such a scenario, the defect in basophil function in juvenile mice that we described in this article could be seen as an evolutionary adaptation to downregulate potential hyperactivation of type 2 responses evoked by exposure to a plethora of type 2-inducing parasites at a young age. In this scenario, infants evolved with a defect in basophil function would be protected when challenged with helminth type 2 allergens, whereas those with an intact basophil response would have succumbed to fatal anaphylaxis and, thus, have been selected out from the gene pool. Although the recent increased acceptance for significant roles for basophils in allergic inflammation and humoral memory are based on studies in adult animals, the data presented in this article suggest that it is important to address the functions of basophils in children. Our results clearly showed a distinct alteration in the innate basophil response between adult and juvenile mice, which opens new avenues for further investigation into any potential effect that this may have in humans.

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Disclosures
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References


