Impaired basophil induction leads to an age-dependent innate defect in type 2 immunity during helminth infection in mice

Running title:

Age-dependent innate basophil type 2 immune defect

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[*]

[*]Institute of Molecular Medicine, Trinity College Dublin, Dublin 8, Ireland. [†] Department of Immunobiology, Yale University School of Medicine and Howard Hughes Medical Institute, PO Box 208011, New Haven, CT 06520-8011. [‡] Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka 5650871, Japan. [§] MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, UK.

Correspondence should be addressed to:

Padraic G. Fallon, Tel: +353 1 8963267, Fax: +353 1 8964040, *email: pfallon@tcd.ie*

Abstract

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. Here we have investigated, using adult (6-8 weeks old) and juvenile (21-28 days old) mice, the age dependent genesis of helminth-induced type 2 immune responses. 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 antibody responses, mMCP-1 and intestinal goblet cell induction. This innate immune defect in juvenile mice was independent of toll-like receptor signaling, dendritic cells or $CD4^+$ cell function. Using IL-4-eGFP (4Get) mice it was demonstrated that in naïve mice the numbers of IL-4 producing basophil and eosinophils were comparable in young and adult mice, but following helminth infection the early induction of these cells was impaired in juvenile mice relative to older animals. In non-helminth models there was an innate *in vivo* defect in activation of basophils, but not eosinophils, in juvenile mice compared to adult animals. The specific role for basophils in this innate defect in helminth-induced type 2 immunity was confirmed by adoptive transfer of adult-derived basophils, but not eosinophils, restoring the ability of juvenile mice to expel *N. brasiliensis*. The defect in juvenile mice in helminth-induced innate basophil mediated type 2 response is relevant to allergic conditions.

Introduction

Approximately 1/3 of the worlds population, or ~2 billion people, are currently 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 is elegantly exemplified by a recent study showing that helminth infections exerted the dominant pressure, compared to viruses, fungi or bacteria, on the selection of the interleukin 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 has 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 ($CD4^-$) cellular 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 adults with increased worm burden. However, in the case of human helminth

infection this phenomenon is less clear. 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 trematode parasite *Schistosoma mansoni* contradictory results on age-prevalence exist, with some studies suggesting juveniles are more susceptible and others suggesting prevalence increases in adulthood, however, this may be in part due to varying levels of exposure to the helminth in daily activities (19, 20).

It remains to be elucidated if 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 if adults have fewer worms due to their ability to evoke adaptive type 2 anti-helminth immunity. This age-dependent phenomenon has previously been 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 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 helminth induced type 2 response are unknown.

In this study we have used mouse helminth models to compare the generation of type 2 responses in adult and juvenile mice and, subsequently, address the mechanisms underlying the apparent age-associated defect. The data presented herein 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-eGFP (enhanced green fluorescent protein) 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, USA) 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-mRFP (monomeric red fluorescent protein) reporter mice (23) were bred in-house. TLR2$^{-/-}$ mice were from Jackson Laboratories (USA) and bred in-house. TLR4$^{-/-}$, TIRAP$^{-/-}$, MyD88$^{-/-}$ and TRIF$^{-/-}$ knockout mice were originally from S. Akira. All TLR knockout mice were on a C57BL/6 background, except TRIF$^{-/-}$ where heterozygous F2 (129/C57) mating was used to generate TRIF$^{-/-}$ and $^{+/+}$ wild-type control littermates. Female Wistar rats were obtained from BioResources, Trinity College Dublin. In all studies, juvenile mice and rats were 21-28 days of age and adults were from 7-9 weeks of age. Animals were housed in a specific pathogen-free facility in individually ventilated and filtered cages under positive pressure. All animal experiments were performed in compliance with Irish Department of Health and Children regulations and approved by the Trinity College Dublin's BioResources ethical review board.

N. brasiliensis infections and ES production

Mice were infected subcutaneously with 500 viable third-stage larvae (L3) from the rat-adapted strain of *N. brasiliensis*. For re-infection studies, mice were initially given a 500 L3 primary infection followed on day 20 with a similar 500 L3 re-infection. Worm and fecal egg

counts were as described (24). Rats were infected subcutaneously with 1,500 viable third-stage larvae from a rat-adapted strain of *N. brasiliensis*. The infected rats were killed on days 5, 7, 9 and 12 post-infection and worm counts and fecal egg counts performed (24). *N. brasiliensis* excretory-secretory (NbES) antigens were prepared as previously reported (25) with slight modification. NbES protease activity was determined by gelatin zymography and adjusted to protease activity per mg protein. For heat inactivation, NbES was incubated for 1 hour at 100 °C. For cysteine-specific protease inactivation, the NbES was incubated for 1 hour at a ratio of 10μM to 1μM E-64 (Sigma). NbES batches were screened for endotoxin contamination, and only batches with <0.5 endotoxin units per mg protein were used.

Flow cytometry

Surface marker expression and detection of IL-4 or IL-13 (eGFP) and Foxp3 (mRFP) in reporter mice were assessed by flow cytometry with data collection on a FACSCalibur, LSRII (BD Biosciences) or CyAn (Beckman Coulter). Cells were stained with BD Biosciences mAbs; PerCP anti-CD4 (RM4-5), PerCP-Cy5.5 anti-CD19 (1D3), PerCP anti-CD8a (53-6.7) and PE anti-Siglec-F (E50-2440). Caltag mAbs; APC anti-CD25 (PC61 5.3). eBiosciences mAbs; PE anti-FcεR1 (MAR-1), APC anti-CD117 (c-kit; 2B8), APC anti-CD49b (DX5), Pacific Blue anti-CD16/CD32 (Fcγ III/II receptors), AlexaFluor 647 anti-IL-4 (11B11), AlexaFluor 647 anti-IL-13 (eBIO 13A). R&D Systems; PE anti-CCR3 (83101). Flow buffers contained 2mM EDTA to exclude doublets. Using appropriate isotype-controls, quadrants were drawn and data were plotted on logarithmic scale density- or dot-plots. Data were analyzed using CellQuest software (BD Biosciences) or FlowJo (Tree Star).

Cytokine and antibody 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-born proteins. Total TGF-β (acidified samples) was measured using a commercial kit (Promega) according to the manufacturers instructions. Antibodies and reagents for detection of IL-4 and IFN-γ were obtained from BD Pharmingen, while 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 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). 1 x 10^6 $CD4^+$ cells were cultured for 4 days under following conditions: Th1 differentiation, 10 ng/ml IL-2 (R&D Systems), 1 ng/ml IL-12 (R&D Systems) and 10 ng/ml anti-IL-4 (R&D Systems); Th2 differentiation, 10 ng/ml IL-2 (R&D Systems), 10 ng/ml IL-4 (R&D Systems) and anti-IFNγ (R&D Systems); Th17 differentiation, 100 ng/ml IL-6 (R&D Systems), 1 ng/ml TGF-β (R&D Systems), 1 ng/ml anti-IL-4 (R&D Systems) and 1 ng/ml anti-IFN-γ (R&D Systems). Cells were washed and re-stimulated in the presence of 20 ng/ml PMA (Sigma) and 1 μg/ml Ionomycin (Sigma). After

12 hours supernatants and cells were harvested for cytokine ELISA analysis or RNA isolation for gene expression analysis, respectively.

Adoptive $CD4^+$ cell transfer assay

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

Real-time PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen) and reverse transcribed with Quantitect Reverse Transcription kit (Qiagen) to obtain cDNA. Applied Biosystem's TaqMan-labeled probes for T-bet (Th1 transcription factor), GATA3 (Th2 transcription factor) and RORγT (Th17 transcription factor) using 18S as a reference gene were analyzed on an ABI7900 Real-Time PCR cycler (Applied Biosystems) according to the manufacturers protocols. Levels of Th1-, Th2- and Th17- specific transcription factors were quantified employing the ΔCt method of relative quantification after real-time PCR.

Dendritic Cells (DC) analysis

For DC functional analysis, CD11c expressing DCs were isolated from collagenase digested cell suspension from spleen from juvenile and adult BALB/c mice. Spleen DCs were isolated using Automacs CD11c columns (Miltenyi Biotech), and were >95% $CD11c^+$ in purity. DCs were cultured *in vitro* with either $OVA_{(323-339)}$ peptide (Cambridge Research Biochemicals, Cleveland, UK; <0.01 Endotoxin Units per mg protein) or PBS for 6 hours. DCs were harvested, washed and injected i.p. into recipient BALB/c strain mice, that had been injected

the previous day with 3 x 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 PLN were removed. Flow cytometric analysis was performed to identify the presence of OVA ($KJI\text{-}26^+$) specific $CD4^+$ cells present, as described (26), in the PLN.

PLN cells cultured under increasing OVA concentrations for 72 hours and pulsed with 1 μCi of [^{3}H] thymidine for the last 16 hours of culture. Thymidine incorporation was measured by Microbeta liquid scintillation system (1450 Microbeta plus liquid scintillation counter, Wallac) as counts per minute (CPM). Cytokine ELISA measurements of supernatants from OVA-stimulated PLN cell cultures from recipients of juvenile or adults DCs were performed to detect IFN-γ, IL-4, IL-10 and IL-17. Antibodies and reagents for detection of IL-4 and IFN-γ were obtained from BD Pharmingen, while 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, MLN or PLN and depleted of erythrocytes with ammonium chloride solution (if required) as described (27). Purified $CD4^+$ cells were isolated using either 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 non-specific stimulation, cells were cultured with plate bound anti-CD3 (145-2C11; BD Biosciences) and soluble anti-CD28 mAb (37.51; BD Biosciences). Cultures

were incubated at 37°C and 5% CO_2 and supernatants for cytokine analysis by ELISA were harvested after 72 hours.

Isolation of basophils

Spleens, bone marrow and blood were harvested from naïve adult or juvenile 4Get mice and single cell suspensions prepared. Flow buffers used contained 2mM EDTA to exclude doublets. $CD49b^+$ (DX5) cells were enriched by positive selection using magnetic microbeads according to the manufacturers instructions (Miltenyi Biotech). The cells were further incubated with anti-mouse ckit- and CCR3-fluorochrome conjugated mAbs before basophils were purified using a MoFlow cytometer (IL-4-$eGFP^+ckit^-CCR3^-$). 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 cytospin.

Adoptive basophil cell transfer assay

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

Isolation of eosinophils and adoptive cell transfer

Spleens, bone marrow and blood were harvested from naïve adult or juvenile 4Get mice and single cell suspensions were prepared. Flow buffers used contained 2mM 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^{+}NBNT^{-}SiglecF^{+}). The purity of sorted eosinophils was confirmed by flow cytometry to be >96% and visually validated with cytospins. Adult and juvenile BALB/c mice were infected with *N. brasiliensis* before subsequent i.v. adoptive transfer of sorted 0.2-0.5 x 10^{6} eosinophils on days 0, 2 and 4 post-infection.

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 anaesthesia while air pouches were raised by 2 injections, 3 days apart, of sterile air (5 ml) subcutaneously into the dorsal area of the mouse (Fig. 6B). Two days after the second injection, mice were injected i.v. with 300 ng of recombinant mouse IL-5 (R&D Systems) followed 30 minutes later by injection of 2 μg of recombinant mouse eotaxin-1 (R&D Systems) into the air pouch lumen. The air pouches were lavaged 4 hours later with 5ml cold PBS and the recovered cells quantified by flow cytometry, as above, and differential cell counts.

Differential cell counting

Cytopsins (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 days to induce nuocyte activation (11, 27). The nuocyte population was identified in the MLN by flow cytometry. These cells are determined as Lin^- IL-13-$eGFP^+$ c-kit^+ FcεR1$^-$ (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's *t* test with 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, as *S. mansoni* infection takes > 5weeks to establish *in vivo* (32), by this stage juvenile mice would have matured and could no longer be considered juvenile. We therefore used *N. brasiliensis,* a well-characterized type 2 inducing mouse model of a primary helminth infection (33), which is rapidly rejected in 5-7 days after infection 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. 1*A*), with no parasite eggs detected by day 8 (Fig 1*B*). In contrast, juvenile mice demonstrated greater susceptibility than adult mice with more worms ($P<0.001$) and eggs ($P<0.05$) at day 5 post-infection, and a significant delay in the expulsion of the worms (Fig. 1*A*, *B*). When mice first infected with *N. brasiliensis* as juveniles were re-challenged, they developed acquired resistance to secondary infection (Fig. 1*C*, *D*). Therefore, juvenile mice are innately more susceptive to a primary helminth infection with delayed worm expulsion, but do develop resistance to re-infection. This effect is not specific to the BALB/c mice, and was also apparent in juvenile C57BL/6J mice in response to *N. brasiliensis* infection (Fig. 1*E*).

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 mesenteric lymph nodes (MLN) demonstrated a progressive increase in type 2 cytokine expression upon *in vitro* re-stimulation, peaking at approximately day 9 (Fig. 2*A*). 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 comparable Th2 cytokine production as seen in adult mice after worm expulsion on day 12 (Fig. 1*A-C*). There were no significant differences in levels of cytokines associated with either a type 1 (IFN-γ), Th17 (IL-17) or regulatory cell phenotypes (TGF-β and IL-10) between juvenile and adult animals (Supplemental Fig. 2*A-D*).

Consistent with a defect in initial type 2 cytokine responses in juvenile relative to adult mice following primary infection, there was delayed generation of characteristic type 2 phenotypes, including levels of mouse mast cell protease 1 (mMCP-1) (Fig. 2*B*), serum IgE (Fig. 2*C*), and numbers of intestinal goblet cells (Fig. 2*D*). However, the intact resistance of juvenile mice to secondary infection (Fig. 1*C*, *D*) was associated with generation of a functional adaptive type 2 response with mMCP-1 (Fig. 2*B*), serum IgE (Fig. 2*C*) and goblet cells (Fig. 2*D*) raised to levels comparable with re-infected adult mice. These data suggest that juvenile mice are more susceptive 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 dendritic cell functions with no role evident for TLRs. As rejection of *N. brasiliensis* infection is $CD4^+$ T cell mediated (35), the delay in type 2 immune responses in juvenile mice during primary helminth infection raises questions

regarding the development and functionality of $CD4^+$ T(h) helper 2 cells in juvenile animals. $CD4^+$ T cells isolated from juvenile mice had capable capacity to differentiate *in vitro* into Th1, Th2 and Th17 cells as did cells from adult animals, based on both relevant cytokine protein production (Fig. 3*A*-C) or transcription factor expression (Fig. 3*D*). 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. 3*E*). Additionally, we found comparable frequencies of $FoxP3^+$ regulatory T cells (Treg) 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 toll like receptor (TLR) signaling or dendritic cell functionality. In order to investigate the role of TLRs in helminth infection, we infected adult and juvenile $TLR2^{-/-}$ or $TLR4^{-/-}$ mice, as well mice deficient in the adaptor proteins MyD88, TIRAP 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*A, B*). Extensive studies revealed that juvenile mouse DCs are not defective *in vivo* and are capable of eliciting functional antigen-specific $CD4^+$ cell responses comparable to DCs from adult animals (Supplemental Fig. 3*A*-*C*). Collectively these data suggest 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 co-expressing an enhanced green fluorescent protein (eGFP) with IL-4 mRNA, designated 4Get mice (36), were used. Following *N. brasiliensis* infection of adult mice, there is an early expansion of the innate type 2 $CD4^{-}IL\text{-}4^{+}$ cell populations prior to generation of the adaptive $CD4^{+}IL\text{-}4^{+}$ Th2 cells (Fig. 5*A*). In contrast, in juvenile mice there is an initial absence of $CD4^{-}$IL-4-producing cell populations up to day 9 after infection, with a secondary delayed generation of Th2 cells (Fig. 5*B*). The data indicates that in juvenile mice an early deficiency in the generation of $CD4^{-}IL\text{-}4^{+}$ cell population(s) precedes the delayed expansion of Th2 cell 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 $CD4^{-}IL\text{-}4^{+}$ population, in particular basophils and eosinophils. FACS profiles used for detection of basophils [non-lymphocytic cells, designated $CD4^{-}/CD8^{-}/CD19^{-}$ (NBNT), $IL\text{-}4^{+}$, $Fc\varepsilon R1^{+}$, CD49b $(DX5)^{+}$, Fcγ Receptor IIIB/IIA $(CD16/CD32)^{+}$, c-kit^{-} and chemokine receptor 3 $(CCR3)^{-}$], and eosinophils [NBNT, $IL\text{-}4^{+}$, $CCR3^{+}$, $SiglecF^{+}$, $ckit^{-}$ and $Fc\varepsilon R1^{-}$] included a gated cell population confirmed to be Side Scatter $(SSC)^{low}$, contrasting with gated eosinophils being SSC^{hi} (Supplemental Fig. 4*A, B*). 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). In adult mice there is a marked increase in basophils, with levels peaking at day 7 post-infection (Fig. 5*C*). Importantly, even though 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 uninfected adult mice to have significantly higher baseline levels of basophils in bone marrow when compared to

juvenile mice. However, in the spleen, MLN and the periphery adult and juvenile mice have similar basal levels of basophils (data not shown).

Recently Sokol *et al.* identified basophils as major cells in the initiation of type 2 immune responses using the cysteine protease, papain, as a model allergen (9). It is also known that certain helminth species require secreted proteases to establish infection (37). Using the model of Sokol *et al.*, proteolytically active excretory-secretory (ES) antigens from *N. brasiliensis* (NbES; protease activity 0.64 μg/ml) or with 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. 5*D*). Consistent with a role for protease activity in the activation of basophils, protease-inactive NbES (heat- or E-64-inactivation) did not cause basophil recruitment in any mice (Fig. 5*D*). These data indicate that the induction and recruitment of basophils by helminth-ES proteins act via a protease-specific pathway and furthermore validate the presence of a defective basophil response in juvenile mice.

Apart from basophils, *N. brasiliensis* infection in mice is also 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, in juvenile mice there was a significant delay in eosinophil expansion, but this delayed expansion is subsequent to the initial impaired basophil response (Fig. 6A). Baseline eosinophil levels in the bone marrow, spleen and MLN were not significantly different between adult and juvenile mice, although naïve juveniles presented with slightly higher levels in the periphery (data not shown). To further address if the delayed recruitment of eosinophils into the lungs of helminth-infected juvenile mice was due to an innate

eosionophil 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-eGFP^{+} SiglecF^{+}) cells into the air pouches of juvenile mice relative to adult animals (Fig. 6C). These data imply 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^{-}, IL-13^{+}, ckit^{+}, FcεR1^{-}) has also been shown as an early source of IL-13 during *N. brasiliensis* infection, recently termed the nuocyte (11). To address if juvenile animals have a defect in the induction of nuocytes, we administered rIL-25 (0.4 μg) or PBS as control to IL-13-eGFP mice daily for 3 consecutive days, as described (11). Nucoytes were induced in the MLN following rIL-25 treatment at comparable levels in both adult and juvenile mice (Fig. 7*A*). We also examined levels of nuocyte induction in MLN of juvenile and adult mice following *N. brasiliensis* infection. Nuocyte cell numbers increased markedly as early as 4 days post-infection, showing a steady decline on days 6 and 10 with no differences between adult and juvenile mice (Fig. 7*B*). These data demonstrate 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. In order 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 non-activated basophils, as illustrated in Supplemental Fig. 1, and adoptively transferred them into recipient BALB/c juvenile mice every 48 hours during *N. brasiliensis*-infection. While adult animals had mostly expelled the parasites by day 7, juvenile mice had significantly more worms ($P<0.001$) than the older mice (Fig. 8*A*). Juvenile mice that received basophils isolated from adult mice had a worm burden comparable to adult mice, with significantly reduced numbers of worms when compared to juvenile mice that did not received cells ($P<0.01$), (Fig. 8*A*). In contrast, transfer of basophils from juvenile mice did not reduce the number of worms present in juvenile animals (Fig. 8*A*). We examined the blood of recipient mice and could not find any donor 4Get mouse IL-4-eGFP positive basophils, possibly due to the short life expectancy of such cells *in vivo* (28). However, non-donor basophil levels in the blood of juvenile mice that had received adult-derived basophils were elevated significantly ($P<0.05$) above control juvenile mice and were at similar levels as detected in adult mice (Fig. 8*B*).

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 naïve adult mice before and during *N. brasilienses* infection. Seven days after infection adult mice had expelled worms while 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 appears to be age-associated (16). Previously, using rhesus monkeys it was shown 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 immune response to helminths is not restricted to rhesus monkeys, but 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 due to an age-dependent defect in the genesis of type 2 immunity? In juvenile 21-28 day 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 have identified that juvenile mice, both BALB/c and C57BL/6J strains, have a defect in basophil induction following helminth challenge. We have 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. Though there is also a delay in eosinophil recruitment to lungs of helminth-infected juvenile mice, we demonstrate 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, while transfer of eosinophils from

adult mice to juvenile mice during infection restored the levels of blood eosinophilia to that detected in infected adult mice, this increase in eosinophils did not lead to expulsion of the worms. These data are consistent 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 contribute 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 for 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 antigen 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 generation of type 2 responses is illustrated by the expansion of eosinophils after *N. brasiliensis* infection being dependent on an initial basophil response (28). Indeed, in support of this, the defect in basophils generation in juvenile mice reported herein precedes the delayed expansion of eosinophils and also Th2 cells in younger animals.

Using helminth infection as a model we have identified an apparently previously unrecognized age-dependent deficiency in innate induction of basophils. Further definition of this phenotype was achieved using helminth excretory/secretory proteases, which again results in the induction of basophils in adult mice, but not juvenile animals. Our data is consistent with a mechanism involving helminth proteases evoking type 2 immune responses through activation of basophils (9), as inactivation of such helminth proteases ablated the observed basophil recruitment to PLN in adult mice. In separate experiments we confirmed

that adult and juvenile basophils expand equally in response to external IL-3 administration, both *in vivo* and *in vitro* (43) (data not shown). A number of recent publications have also identified new innate type 2 cells that play an important role in innate immunity (11, 44-46). Following helminth infection using novel IL-13-eGFP mice we found 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.

Currently the emerging paradigms on the mechanisms and functions of basophils are derived from studies on cells from adult experimental animals or humans (39, 41, 47, 48). Data presented herein outline a clear defect in innate induction of basophils in juvenile animals, thus suggesting the need to assess basophil levels in infants and children. Given that we focus on juvenile 21-28 day old mice, it is prudent to mention that it is known that neonate mice have an impaired development of Th function (49-51). For example, $CD4^+$ cells isolated from neonatal murine lymph nodes have a Th2-skewed primary immune response that induces a Th2-biased memory response towards secondary insults (52). In contrast, 3 week old juvenile mice were used herein in models of helminth infection, demonstrating decreased or delayed type 2 immune responses compared to adult animals.

It was recently identified that basophils play a role in enhancing humoral memory responses, through their ability to bind antigen on their surface (53). In these studies, depletion of basophils in adult mice led to impaired generation of allergen-specific antibody responses, with B cell function enhanced by activated basophils (53). Thus, it is interesting that in this study juvenile mice have a defective capacity, relative to adult mice, in the generation of antigen-specific IgE following helminth infection. The humoral defect in juvenile mice is therefore consistent with impaired basophil function.

A number of groups have recently demonstrated that basophils in adult mice can present antigen to Th2 cells under specific experimental conditions (54-56), a phenomenon with potential relevance to this study. However, recent publications highlight some discrepancies in the suggestion that basophils may function as APC; with demonstrations of a redundant role for basophils in Th2 induction (57-60) or the possibility for a co-operative role for DCs and basophils in Th2 induction under certain conditions (61). Once there is a consensus on the mechanisms that basophils utilize in generation of type 2 immunity, such as antigen 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 antigens indirectly though antigen-specific FcεR1-bound IgE could be another important mechanism of 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 (FcεR1) or IgG (FcγRII/III) receptors on basophils. It is also possible that juvenile mouse basophils are in some other way immature or defective in they ability to bind IgE or IgG antibodies accompanied by insufficient or an inability to induce intracellular signal. We have not as yet 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 co-existed with humans and human ancestors by evolving to establish and maintain chronic infections (2, 62). Indeed, it is widely postulated that the escalation in allergic disease in Westernized societies is linked to the absence of helminth immune modulation (3, 63). As 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 we have described here, could be seen as an evolutionary adaptation to down regulate potential hyper activation 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. Whereas 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 here suggest it is of importance to address the functions of basophils in children. Our results clearly show a distinct alteration in the innate basophil response between adult and juvenile mice, which opens new avenues for further investigation into any potential effect this may have in humans.

Acknowledgements

Poom Adisakwattana and Bina Mistry kindly assisted with experiments. We are grateful for Kenji Nakanishi and Richard Grencis for providing reagents. Fred Finkelman is thanked for reagents and comments on the manuscript. The National Children's Research Centre (Crumlin, Ireland) kindly provided essential equipment.

References

1. WHO. 2005. Deworming for health and development: Report of the third global meeting of the partners for parasite control. Geneva: World Health Organization.

2. Fumagalli, M., U. Pozzoli, R. Cagliani, G. P. Comi, S. Riva, M. Clerici, N. Bresolin, and M. Sironi. 2009. Parasites represent a major selective force for interleukin genes and shape the genetic predisposition to autoimmune conditions. *J. Exp. Med.* 206:1395-1408.

3. Fallon, P. G., and N. E. Mangan. 2007. Suppression of TH2-type allergic reactions by helminth infection. *Nat. Rev. Immunol.* 7:220-230.

4. Shinkai, K., M. Mohrs, and R. M. Locksley. 2002. Helper T cells regulate type-2 innate immunity *in vivo*. *Nature* 420:825-829.

5. Min, B., M. Prout, J. Hu-Li, J. Zhu, D. Jankovic, E. S. Morgan, J. F. Urban, Jr., A. M. Dvorak, F. D. Finkelman, G. LeGros, and W. E. Paul. 2004. Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J. Exp. Med.* 200:507-517.

6. Khodoun, M. V., T. Orekhova, C. Potter, S. Morris, and F. D. Finkelman. 2004. Basophils initiate IL-4 production during a memory T-dependent response. *J. Exp. Med.* 200:857-870.

7. Voehringer, D., K. Shinkai, and R. M. Locksley. 2004. Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production. *Immunity* 20:267-277.

8. Voehringer, D., T. A. Reese, X. Huang, K. Shinkai, and R. M. Locksley. 2006. Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. *J. Exp. Med.* 203:1435-1446.

9. Sokol, C. L., G. M. Barton, A. G. Farr, and R. Medzhitov. 2008. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat. Immunol.* 9:310-318.

10. van Panhuys, N., S. C. Tang, M. Prout, M. Camberis, D. Scarlett, J. Roberts, J. Hu-Li, W. E. Paul, and G. Le Gros. 2008. *In vivo* studies fail to reveal a role for IL-4 or STAT6 signaling in Th2 lymphocyte differentiation. *Proc. Natl. Acad. Sci. U S A* 105:12423-12428.

11. Neill, D. R., S. H. Wong, A. Bellosi, R. J. Flynn, M. Daly, T. K. Langford, C. Bucks, C. M. Kane, P. G. Fallon, R. Pannell, H. E. Jolin, and A. N. McKenzie. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464:1367-1370.

12. Moro, K., T. Yamada, M. Tanabe, T. Takeuchi, T. Ikawa, H. Kawamoto, J. Furusawa, M. Ohtani, H. Fujii, and S. Koyasu. 2010. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* 463:540-544.

13. Saenz, S. A., M. C. Siracusa, J. G. Perrigoue, S. P. Spencer, J. F. Urban, Jr., J. E. Tocker, A. L. Budelsky, M. A. Kleinschek, R. A. Kastelein, T. Kambayashi, A. Bhandoola, and D. Artis. 2010. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature* 464:1362-1366.

14. Price, A. E., H. E. Liang, B. M. Sullivan, R. L. Reinhardt, C. J. Eisley, D. J. Erle, and R. M. Locksley. 2010. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc. Natl. Acad. Sci. U S A.*

15. Paul, W. E., and J. Zhu. 2010. How are T(H)2-type immune responses initiated and amplified? *Nat. Rev. Immunol.* 10:225-235.

16. Hotez, P. J., D. A. Bundy, K. Beegle, S. Brooker, and L. Drake. 2006. Helminth infections: soil-transmitted helminth infections and schistosomiasis. In *Disease control priorities in developing countries*, 2nd Edition ed. J. G. B. Dean T. Jamison , Anthony R. Measham , George Alleyne , Mariam Claeson , David B. Evans , Prabhat Jha , Anne Mills , Philip Musgrove, eds. Oxford University Press. 467-482.

17. Hotez, P. J., S. Brooker, J. M. Bethony, M. E. Bottazzi, A. Loukas, and S. Xiao. 2004. Hookworm infection. *N. Engl. J. Med.* 351:799-807.

18. Hotez, P. J., S. Arora, J. Bethony, M. E. Bottazzi, A. Loukas, R. Correa-Oliveira, and S. Brooker. 2005. Helminth infections of children: Prospects for control. Hot Topics in Infection and Immunity in Children II. In *Advances in Experimental Medicine and Biology*, A. J. Pollard and A. Finn, eds. Springer, New York. 568:135-144.

19. Pereira, W. R., H. Kloos, S. B. Crawford, J. G. Velasquez-Melendez, L. F. Matoso, R. T. Fujiwara, G. G. Cancado, P. T. Loverde, R. Correa-Oliveira, and A. Gazzinelli. 2010. *Schistosoma mansoni* infection in a rural area of the Jequitinhonha Valley, Minas Gerais, Brazil: analysis of exposure risk. *Acta Trop.* 113:34-41.

20. Jardim-Botelho, A., S. Brooker, S. M. Geiger, F. Fleming, A. C. Souza Lopes, D. J. Diemert, R. Correa-Oliveira, and J. M. Bethony. 2008. Age patterns in undernutrition and helminth infection in a rural area of Brazil: associations with ascariasis and hookworm. *Trop. Med. Int. Health* 13:458-467.

21. Fallon, P. G., J. Gibbons, R. A. Vervenne, E. J. Richardson, A. J. C. Fulford, S. Kiarie, R. F. Sturrock, P. S. Coulson, A. M. Deelder, J. A. M. Langermans, A. W. Thomas, and D. W. Dunne. 2003. Juvenile rhesus monkeys have lower type 2 cytokine responses than adults after primary infection with *Schistosoma mansoni*. *J. Infect. Dis.* 187:939-945.

22. Bogers, J. J. P. M., S. Chatterjee, W. Jacobs, P. G. Fallon, D. W. Dunne, J. A. M. Langermans, A. M. Deelder, A. W. Thomas, and E. A. E. Van Marck. 2004. Juvenile

rhesus monkeys have more colonic granulomas than adults after primary infection with *Schistosoma mansoni*. *Virchows Archive* 445:285-291.

23. Wan, Y. Y., and R. A. Flavell. 2005. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc. Natl. Acad. Sci. U S A* 102:5126-5131.

24. Fallon, P. G., H. E. Jolin, P. Smith, C. L. Emson, M. J. Townsend, R. Fallon, P. Smith, and A. N. J. McKenzie. 2002. IL-4 Induces characteristic Th2 responses even in the combined absence of IL-5, IL-9, and IL-13. *Immunity* 17:7-17.

25. Yamada, M., M. Nakazawa, Y. Matsumoto, and N. Arizono. 1991. IgE antibody production in rats against multiple components of excretory-secretory products of the nematode *Nippostrongylus brasiliensis*. *Immunology* 72:104-108.

26. Mangan, N. E., A. Dasvarma, A. N. McKenzie, and P. G. Fallon. 2007. T1/ST2 expression on Th2 cells negatively regulates allergic pulmonary inflammation. *Eur. J. Immunol.* 37:1302-1312.

27. Fallon, P. G., S. J. Ballantyne, N. E. Mangan, J. L. Barlow, A. Dasvarma, D. R. Hewett, A. McIlgorm, H. E. Jolin, and A. N. McKenzie. 2006. Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J. Exp. Med.* 203:1105-1116.

28. Ohnmacht, C., and D. Voehringer. 2009. Basophil effector function and homeostasis during helminth infection. *Blood* 113:2816-2825.

29. Smith, P., R. E. Fallon, N. E. Mangan, C. M. Walsh, M. Saraiva, J. R. Sayers, A. N. McKenzie, A. Alcami, and P. G. Fallon. 2005. *Schistosoma mansoni* secretes a chemokine binding protein with antiinflammatory activity. *J. Exp. Med.* 202:1319-1325.

30. Okigami, H., K. Takeshita, M. Tajimi, H. Komura, M. Albers, T. E. Lehmann, T. Rolle, and K. B. Bacon. 2007. Inhibition of eosinophilia *in vivo* by a small molecule inhibitor of very late antigen (VLA)-4. *Eur. J. Pharmacol.* 559:202-209.

31. Fallon, P. G. 2000. Immunopathology of schistosomiasis: a cautionary tale of mice and men. *Immunol. Today* 21:29-35.

32. Pearce, E. J., and A. S. MacDonald. 2002. The immunobiology of schistosomiasis. *Nat. Rev. Immunol.* 2:499-511.

33. Anthony, R. M., L. I. Rutitzky, J. F. Urban, Jr., M. J. Stadecker, and W. C. Gause. 2007. Protective immune mechanisms in helminth infection. *Nat. Rev. Immunol.* 7:975-987.

34. Finkelman, F. D., T. Shea-Donohue, S. C. Morris, L. Gildea, R. Strait, K. B. Madden, L. Schopf, and J. F. Urban, Jr. 2004. Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunol. Rev.* 201:139-155.

35. Fowell, D. J., J. Magram, C. W. Turck, N. Killeen, and R. M. Locksley. 1997. Impaired Th2 subset development in the absence of CD4. *Immunity* 6:559-569.

36. Mohrs, M., K. Shinkai, K. Mohrs, and R. M. Locksley. 2001. Analysis of type 2 immunity *in vivo* with a bicistronic IL-4 reporter. *Immunity* 15:303-311.

37. McKerrow, J. H., C. Caffrey, B. Kelly, P. Loke, and M. Sajid. 2006. Proteases in parasitic diseases. *Annu. Rev. Pathol.* 1:497-536.

38. Min, B., and W. E. Paul. 2008. Basophils: in the spotlight at last. *Nat. Immunol.* 9:223-225.

39. Karasuyama, H., K. Mukai, Y. Tsujimura, and K. Obata. 2009. Newly discovered roles for basophils: a neglected minority gains new respect. *Nat. Rev. Immunol.* 9:9-13.

40. Wynn, T. A. 2009. Basophils trump dendritic cells as APCs for T(H)2 responses. *Nat. Immunol.* 10:679-681.

41. Sullivan, B. M., and R. M. Locksley. 2009. Basophils: a nonredundant contributor to host immunity. *Immunity* 30:12-20.

42. Falcone, F. H., G. Telford, D. Hooi, A. P. Brown, R. Seabra, J. Feary, A. Venn, J. Britton, and D. I. Pritchard. 2009. Antigen-driven basophil activation is indicative of early *Necator americanus* infection in IgE-seronegative patients. *J. Allergy. Clin. Immunol*. 124:1343-50.

43. Ohmori, K., Y. Luo, Y. Jia, J. Nishida, Z. Wang, K. D. Bunting, D. Wang, and H. Huang. 2009. IL-3 induces basophil expansion *in vivo* by directing granulocyte-monocyte progenitors to differentiate into basophil lineage-restricted progenitors in the bone marrow and by increasing the number of basophil/mast cell progenitors in the spleen. *J. Immunol.* 182:2835-2841.

44. Price, A. E., H. E. Liang, B. M. Sullivan, R. L. Reinhardt, C. J. Eisley, D. J. Erle, and R. M. Locksley. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc. Natl. Acad. Sci. U S A* 107:11489-11494.

45. Saenz, S. A., M. C. Siracusa, J. G. Perrigoue, S. P. Spencer, J. F. Urban, Jr., J. E. Tocker, A. L. Budelsky, M. A. Kleinschek, R. A. Kastelein, T. Kambayashi, A. Bhandoola, and D. Artis. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature* 464:1362-1366.

46. Moro, K., T. Yamada, M. Tanabe, T. Takeuchi, T. Ikawa, H. Kawamoto, J. Furusawa, M. Ohtani, H. Fujii, and S. Koyasu. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* 463:540-544.

47. Galli, S. J., and C. B. Franco. 2008. Basophils are back! *Immunity* 28:495-497.

48. Min, B. 2008. Basophils: what they 'can do' versus what they 'actually do'. *Nat. Immunol.* 9:1333-1339.

49. Siegrist, C. A. 2000. Vaccination in the neonatal period and early infancy. *Int. Rev. Immunol.* 19:195-219.

50. Garcia, A. M., S. A. Fadel, S. Cao, and M. Sarzotti. 2000. T cell immunity in neonates. *Immunol Res* 22:177-190.

51. Adkins, B. 2000. Development of neonatal Th1/Th2 function. *Int. Rev. Immunol.* 19:157-171.

52. Adkins, B., Y. Bu, V. Vincek, and P. Guevara. 2003. The primary responses of murine neonatal lymph node CD4+ cells are Th2-skewed and are sufficient for the development of Th2-biased memory. *Clin. Dev. Immunol.* 10:43-51.

53. Denzel, A., U. A. Maus, M. Rodriguez Gomez, C. Moll, M. Niedermeier, C. Winter, R. Maus, S. Hollingshead, D. E. Briles, L. A. Kunz-Schughart, Y. Talke, and M. Mack. 2008. Basophils enhance immunological memory responses. *Nat. Immunol.* 9:733-742.

54. Perrigoue, J. G., S. A. Saenz, M. C. Siracusa, E. J. Allenspach, B. C. Taylor, P. R. Giacomin, M. G. Nair, Y. Du, C. Zaph, N. van Rooijen, M. R. Comeau, E. J. Pearce, T. M. Laufer, and D. Artis. 2009. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat. Immunol.* 10:697-705.

55. Sokol, C. L., N. Q. Chu, S. Yu, S. A. Nish, T. M. Laufer, and R. Medzhitov. 2009. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat. Immunol.* 10:713-720.

56. Yoshimoto, T., K. Yasuda, H. Tanaka, M. Nakahira, Y. Imai, Y. Fujimori, and K. Nakanishi. 2009. Basophils contribute to T(H)2-IgE responses *in vivo* via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat. Immunol.* 10:706-712.

57. Kim, S., M. Prout, H. Ramshaw, A. F. Lopez, G. LeGros, and B. Min. 2010. Cutting edge: basophils are transiently recruited into the draining lymph nodes during helminth infection via IL-3, but infection-induced Th2 immunity can develop without basophil lymph node recruitment or IL-3. *J. Immunol.* 184:1143-1147.

58. Ohnmacht, C., C. Schwartz, M. Panzer, I. Schiedewitz, R. Naumann, and D. Voehringer. 2010. Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. *Immunity* 33:364-374.

59. Phythian-Adams, A. T., P. C. Cook, R. J. Lundie, L. H. Jones, K. A. Smith, T. A. Barr, K. Hochweller, S. M. Anderton, G. J. Hammerling, R. M. Maizels, and A. S. MacDonald. 2010. CD11c depletion severely disrupts Th2 induction and development *in vivo*. *J. Exp. Med.* 207:2089-2096.

60. Hammad, H., M. Plantinga, K. Deswarte, P. Pouliot, M. A. Willart, M. Kool, F. Muskens, and B. N. Lambrecht. 2010. Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J. Exp. Med.* 207:2097-2111.

61. Tang, H., W. Cao, S. P. Kasturi, R. Ravindran, H. I. Nakaya, K. Kundu, N. Murthy, T. B. Kepler, B. Malissen, and B. Pulendran. 2010. The T helper type 2 response to cysteine proteases requires dendritic cell-basophil cooperation via ROS-mediated signaling. *Nat. Immunol.* 11:608-617.

62. Dunne, D. W., and A. Cooke. 2005. A worm's eye view of the immune system: consequences for evolution of human autoimmune disease. *Nat. Rev. Immunol.* 5:420-426.

63. Yazdanbakhsh, M., P. G. Kremsner, and R. van Ree. 2002. Allergy, parasites, and the hygiene hypothesis. *Science* 296:490-494.

Footnotes

1. This work was supported by Science Foundation Ireland (07/IN.1/B902) and Health Research Board (HRA/2009/154).

2. Address correspondence to Padraic G. Fallon, Institute of Molecular Medicine, Trinity College Dublin, Dublin 8, Ireland. Email address: pfallon@tcd.ie

3. Abbreviations used in this paper: CPM = counts per minute; DC = dendritic cell; eGFP = enhanced green fluorescent protein; EPG = eggs per gram feces; mMCP-1 = mouse mast cell proteases 1; MLN = mesenteric lymph node; mRFP = monomeric red fluorescent protein; MyD88 = myeloid differentiation primary response gene 88; NbES = *Nippostrongylus brasiliensis* excretory-secretory; NBNT = Non-B non-T cell; ND = not detected; NS = Not significant; OVA = ovalbumin; PLN = popliteal lymph node; SSC = side scatter; TIR = toll-interleukin 1 receptor; TIRAP = TIR-domain containing adaptor protein; TLR= toll like receptor; Treg = regulatory T cell; TRIF = TIR-domain-containing adapter-inducing interferon-β.

Figure Legends

Figure 1. Juvenile mice are more susceptible to *N. brasiliensis* infection than adults with delayed type 2 immunity. (A and B) Intestinal worm and egg per gram feces (EPG) counts during primary *N. brasiliensis* infection of juvenile (21-23 day old) and adult (6-8 weeks old) BALB/c mice. (C and D) Intestinal worm and egg counts 5 days after a secondary *N. brasiliensis* re-infection. Re-infections were administered 20 days 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 per group (Two-tailed Student's *t*-test: * $P<0.05$, ** $P< 0.001$, ND = Not detected).

Figure 2. Juvenile mice have a delayed type 2 cytokine immune response to *N. brasiliensis* infection compared to adults. (A) MLN cells removed at indicated days from juvenile and adult *N. brasiliensis* infected mice were re-stimulated with anti-CD3 mAb and supernatants analyzed by ELISA for the type 2 cytokines IL-4, IL-5 and IL-13 (mean and SD). (B) Serum levels of mMCP-1 (mean and SEM), (C) total IgE (mean and SD) and (D) intestinal goblet cell numbers per villous crypt units (VCU) (mean and SEM) were measured on the indicated time points. Data are from at least three separate experiments with 7-14 mice per group (Two-tailed Student's *t*-test: * $P<0.05$, ** $P<0.001$).

Figure 3. Intact Th cell differentiation of $CD4^+$ cells from juvenile mice *in vitro*. (A) Production of IL-4, IL-5, IL-13 in supernatant of splenic $CD4^+$ cells from juvenile and adult mice cultured *in vitro* for 4 days under Th2 cell differentiation conditions. Cells were washed and restimulated with PMA/Ionomycin and supernatants analyzed by ELISA (B) IFN-γ and

(C) IL-17 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 days after infection with *N. brasiliensis*. RAG-$1^{-/-}$ mice were untreated (-) or received $CD4^{+}$ splenocytes isolated from juvenile or adult mice. Data are from at least two separate experiments (Two-tailed Student's t-test: * $P>0.05$, ** $P<0.001$, NS = Not significant).

Figure 4. Delayed clearance of *N. brasiliensis* in infected juvenile with TLR and TLR signaling mechanism defects compared to corresponding adult mice. Worm recovery of juvenile and adult mice defective in (A) $TLR2^{-/-}$ and $TLR4^{-/-}$ and (B) $MyD88^{-/-}$, $TIRAP^{-/-}$ and $TRIF^{-/-}$, 8 days after *N. brasiliensis* infection. Data are presented as mean and SEM from 5-8 mice per group (Two-tailed Student's t-test: ND = not detected).

Figure 5. Impaired basophil responses in juvenile mice after helminth infection and injection with helminth-derived antigens. (A) Total numbers of $CD4^{+}IL\text{-}4^{+}$ (Th2) and $CD4^{-}IL\text{-}4^{+}$ cells in MLN of adult 4Get mice during *N. brasiliensis* infection. (B) Total numbers of $CD4^{+}IL\text{-}4^{+}$ (Th2) and $CD4^{-}IL\text{-}4^{+}$ cells in MLN of juvenile 4Get mice during *N. brasiliensis* infection. (C) Flow cytometry detection of the total numbers of basophils (SSC^{low}, $IL\text{-}4^{+}$, non-B, non-T cell, $CCR3^{-}$, $c\text{-}kit^{-}$, $CD49b^{+}$, $Fc\varepsilon R1^{+}$) in lungs of juvenile and adult 4Get mice during *N. brasiliensis* infection. (D) Numbers of basophils in popliteal lymph nodes of adult and juvenile 4Get mice 3 days after footpad injection with *N. brasiliensis* excretory/secretory (NbES) antigens or inactivated NbES (E-64). Control animals were injected with PBS. Data

are mean and SD (A, B) or SEM (C-D) from at least three separate experiments with 5 mice per group (Two-tailed Student's *t*-test: * $P<0.05$, ** $P<0.001$).

Figure 6. Juvenile mice have delayed eosinophil expansion following infection but normal eosinophil recruitment in an air pouch model. (A) Flow cytometric detection of the total numbers of eosinophils (SSC^{hi}, IL-4^{+}, non-B, non-T cell, ckit^{-}, $CCR3^{+}$, $SiglecF^{+}$) in lungs of juvenile and adult 4Get mice during *N. brasiliensis* infection. (B) Regime of air pouch model to test eosinophil induction and recruitment. (C) Flow cytometric detection of the percentage and total number of eosinophils recruited into the air pouch of IL-5/eotaxin-1-treated adult and juvenile 4Get mice. There were no difference in baseline numbers of eosinophils in air pouch of PBS-injected adult or juvenile mice and data are presented as expansion over PBS-treated mice. Data are mean and SEM from two separate experiments with 3-4 mice per group (Two-tailed Student's *t*-test: * $P<0.05$, ** $P<0.001$).

Figure 7. Functional nuocyte responses in juvenile mice. (A) Flow cytometry detection of the nuocyte population (IL-13-$eGFP^{+}$, Lin^{-}, c-kit^{+}, FcεR1^{-}) in the MLN of adult and juvenile IL-13-eGFP mice in response to treatment with IL-25 (0.4 μg, days 1-3) and PBS controls. (B) Flow cytometry detection of total numbers of nuocytes in MLN of adult and juvenile IL-13-eGFP mice during *N. brasiliensis* infection. Data are mean and SEM from two or three separate experiments with 4 - 5 mice per group (Two-tailed Student's *t*-test: * $P<0.05$, ** $P<0.001$).

Figure 8. Reconstitution of juvenile mice with adult mouse-derived basophils restores resistance to helminth infection. (A) Day 7 intestinal worm counts and (B) percentages blood

basophil from *N. brasiliensis*-infected adult and juvenile mice after adoptive transfer of isolated basophils from naïve adult or juvenile mice on days 0, 2 and 4 post-infection. Data are presented as mean and SEM from 5-7 animals per group (Two-tailed Student's *t*-test: * $P<0.05$, ** $P<0.001$).

Fig 1

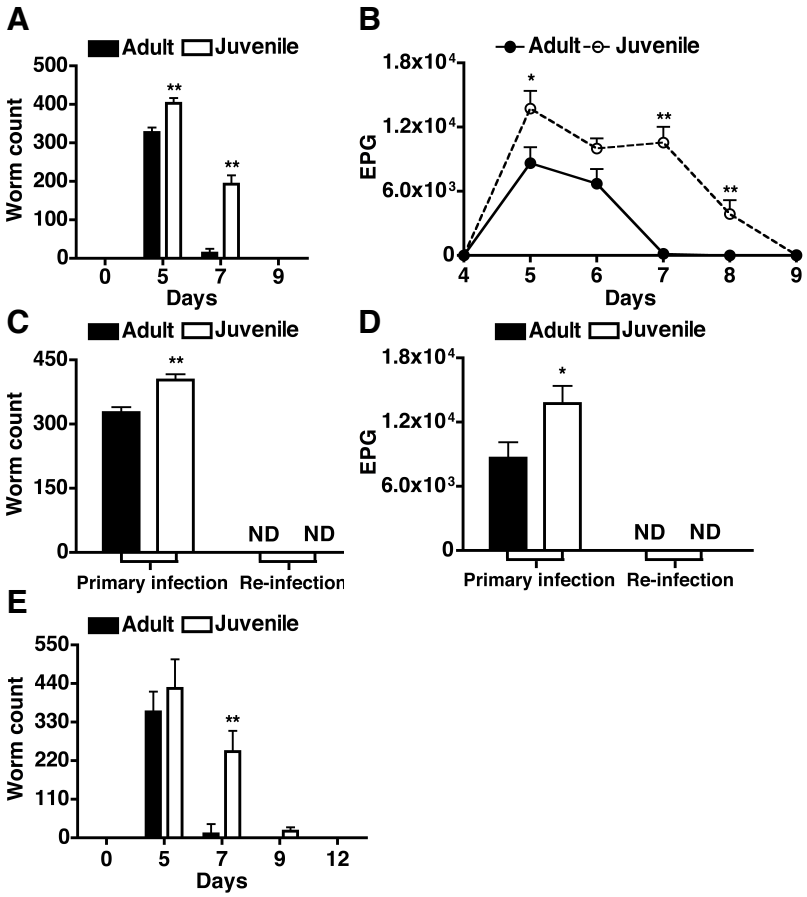

A
Adult
Juvenile
Worm count
500
400
300
200
100
0
**
**
0
5
7
9
Days
B
Adult
Juvenile
EPG
1.8x10⁴
1.2x10⁴
6.0x10³
0
*
**
**
4
5
6
7
8
9
Days
C
Adult
Juvenile
Worm count
450
300
150
0
**
ND
ND
Primary infection
Re-infection
D
Adult
Juvenile
EPG
1.8x10⁴
1.2x10⁴
6.0x10³
0
*
ND
ND
Primary infection
Re-infection
E
Adult
Juvenile
Worm count
550
440
330
220
110
0
**
0
5
7
9
12
Days

Fig 2

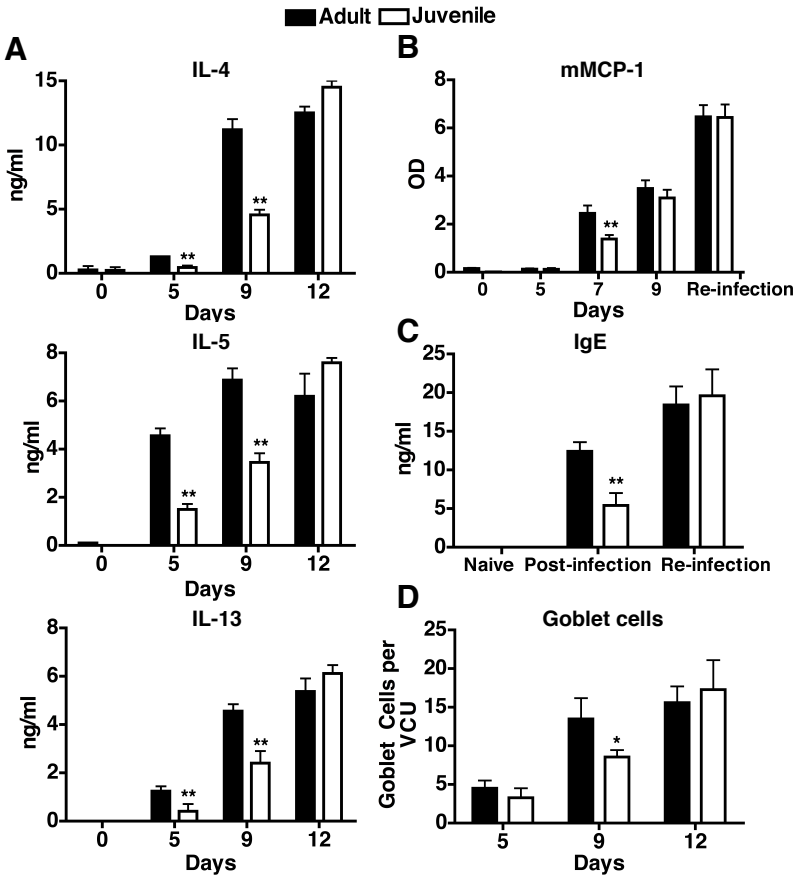

Adult
Juvenile
A
IL-4
ng/ml
Days
B
mMCP-1
OD
Re-infection
IL-5
C
IgE
Naive
Post-infection
Re-infection
IL-13
D
Goblet cells
Goblet Cells per VCU

Fig 3

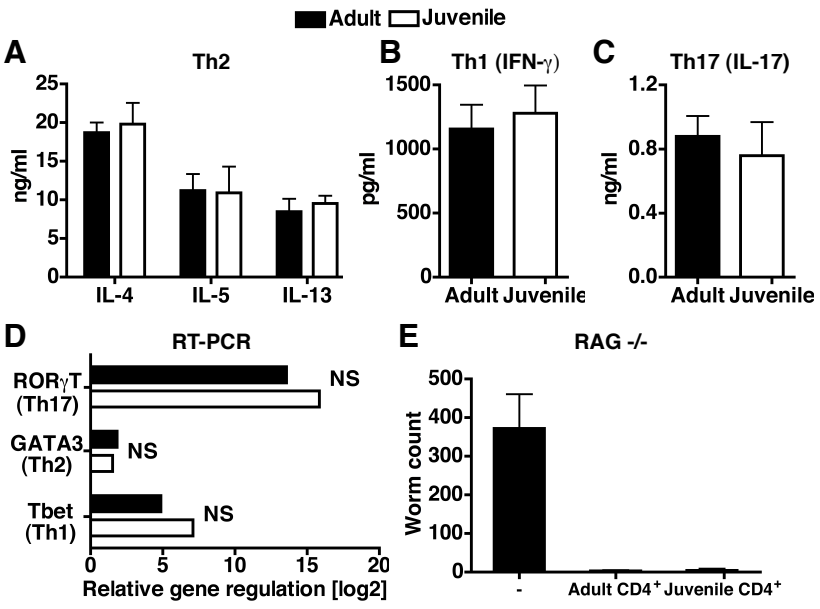

Adult
Juvenile
A
Th2
ng/ml
25
20
15
10
5
0
IL-4
IL-5
IL-13
B
Th1 (IFN-γ)
pg/ml
1500
1000
500
0
Adult
Juvenile
C
Th17 (IL-17)
ng/ml
1.2
0.8
0.4
0.0
Adult
Juvenile
D
RT-PCR
RORγT
(Th17)
NS
GATA3
(Th2)
NS
Tbet
(Th1)
NS
0
5
10
15
20
Relative gene regulation [log2]
E
RAG -/-
Worm count
500
400
300
200
100
0
-
Adult CD4+
Juvenile CD4+

Fig 4

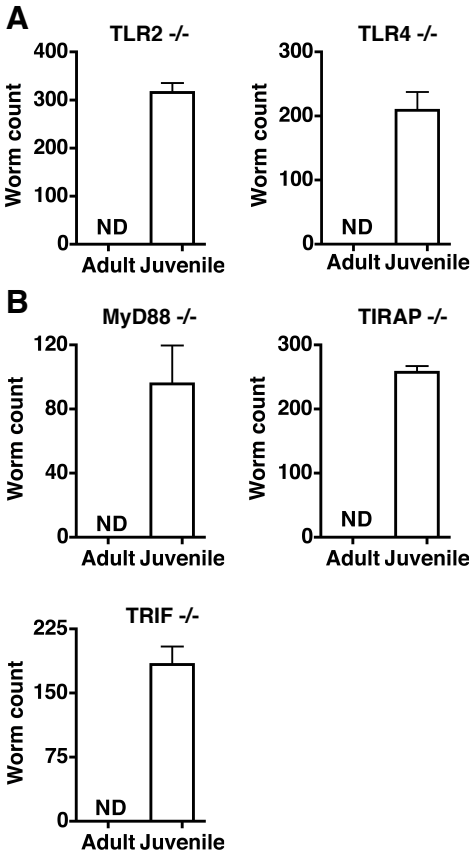

A
TLR2 -/-
400
300
200
100
0
Worm count
ND
Adult Juvenile
TLR4 -/-
300
200
100
0
Worm count
ND
Adult Juvenile
B
MyD88 -/-
120
80
40
0
Worm count
ND
Adult Juvenile
TIRAP -/-
300
200
100
0
Worm count
ND
Adult Juvenile
TRIF -/-
225
150
75
0
Worm count
ND
Adult Juvenile

Fig 5

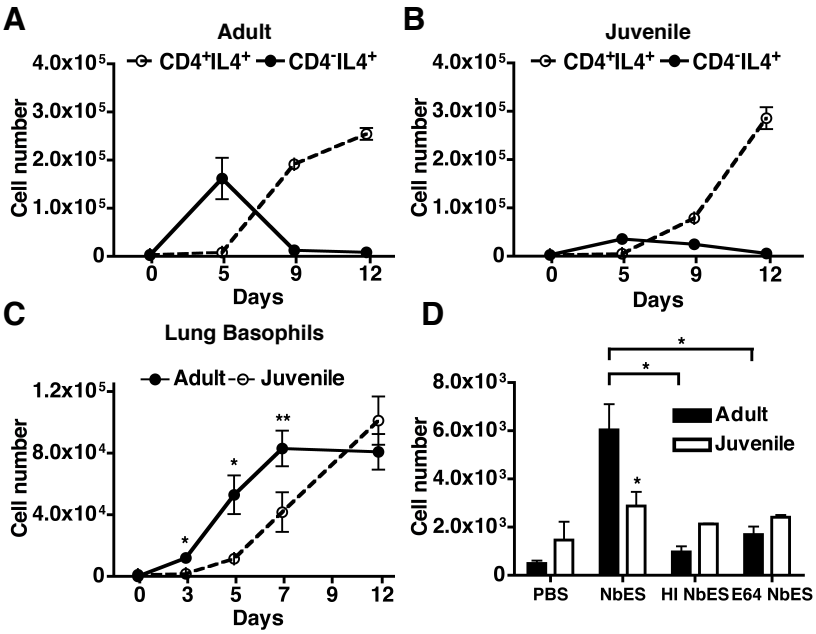

A
Adult
CD4+IL4+
CD4-IL4+
Cell number
Days
B
Juvenile
C
Lung Basophils
Adult
Juvenile
D
Adult
Juvenile
PBS
NbES
HI NbES
E64 NbES

Fig 6

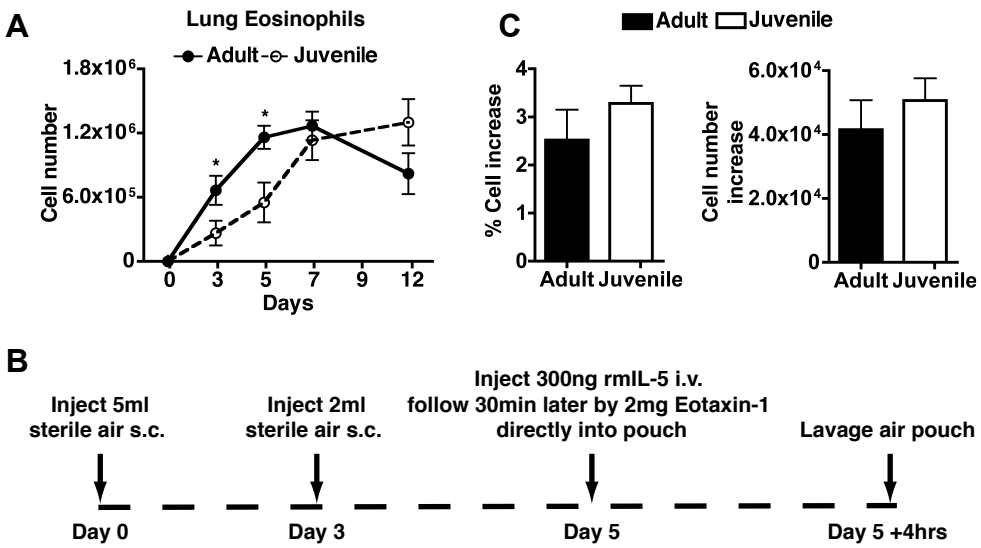

A
Lung Eosinophils
Adult
Juvenile
1.8x10⁶
1.2x10⁶
6.0x10⁵
0
Cell number
0
3
5
7
9
12
Days
*
*
C
Adult
Juvenile
4
3
2
1
0
% Cell increase
Adult
Juvenile
6.0x10⁴
4.0x10⁴
2.0x10⁴
0
Cell number increase
Adult
Juvenile
B
Inject 5ml sterile air s.c.
Inject 2ml sterile air s.c.
Inject 300ng rmIL-5 i.v. follow 30min later by 2mg Eotaxin-1 directly into pouch
Lavage air pouch
Day 0
Day 3
Day 5
Day 5 +4hrs

Fig 7

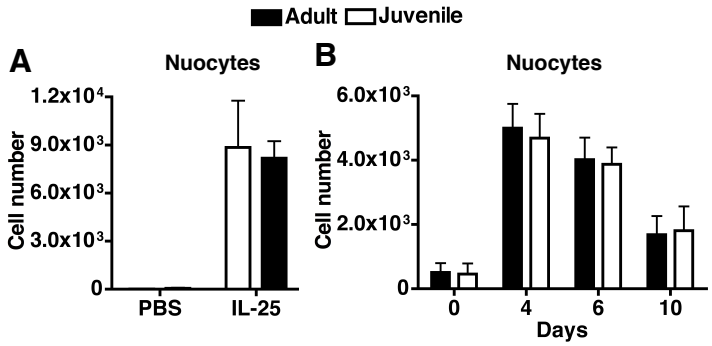
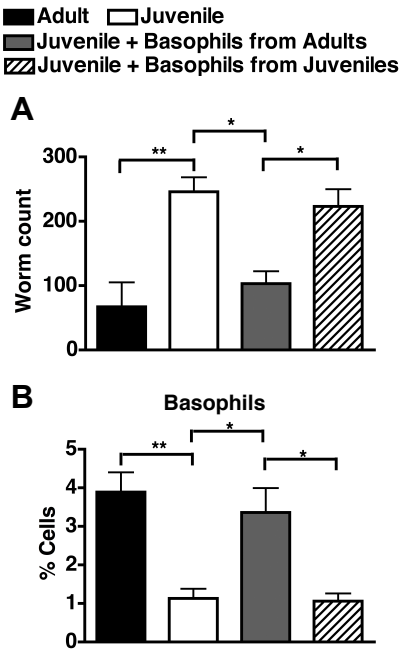
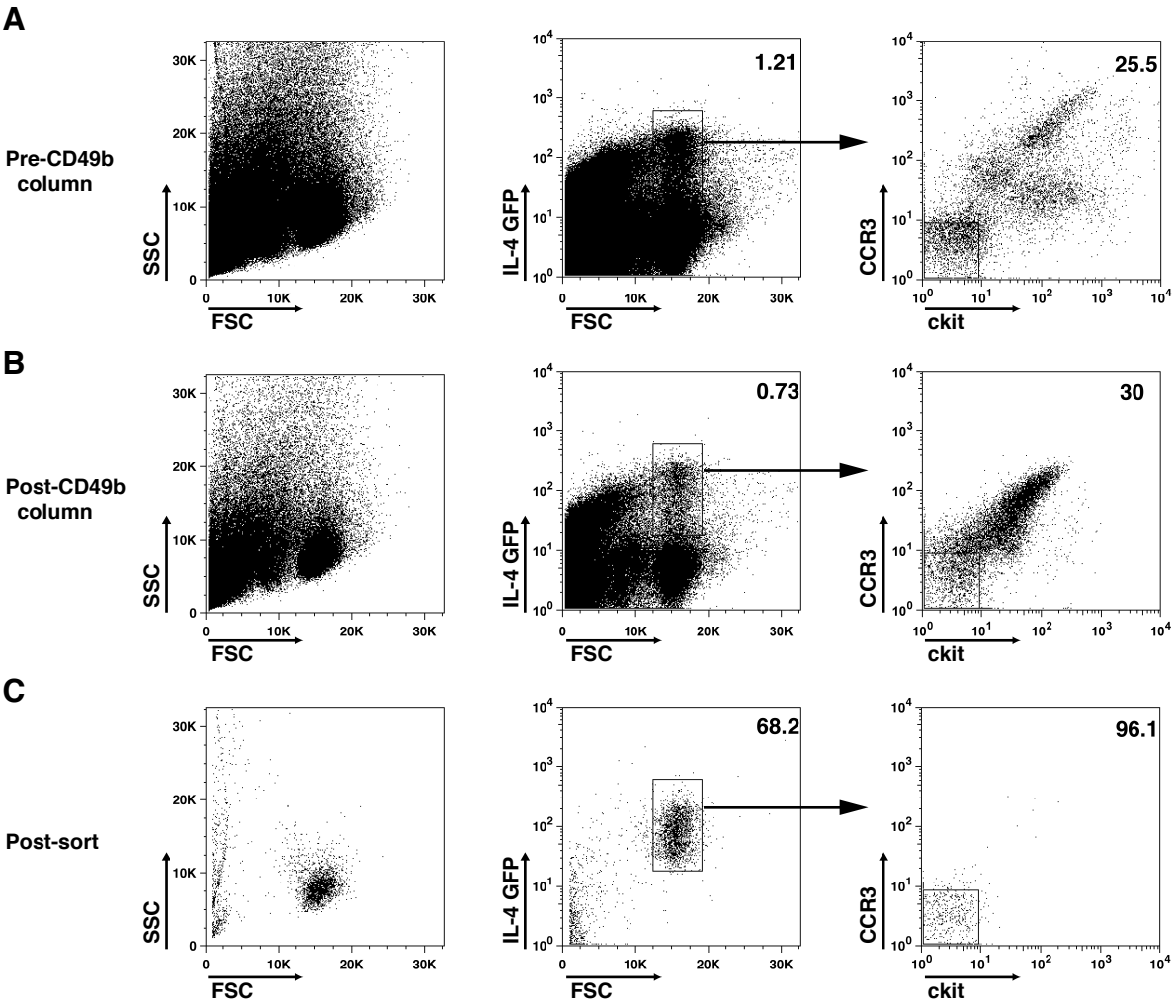

Adult
Juvenile
A
Nuocytes
1.2x10⁴
9.0x10³
6.0x10³
3.0x10³
0
Cell number
PBS
IL-25
B
Nuocytes
6.0x10³
4.0x10³
2.0x10³
0
Cell number
0
4
6
10
Days

Fig 8

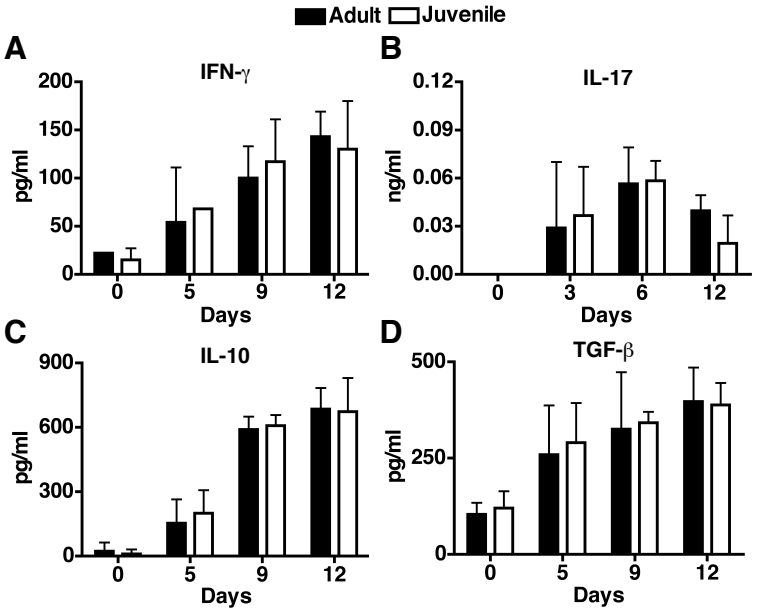

Adult
Juvenile
Juvenile + Basophils from Adults
Juvenile + Basophils from Juveniles
A
Worm count
300
200
100
0
**
*
*
B
Basophils
% Cells
5
4
3
2
1
0
**
*
*

Supplemental Fig 1



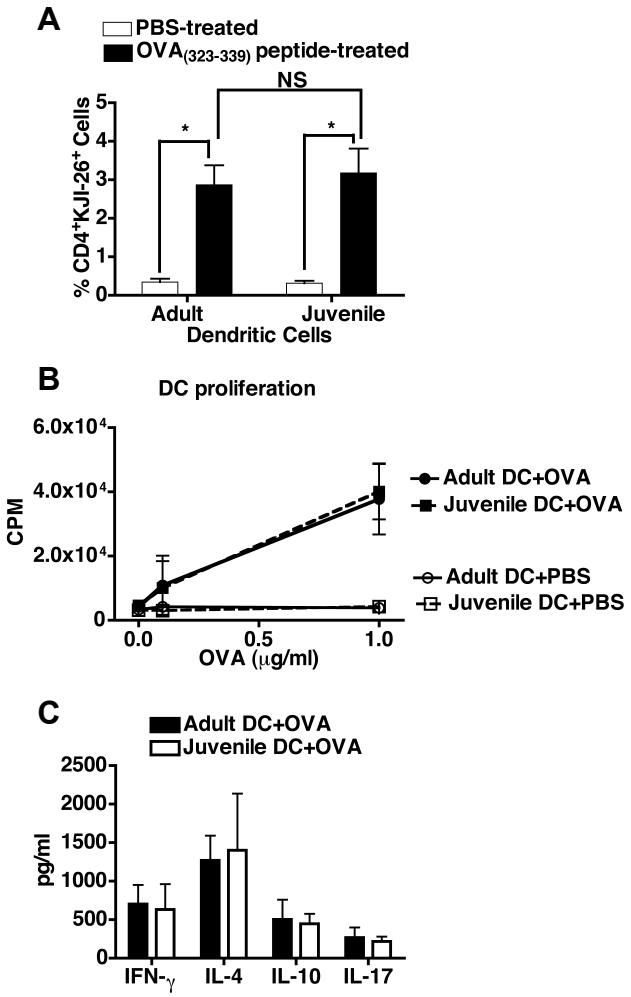
Supplemental Fig 1: Basophil sorting. (A) Representative flow cytometry images of basophis before,(B) after column enrichment and (C) post sorting. Spleen, blood and bone marrow from naïve adult 4Get mice were positively selected for CD49b+ cells by magnetic cell sorting using CD49b MicroBeads. The CD49b+ cells were incubated with anti-CCR3 and anti-ckit antibodies and used in flow cytometric analysis. Viable cells from B were run on a MoFlow flow cytometric sorter and cells sorted as being positive for IL-4-*e*GFP as well as negative for CCR3 and ckit. Sorted basophils (IL-4-*e*GFP+ CCR3- ckit-) are illustrated (C). Sorted cells were confirmed to have the basophil marker profile described in Supplemental fig 4.

Supplemental Fig 2



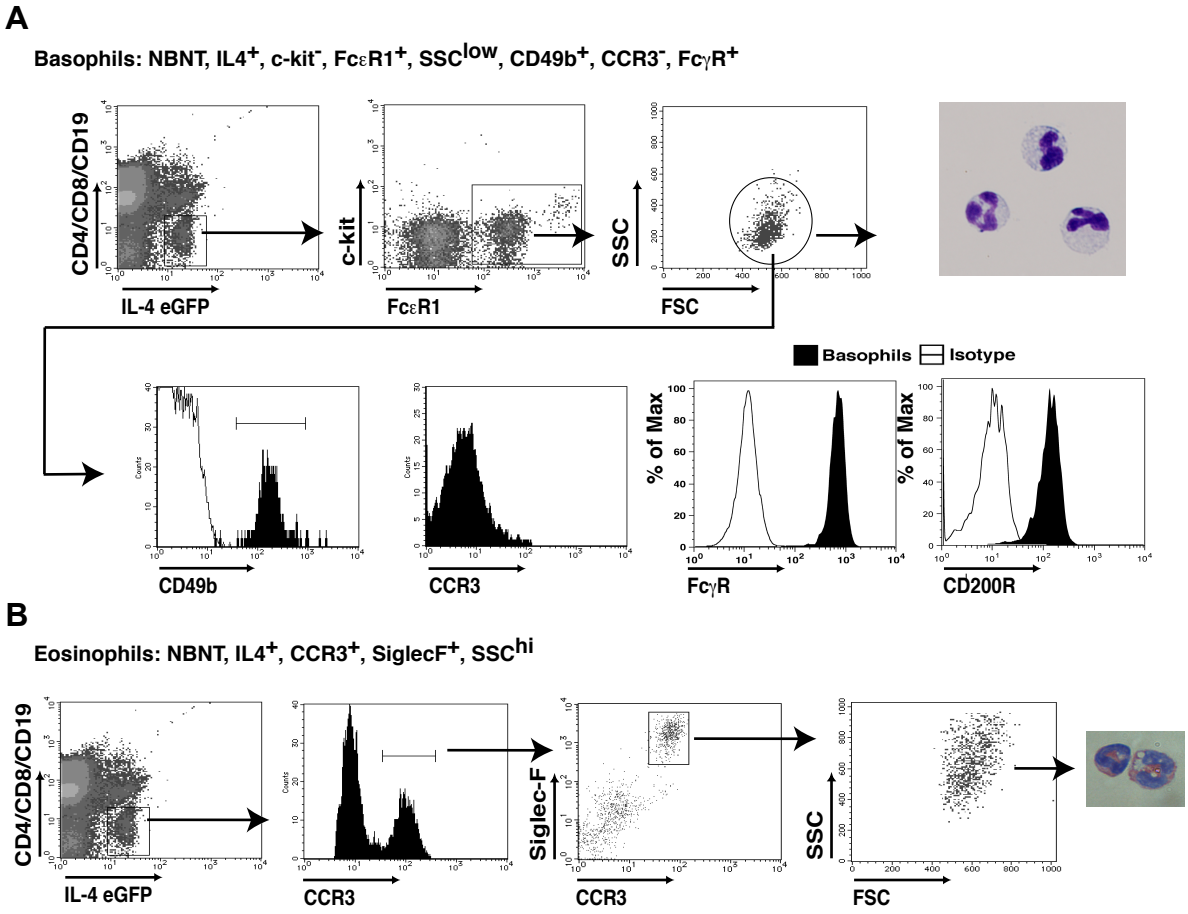
Supplemental Fig 2: Juvenile mice have no defect in type 1, 17 or regulatory cytokine immune response to *N. brasiliensis* infection. MLN cells removed at indicated days from juvenile and adult N. brasiliensis infected mice were re-stimulated with anti-CD3 mAb and supernatants analyzed by ELISA for the cytokines IFN-γ (A), IL-17 (B), IL-10 (C) and TGF-β (D). Data are from at least three separate experiments and are presented as mean and SD with 7-14 mice per group (Two-tailed Student's t-test: * P<0.05, ** P< 0.001, NS = Not significant).

Supplemental Fig 3



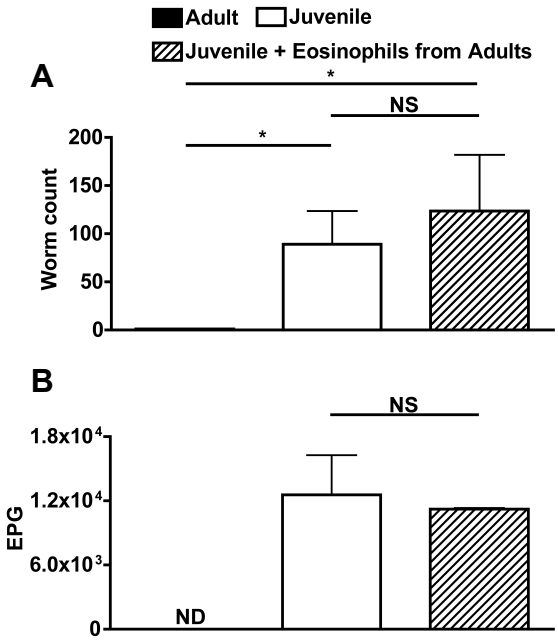
Supplemental Fig 3: Dendritic cells (DC) from juvenile mice are functional. (A) Percentage of OVA-specific CD4+KJI-26+ cells in popliteal lymph node of mice injected with PBS- or $OVA_{323-339}$-primed DCs from juvenile or adult mice (mean and SEM indicated). (B) OVA-specific cell proliferation of spleen cells from mice injected with DCs prepared from juvenile and adult mice that had been stimulated with OVA or PBS *in vitro*. (C) Production of IFN-γ, IL-4, IL-10 and IL-17 by spleen cells from mice injected with DCs prepared from juvenile and adult mice and pre-cultured in vitro with OVA. Data are mean and SD from 3 mice per group (Two-tailed Student's t test: * $P<0.05$, NS = Not significant)

Supplemental Fig 4



Supplemental Fig 4: Basophil and Eosinophil flow cytometry profile. (A) Representative flow cytometry images of basophils in 4Get mice as: non-B and non-T (NBNT; CD4-, CD8-, CD19-), IL-4+, c-kit-, FcεR1+, CD49b+, CCR3-, FcγR+ and SSClow cells. (B) Representative flow cytometry images of eosinophils in 4Get mice as: non-B and non-T (NBNT; CD4-, CD8-, CD19-), IL-4+, CCR3+, Siglec-F+ and SSChi cells. Also illustrated is Wright-Giemsa-stained cytospin images of basophils and eosinophils sorted from peripheral blood on a MoFlow flow cytometer utilizing the above described marker profiles.

Supplemental Fig 5



Supplemental Fig 5: Reconstitution of juvenile mice with adult mouse-derived eosinophils does not restore resistance to N-brasiliensis infection. (A) Day 7 intestinal worm counts and (B) eggs per gram feces (EPG) from helminth-infected adult and juvenile mice and juvenile mie after adoptive transfer of isolated eosinophils from naïve adult mice. Data are from 2 separate experiments with 4-5 animals per group and are presented as mean and SEM (Two-tailed Student's t-test: * P<0.05, NS = Not significant, ND = ND detected).