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This information is current as of September 3, 2014.

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J Immunol published online 29 August 2014

<http://www.jimmunol.org/content/early/2014/08/28/jimmunol.1401155>

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T Cell–Derived IL-4/IL-13 Protects Mice against Fatal *Schistosoma mansoni* Infection Independently of Basophils

Christian Schwartz,^{*,1} Katharina Oeser,^{*,1} Clarissa Prazeres da Costa,[†] Laura E. Layland,[‡] and David Voehringer^{*}

Schistosomiasis is a severe and chronic disease caused by the parasitic trematode *Schistosoma mansoni* after deposition of eggs in the liver and intestines. The immune response to *S. mansoni* eggs is characterized by increased Th2 cells, eosinophilia, and high serum IgE levels. Granulomas are formed around the eggs to protect the organs against tissue damage caused by toxic products that are secreted from the eggs. Egg-derived components have further been shown to activate the IgE-mediated release of IL-4 and IL-13 from basophils, suggesting that basophils could be involved in protection against a fatal course of infection. Using T cell–specific IL-4/IL-13–deficient mice and basophil-deficient Mcpt8Cre mice, we determined the contribution of Th2 cells and basophils for protective immunity against *S. mansoni* egg–induced pathology during the patent stage of infection. Our results demonstrate that T cell–derived IL-4/IL-13 was essential for granuloma formation, IgE production, basophilia, differentiation of alternatively activated macrophages, and protection against fatal infection. Although basophils were recruited into liver granulomas, they appeared to be dispensable as a source of IL-4/IL-13 both for differentiation of Th2 cells and for prevention of weight loss and mortality. *The Journal of Immunology*, 2014, 193: 000–000.

Schistosomiasis is a tropical disease caused by parasitic trematodes with >200 million people infected worldwide (1). Waterborne *Schistosoma mansoni* cercariae infect the mammalian host via the skin and migrate first to the lung and then to the hepatic portal vein, where the adult worms mate. Worms then migrate to the distal mesenteric veins and produce eggs. These eggs eventually transverse the intestinal epithelium into the gut lumen and leave the host's body. Eggs are also trapped in the intestinal wall and liver where they can cause severe pathology characterized by tissue damage and fibrosis. The immune response to *S. mansoni* is studied extensively in both humans and mice (2–4). During the prepatent phase of infection, a moderate Th1-biased immune response is established. With the onset of egg production by the adult worms ~6–8 wk postinfection a switch to a Th2-biased immune response occurs (5). This type 2 immune response is essential for the prevention of an early lethal course of infection, although it can cause tissue fibrosis during the later stages of infection (6).

S. mansoni eggs produce numerous tissue-destructive enzymes and toxic compounds (7–9). To prevent excessive tissue damage, the eggs are shielded off by formation of granulomas that are mainly composed of eosinophils, macrophages, and T cells, among which, CD4⁺ T cells were shown to play an important role for granuloma formation (10–13). In addition, granuloma formation and survival was impaired in IL-4–deficient mice (14) and in IL-4/IL-13–deficient mice (15). Furthermore, IL-4/IL-10–deficient mice have been shown to be highly susceptible to fatal *S. mansoni* infection, indicating that IL-10 plays an important protective function (16). At present, it is not known whether Th2 cells are the critical source of IL-4/IL-13 because both cytokines can also be produced by various innate cell types, including eosinophils, basophils, mast cells, and type 2 innate lymphoid cells (ILC2). Some of these innate IL-4/IL-13–expressing cells can have important functions during protective immune responses against helminths. For example, it has been shown that ILC2s are a critical source of IL-13 for worm expulsion during primary infection of mice with *Nippostrongylus brasiliensis* (17). Our current findings also indicate that IL-4/IL-13 release from basophils mediates protection against secondary infections with *N. brasiliensis* and *Heligmosomoides polygyrus* (C. Schwartz, A. Turqueti-Neves, S. Hartmann, P. Yu, F. Nimmerjahn, and D. Voehringer, submitted for publication). With regard to schistosome infections, mice have increased mortality if macrophages are rendered unresponsive to IL-4/IL-13. These data indicated that IL-4/IL-13–induced differentiation of so-called alternatively activated macrophages (AAMs) was critical for the prevention of lethal infection (18). Interestingly, granuloma formation and protection against fatal infection are not directly linked because IL-4R α on nonhematopoietic cells was demonstrated to be critical for granuloma formation but not for survival (19).

In addition to the induction of AAM differentiation, IL-4/IL-13 are important for class switch recombination to IgE and IgG1 in B cells (20, 21). Schistosomiasis is accompanied by increased serum IgG1 and IgE levels and resistance to infection has been correlated to high IgE levels directed against adult worm Ags (22). Moreover, passive immunization of CD4⁺ T cell–deprived mice with antiserum was

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Received for publication May 5, 2014. Accepted for publication July 31, 2014.

This work was supported in part by Starting Grant PAS_241506 from the European Research Council (to D.V.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AAM, alternatively activated macrophage; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ILC2, type 2 innate lymphoid cell; IPSE/ α 1, IL-4–inducing principle of schistosoma eggs/ α -1; mLN, mesenteric lymph node; NOS-2, NO synthase-2; PBGD, porphobilinogen deaminase; T_{reg}, regulatory T; WT, wild-type.

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shown to neutralize egg toxins and to ameliorate hepatic cytotoxicity (23–25). Furthermore, mice lacking a functional B cell response develop an excessive chronic phase of disease (26–28). IgE-deficient mice are highly susceptible to *S. mansoni* infection and develop smaller granulomas compared with wild-type (WT) mice (29). Basophils and mast cells are the main effector cells that are activated by Ag-mediated crosslinking of IgE bound to FcεRI, the high-affinity receptor for IgE. We and others have shown that basophils are involved in protective immunity against other helminths and ticks, suggesting that basophils may also contribute to immunity against *S. mansoni* (30–33). Basophils have been shown to get recruited to the liver and produce IL-4 upon infection with *N. brasiliensis* (34). Interestingly, the secretory egg Ag IL-4-inducing principle of schistosoma eggs/α-1 (IPSE/α1) can trigger the release of IL-4 from basophils by crosslinking the FcεRI-bound IgE, independently of the Ab's specificity (35). In addition, previous studies have demonstrated that basophils can act as APCs in a schistosome egg Ag immunization model (30) but Ab-mediated depletion of basophils was dispensable for Th2 polarization (36, 37). To elucidate the relative role of Th2 cells and basophils during *S. mansoni* infection, we compared the immune response using three mouse strains lacking distinct cellular sources of IL-4/IL-13: complete IL-4/IL-13-deficient mice, T cell-specific IL-4/IL-13-deficient mice, and basophil-deficient Mcpt8Cre mice. We demonstrate that T cell-derived IL-4/IL-13 is essential for IgE and IgG1 production, granuloma formation, basophilia, generation of AAMs, and prevention of an early mortality during the patent phase of infection. These effects were not dependent on IgE-mediated activation of basophils, as genetically basophil-depleted mice showed normal Th2 polarization, AAM differentiation, granuloma formation, and survival.

Materials and Methods

Mice

BALB/c and C57BL/6 mice were obtained from Charles River Laboratories (Sulzfeld, Germany). IL-4/IL-13-deficient mice (4-13ko) (38) were kindly provided by A. N. McKenzie (MRC Laboratory of Molecular Biology, Cambridge, U.K.). Mice with a loxP-flanked IL-4/IL-13 allele (39) were crossed to CD4Cre mice (40) to generate mice with selective deletion of both cytokines in T cells (4-13Tko mice) and backcrossed for >10 generations onto the BALB/c background. IL-4/eGFP reporter mice (4get mice) (41) were kindly provided by R. M. Locksley (Howard Hughes Medical Institute, University of California, San Francisco). Mcpt8Cre BAC-transgenic mice were generated on C57BL/6 background (31). In brief, these mice show a selective and constitutive deletion of >90% of basophils due to high expression levels of the Cre recombinase in this cell lineage. All mice were housed according to institutional guidelines and used between 6 and 12 wk of age. The animal experiments were approved by the local government and performed in accordance with the German animal protection law and European Union guidelines 86/809.

Experimental infections and determination of egg burden

Cercariae from a Brazilian strain of *S. mansoni* were isolated from *Biomphalaria glabrata* snails (Brazilian origin), and mice were infected i.p. with 100–140 cercariae diluted in tap water. Mice were analyzed 9–12 wk postinfection. Determination of egg burden in liver and small intestine was performed as described (42). Fecal egg burden was determined after fixation of stool samples with 4% formalin. Samples were washed and passed through two layers of gaze, followed by centrifugation and microscopic examination of the sediment (42).

Flow cytometry

Spleen, liver, and mesenteric lymph nodes (mLNs) were mechanically disrupted in a 70-μm nylon strainer (BD Biosciences, Franklin Lakes, NJ) to obtain single-cell suspensions. Cells were washed with FACS buffer (PBS and 2% FCS) and incubated with anti-CD16/CD32 blocking Ab (clone 2.4G2; Bio-XCell, West Lebanon, NH) for 5 min at room temperature followed by staining with Ab mixtures at 4°C. The following Abs were used for membrane staining: PerCP-Cy5.5-labeled anti-CD4 (clone RM4-5), allophycocyanin-labeled anti-CD4 (clone RM4-5), and biotinylated anti-FcεRI (clone MAR-1) were purchased from eBioscience (San Diego, CA); PE-labeled anti-Ly6G (clone 1A8)

and PE-labeled and Brilliant Violet 421-labeled anti-Siglec-F (clone E50-2440) were purchased from BD Biosciences; Alexa Fluor 647 (A647)-labeled anti-CD49b (clone HMα2), PE-labeled anti-CD200R3 (clone Ba13), and Alexa Fluor 488-labeled anti-CD11b (clone M1/70) were purchased from BioLegend (San Diego, CA); FITC-labeled antiCD200R3 (clone Ba103) was purchased from Hycult Biotech (Uden, The Netherlands). PE-labeled anti-Gata3 (clone TWAJ) from eBioscience was used for intranuclear staining after cells had been fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. PE-Cy7-labeled streptavidin (BD Biosciences) was used to detect biotinylated Abs. Dead cells were excluded by staining with DAPI (Sigma-Aldrich, Steinheim, Germany) or fixable viability dye eFluor 780 (eBioscience). Samples were acquired using an FACSanto II (BD Biosciences) or MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). Doublets were discriminated by electronic gating. Data were analyzed by FlowJo software (Tree Star, Ashland, OR).

Restimulations of mLN cells and measurement of cytokines in the supernatant

A total of 5×10^5 cells/well were plated on a 96-well plate that had been coated before with 1 μg/ml anti-CD3 and anti-CD28 Abs (eBioscience). After 3 d of restimulation, the concentrations of different cytokines in the supernatant were detected by MILLIPLEX Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore, Billerica, MA) and by standard ELISA. The MILLIPLEX assay was performed according to the manufacturer's instructions, by which magnetic beads were used to detect the following cytokines: IL-4, IL-5, IL-10, IL-13, IL-17, IFN-γ, GM-CSF, and TNF-α. Samples were measured on MAGPIX, and data were analyzed by xPONENT software (both Millipore). Alternatively, IL-4, IL-5, and IL-13 concentrations were also determined by ELISA. IL-4 was measured with the anti-IL-4 mAbs 11B11 for coating and BVD6-24G2 (BioLegend) for detection. IL-5 and IL-13 were measured with ELISA kits from BD Biosciences and PeproTech (Hamburg, Germany), respectively.

Measurement of Ab and aspartate aminotransferase/alanine aminotransferase levels in the serum

The levels of different Ab isotypes in the serum were measured by standard ELISA. Serum IgE levels were determined with purified anti-IgE (clone R35-72; BD Biosciences) for coating and biotinylated anti-IgE (clone R35-118; BD Biosciences) for detection. IgG1 and IgG2a concentrations were determined using a commercial ELISA kit (Southern Biotechnology Associates, Birmingham, AL). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined by routine testing at the Central Laboratory of the University Clinic Erlangen.

Histology, immunofluorescence, and determination of granuloma size

The 4-μm sections of paraffin embedded liver samples from individually infected mice were stained with H&E or Masson's Blue and microscopically examined for the percentage of eosinophils among total cells in granulomas, which was determined in six randomly selected granulomas per section from each mouse. The size of the granulomas and the presence of alternatively activated macrophages was determined in cryosections (6-μm thick) of 4% paraformaldehyde-fixed liver samples, which were first blocked with TNB-buffer (TSA amplification kit; PerkinElmer) supplemented with 1% rat serum, 1% mouse serum, and 1% CD16/32-blocking Ab. Sections were then stained with rabbit anti-mouse Ym1 Ab (StemCell Technologies, Grenoble, France), which was detected by Cy3-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). For detection of basophils, slides were pretreated with sodium citrate buffer (10 mM sodium citrate and 0.05% Tween-20 [pH 6]) for 5 min at 90°C and incubation in 1% H₂O₂ in PBS for 1 h at room temperature. Sections were then stained with purified rat anti-mouse Mcpt8 Ab (BioLegend) followed by HRP-conjugated goat anti-rat Ab (Jackson ImmunoResearch Laboratories) and Tyramide-Cy3 (TSA amplification kit; PerkinElmer). Sections were counterstained with DAPI. Pictures were acquired on an LSM 700 (Carl Zeiss, Jena, Germany). Image analysis and determination of granuloma size was performed using the LSM image browser software.

Quantitative RT-PCR

RNA was extracted from liver samples with the RNeasy Mini Kit (Qiagen, Hilden, Germany). A total of 1 μg total RNA was used for first-strand cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen-Life Technologies, Carlsbad, CA) and oligo(dT) primers. PCR was performed on the 7900HT Fast Real-Time PCR system (Applied Biosystems-Life Technologies) with 5-fold diluted cDNA templates using SYBR Green I (Qiagen) and the following primer

sequences: porphobilinogen deaminase (PBGD) forward 5'-TGGTTGTTCA CTCCCTGAAGG-3', PBGD reverse 5'-AAAGACAACAGCATCACAAGG GT-3'; collagen I forward 5'-ACTGGACTGTCCCAACCC-3', collagen I reverse 5'-TCCCTCGACTCCTACATCTTCTG-3'; collagen III forward 5'-AA CCTGGTTTCTTCTCACCTTC-3', collagen III reverse 5'-ACTCATAGG ACTGACCAAGGTGG-3'; collagen VI forward 5'-CGCCCTTCCCACTGA-CAA-3', collagen VI reverse 5'-GCGTTCCTTTAAGACAGTTGAG-3'; Relm- α forward 5'-CCATAGAGAGATTATCGTGGA-3', Relm- α reverse 5'-TGGTCGAGTCAACGAGTAAG-3'; Foxp3 forward 5'-ACCATTTGGTT-TACTCGCATGT-3', Foxp3 reverse 5'-TCCACTCGCACAAAGCACTT-3'; Ym1/2 forward 5'-CTGATCTATGCCTTTGCTGG-3', Ym1/2 reverse 5'-CACAGATTCTTCCTCAAAAGC-3'; arginase-1 forward 5'-CTCGCAAGC-CAATGTACACG-3', arginase-1 reverse 5'-GTATGACGTGAGAGACCACG-3'; NOS-2 forward 5'-CAGAAGAATGGAAGAGTCAG-3', NOS-2 reverse 5'-CAGATATGCAGGGAGTCACC-3'; and TNF- α forward 5'-GAGCAATGACTCCAAAGTAG-3', TNF- α reverse 5'-CGTAGCAAAC-

CACCAAGTGG-3'. PCR conditions were the following: 15 min initial denaturation at 95°C, 40 cycles with 30 s of denaturation at 95°C, 30 s of annealing at 50°C, and 45 s of elongation at 72°C.

Statistical analysis

Statistics were calculated either by Mann-Whitney *U* test (for RT-PCR data) or by Student *t* test (all other assays) using SigmaPlot software (SPSS).

Results

Role of T cell-derived versus innate IL-4/IL-13 for mobilization and recruitment of effector cells after *S. mansoni* infection

The relative contribution of IL-4/IL-13 derived from T cells or from cells of the innate immune system (eosinophils, basophils, mast

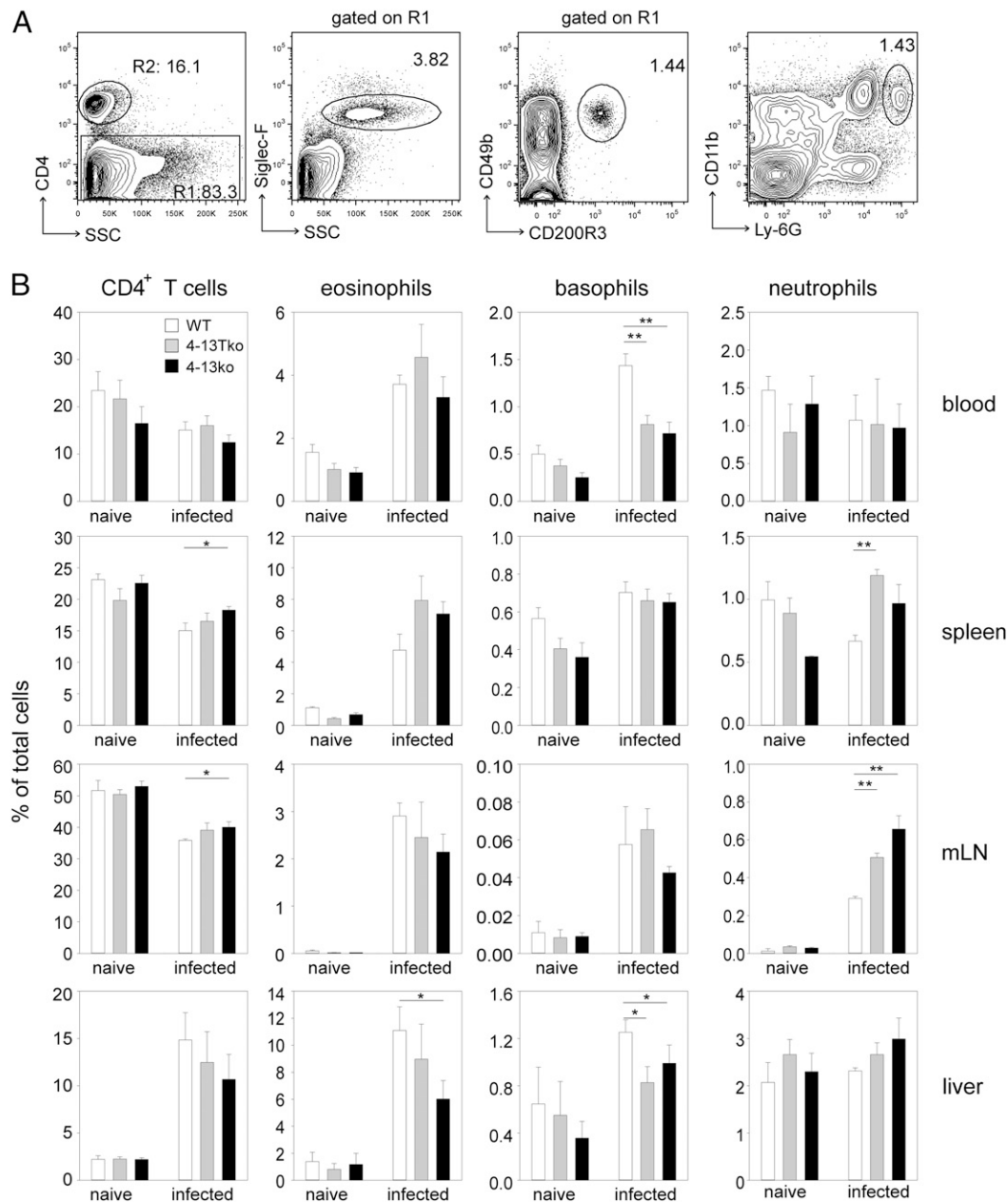


FIGURE 1. Mobilization of CD4 T cells and granulocytes during *S. mansoni* infection in presence or absence of IL-4/IL-13. 4-13ko, 4-13Tko, and WT mice were infected with *S. mansoni* cercariae and analyzed 9 wk later by flow cytometry. **(A)** Gating strategy for identification of CD4⁺ T cells (left panel), CD4⁺Siglec-F⁺SSC^{hi} eosinophils (middle left panel), CD4⁺CD49b⁺CD200R3⁺ basophils (middle right panel), and CD11b⁺Ly-6G^{hi} neutrophils (right panel). **(B)** Bar graphs show the percentage of indicated cell types in blood, spleens, mLNs, and livers of naive and infected WT (white), 4-13Tko (gray), and 4-13ko (black) mice. Bar graphs show the mean + SE of seven to eight individual mice per group from two independent experiments except for neutrophil data, which are from three to four mice per group from one experiment. Statistical analysis was performed to determine significant differences between the groups of infected mice. **p* < 0.05, ***p* < 0.01. SSC, side scatter.

cells, and ILC2s) during *S. mansoni* infection remains unclear. Therefore, WT mice, IL-4/IL-13-deficient mice (4-13ko), and T cell-specific IL-4/IL-13-deficient mice (CD4Cre $\times$ IL-4/IL-13^{F/F}, for short: 4-13Tko) were infected i.p. with *S. mansoni* cercariae and analyzed for mobilization and tissue recruitment of CD4⁺ T cells, eosinophils, basophils, and neutrophils. At 9 wk postinfection, the frequency of basophils was increased in blood and liver of infected WT mice when compared with 4-13Tko and 4-13ko mice (Fig. 1). Eosinophils were comparable between the three groups in blood, spleen, and mLN, and only the eosinophil frequency in the liver was significantly impaired when mice lacked IL-4/IL-13 expression in all cells. In contrast, the frequency of CD4⁺ T cells and neutrophils was slightly increased in spleen and mLN of 4-13ko and 4-13Tko mice, respectively (Fig. 1B). No differences were found for ILC2s in spleen, mLN, and liver of the three different groups of mice (data not shown). The total numbers of CD4⁺ T cells, eosinophils, and basophils were ~2-fold increased in the spleen but not in mLN or liver of WT mice compared with 4-13Tko and 4-13ko mice (Supplemental Fig. 1).

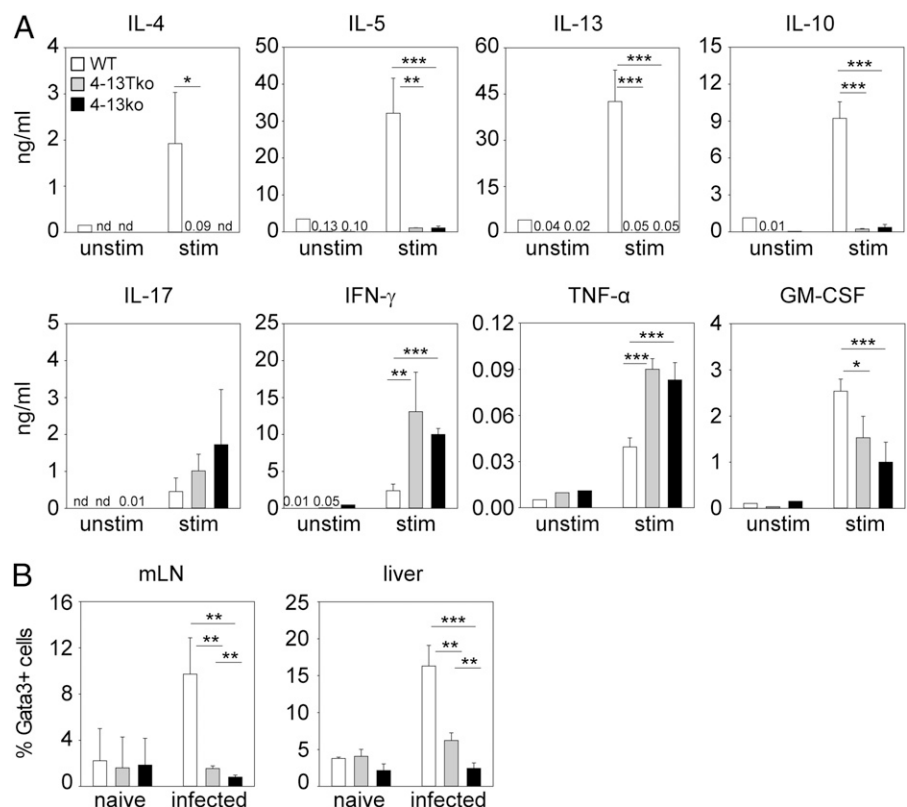
To determine how the deletion of IL-4/IL-13 in T cells of 4-13Tko mice affects T cell polarization, we measured the concentrations of different cytokines in supernatants of restimulated mLN T cells 9 wk postinfection (Fig. 2A). As expected, increased IL-4 and IL-13 levels were only detected in cultures from infected WT mice but not from 4-13Tko and 4-13ko mice. In addition, IL-5 and IL-10 were only produced by T cells of WT mice and these culture supernatants also contained significantly more GM-CSF when compared with T cells from 4-13Tko and 4-13ko mice. This suggested that Th2 polarization might be generally affected in 4-13Tko and 4-13ko mice in this infection model. In contrast, T cells from 4-13Tko and 4-13ko mice produced more of the Th1-associated cytokines IFN- γ and TNF- α when compared with WT T cells but IL-17 levels were comparable between all three groups. These results indicate that the lack of IL-4/IL-13 expression in T cells leads to impaired Th2 polarization, which in turn promotes

expansion of Th1 cells. To further corroborate these findings, we performed intranuclear staining for the Th2 master transcription factor Gata3. Indeed, the expression of Gata3 was strongly induced in CD4⁺ T cells from WT mice both in mLN and liver (Fig. 2B). In addition, a small but significant difference was observed between 4-13Tko and 4-13ko mice, indicating that innate IL-4/IL-13-producing cells can induce some Gata3 expression in CD4⁺ T cells (Fig. 2B). Taken together, these results indicate that selective deletion of IL-4/IL-13 in CD4⁺ T cells results in a Th1-biased immune response after *S. mansoni* infection with lower basophil levels in blood and liver, whereas neutrophils increase in spleen and mLN.

Regulation of the humoral immune response to *S. mansoni* infection by T cell-derived versus innate IL-4/IL-13

The humoral arm of type 2 immunity is characterized by Ig class-switch recombination in B cells to IgE and IgG1. This process is largely dependent on IL-4/IL-13, which can be produced by a variety of cell types, and it remains unknown whether innate IL-4/IL-13-producing cells might be sufficient for the IgE/IgG1 response to *S. mansoni*. To address this issue, the concentrations of different Ab isotypes in the serum were monitored during the course of infection. As expected, levels of IgE and IgG1 strongly increased in WT mice after egg deposition, which occurs at around 6 wk postinfection (Fig. 3). In contrast, IgE remained undetectable in both 4-13Tko and 4-13ko mice. IgG1 slightly increased during the course of infection, and although levels were comparable in 4-13Tko and 4-13ko mice, the amounts were significantly lower in infected WT controls (Fig. 3). Levels of IgG2a remained low in WT mice throughout the experiment, whereas they increased in 4-13Tko and 4-13ko mice from week 6 onwards. This result supports the observed Th1-biased response in 4-13Tko and 4-13ko mice determined by restimulation of T cells from mLN (Fig. 2A). Furthermore, 4-13ko mice showed higher IgG2a levels compared with 4-13Tko mice, suggesting that innate IL-4/

FIGURE 2. Analysis of T cell polarization after *S. mansoni* infection. **(A)** Total of 5×10^5 mLN cells from WT (white), 4-13Tko (gray), and 4-13ko (black) mice at 9 wk after *S. mansoni* infection were left unstimulated (unstim) or activated in vitro (stim) for 72 h with anti-CD3/anti-CD28. Thereafter, the resulting culture supernatant was screened using a MILLIPLEX assay for determination of indicated cytokine concentrations. Bars show the mean $\pm$ SD of two to four individual mice per group from one experiment. Similar results were obtained for IL-4, IL-5, and IL-13 levels in an independent experiment measured by ELISA (data not shown). **(B)** Cells from mLN and livers of naive and infected mice were stained for surface CD4 and intranuclear Gata3. Dead cells (DAPI⁺) and doublets were excluded by electronic gating. Bars show the mean $\pm$ SD of two to four individual mice per group and are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. nd, not detectable.



IL-13-expressing cells can somewhat inhibit the IgG2a response. In general, these findings demonstrate that the induction of IgE and IgG1 during schistosomiasis is highly dependent upon the release of IL-4/IL-13 from CD4⁺ T cells, and innate sources of IL-4/IL-13 are not sufficient.

Protection against fatal S. mansoni infection by T cell-derived versus innate IL-4/IL-13

Next, we investigated whether helminth clearance and liver pathology were altered in the different infected mouse strains. Liver pathology, formation of granulomas around the parasites' eggs, and fibrosis are characteristic features of schistosome infections and provide a platform to quantitatively assess disease progression. Previous work demonstrated that IL-4 promotes the egg passage into the lumen of the intestine (15). We also observed a trend to more eggs in the feces of WT mice but the number of eggs in the small intestine, liver, and feces was not significantly different between WT, 4-13Tko, and 4-13ko mice (Fig. 4A). However, liver granulomas were significantly larger and contained a higher percentage of eosinophils in WT mice compared with 4-13Tko and 4-13ko mice (Fig. 4B, 4C). A previous study has shown that the size of the granulomas was not smaller in *S. mansoni*-infected eosinophil-deficient as compared with WT mice (43). This indicated that eosinophils are not required for granuloma formation, and therefore, we believe that the reduced granuloma size in 4-13Tko and 4-13ko mice is not due to impaired eosinophil recruitment. Granulomas in 4-13Tko mice were slightly larger than

granulomas in 4-13ko mice but contained similar numbers of eosinophils, indicating that innate IL-4/IL-13-expressing cells make only a minor contribution to granuloma formation (Fig. 4B). Therefore, lack of IL-4/IL-13 derived from T cells does not lead to an impaired egg expulsion but severely affects granuloma formation. Normal granulomas are composed of many different cell types including AAMs, which have previously been shown to be dispensable for granuloma formation but were required to protect against fatal outcome of the infection (18). AAM differentiation is largely dependent on IL-4/IL-13, but it remains unclear whether AAMs require Th2 cells or other cell types as source of these cytokines. Immunofluorescence stainings of liver sections with Abs against Ym1, a chitinase-like protein produced by AAMs, revealed that AAMs were only present in granulomas of WT mice but not in 4-13Tko or 4-13ko mice (Fig. 4C). This finding could be confirmed by quantitative RT-PCR analysis because the expression levels of Ym1/2 and two other well-characterized AAM markers, namely Relm- α and arginase 1, were all higher in the livers of infected WT mice when compared with 4-13Tko and 4-13ko mice (Fig. 4D). This also correlated with higher IL-4 and IL-13 mRNA levels in the livers of WT mice (Supplemental Fig. 2). Collagen mRNA levels were lower in 4-13Tko and 4-13ko mice compared with the WT controls, indicating that T cell-derived IL-4/IL-13 promotes liver fibrosis (Fig. 4D). Expression of Foxp3, the master transcription factor for regulatory T (T_{reg}) cells, was increased to similar levels in all three mouse strains excluding a defect of T_{reg} recruitment to the liver in 4-13Tko or 4-13ko mice (Fig. 4D). Finally, the expression of TNF- α and inducible NOS-2 was increased similarly in all three mouse strains, which indicates that impaired AAM differentiation in 4-13Tko and 4-13ko mice did not result in more pronounced differentiation of classically activated macrophages (Fig. 4D).

Serum AST and ALT are enzymes that are used to monitor hepatocyte damage. Therefore, AST/ALT levels were measured in the serum of infected mice, but no significant differences could be detected (Fig. 5A, 5B). 4-13ko and 4-13Tko mice did, however, lose up to 20% body weight when compared with uninfected controls and died between 8 and 12 wk postinfection (Fig. 5C, 5D). These results demonstrate that IL-4/IL-13 from T cells is needed for granuloma formation and protection against a fatal course of infection. Only a minor contribution of IL-4/IL-13 from innate cell types could be observed for protective immunity because a small trend to higher AST levels, more pronounced weight loss, and shorter survival could be observed in 4-13ko mice when compared with 4-13Tko mice. We did not observe increased serum endotoxin levels in 4-13Tko or 4-13ko mice at 9 wk postinfection (data not shown), indicating that mortality was not caused by sepsis.

Role of basophils during the immune response to patent S. mansoni infection

The protective effect of T cell-derived IL-4/IL-13 described above could have been mediated directly (e.g., by promoting collagen production in hepatic stellate cells or differentiation and recruitment of AAMs into granulomas). Alternatively, this effect may have occurred indirectly (e.g., by recruiting basophils into the liver and by promoting IgE and IgG1 production, which then activate basophils to release IL-4/IL-13 or IL-10). The latter would correlate with studies that have shown the protective effect of Abs in adoptive transfer studies (23, 24). Although basophils expressed IL-4, IL-5, and IL-13, we could not detect IL-10 expression by RT-PCR analysis of resting or stimulated basophils (Supplemental Fig. 3). Basophils are efficiently activated by IgE upon binding of IPSE/ α -1, an *S. mansoni* egg-derived glycoprotein, and therefore,

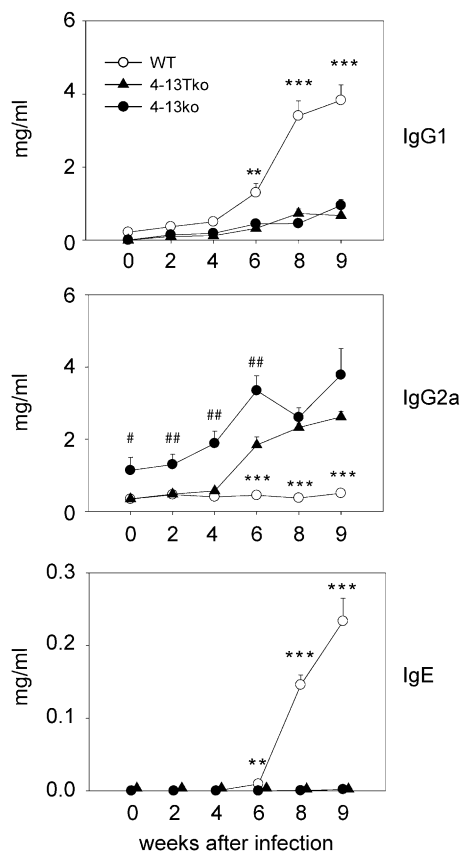


FIGURE 3. T cell-derived IL-4/IL-13 is required for IgE and IgG1 production. IgE, IgG1 and IgG2a levels determined in serum samples by ELISA at the indicated time points after *S. mansoni* infection of WT (○), 4-13Tko (▲), and 4-13ko (●) mice. Graphs show the mean + SE with eight mice per group from two independent experiments. ** $p < 0.01$, *** $p < 0.001$ (WT compared with 4-13Tko and 4-13ko), # $p < 0.05$, ## $p < 0.01$ (4-13Tko versus 4-13ko).

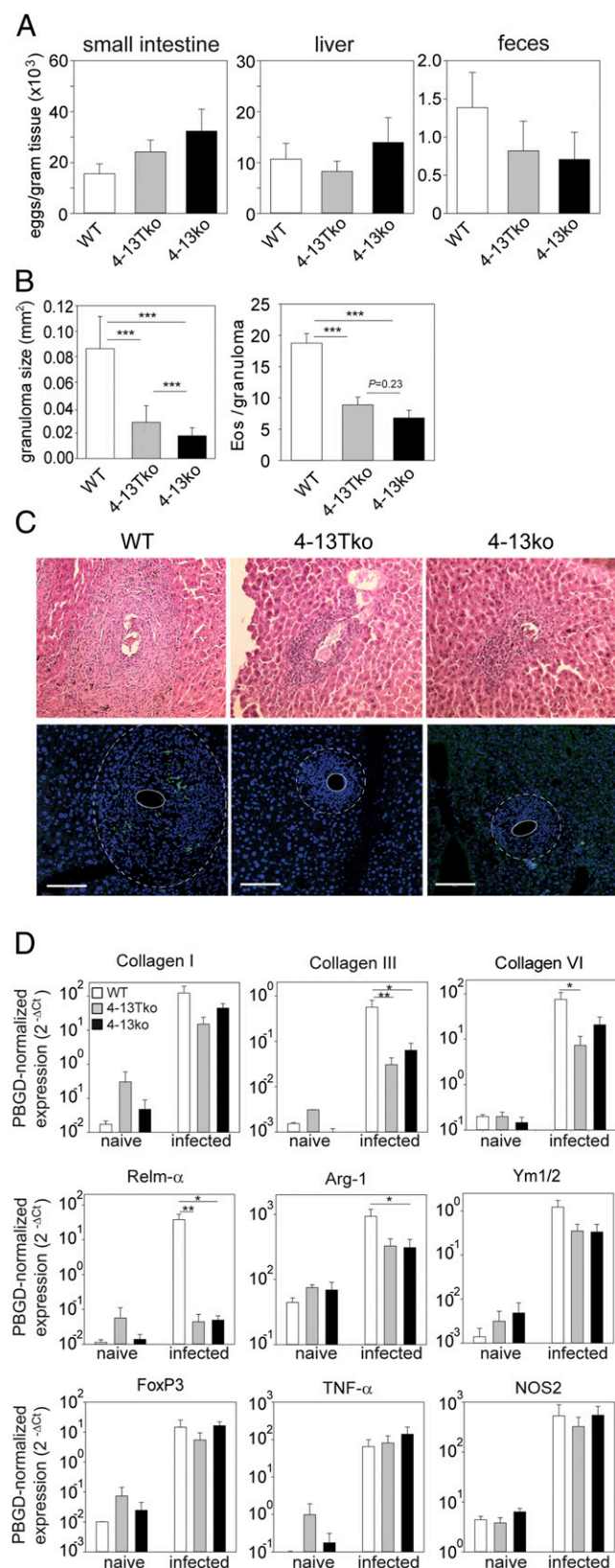


FIGURE 4. Reduced granuloma formation in the absence of IL-4/IL-13 from T cells. **(A)** Number of eggs in small intestine, liver, and feces of week 9 infected WT (white), 4-13Tko (gray), and 4-13ko (black) mice. Bar graphs show the mean $\pm$ SE of seven to eight individual mice per group from two independent experiments (small intestine and liver) or three to four mice per group from one experiment (feces). **(B)** Size of liver granulomas and frequency of eosinophils in granulomas. *Left panel* shows the granuloma size as mean $\pm$ SE of ~ 50 analyzed granulomas from each

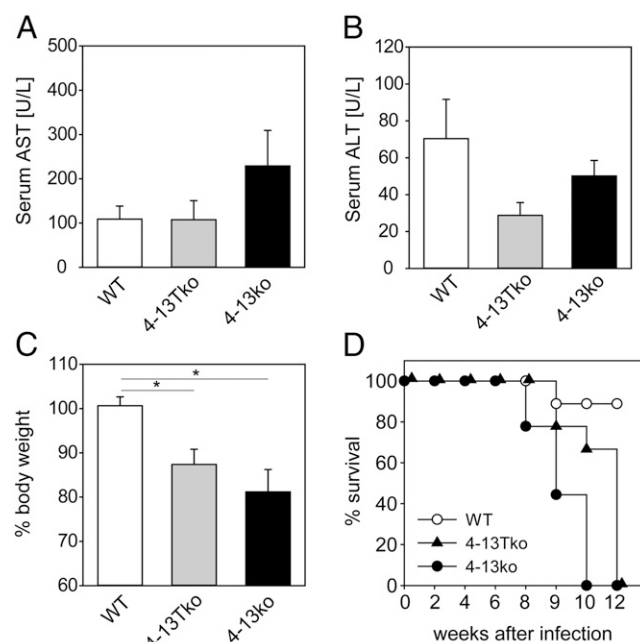


FIGURE 5. IL-4/IL-13 from T cells is required to protect against fatal infection. **(A and B)** Levels of AST and ALT in the serum of week 9 infected mice. **(C)** Body weight in percent of uninfected control mice at week 9 postinfection. **(D)** Survival curve of WT ($\circ$), 4-13Tko ($\blacktriangle$) and 4-13ko ($\bullet$) mice after *S. mansoni* infection. **(A–C)** Eight mice per group from two independent experiments or **(D)** nine mice per group from one experiment were analyzed. $*p < 0.05$.

it seemed plausible that basophils may contribute to the developing type 2 immune response during the patent phase of *S. mansoni* infection (35). To directly address this possibility, the immune profiles postinfection were compared between WT and Mcpt8Cre mice, in which basophils are constitutively deleted due to the recognition of cryptic loxP sites in the genome of basophils by Cre recombinase. Both mouse strains had been crossed to IL-4eGFP reporter mice (41) to facilitate the detection of IL-4-expressing cells.

Flow cytometric analysis revealed that basophils started to increase in frequency in the blood after 8 wk postinfection in WT but not Mcpt8Cre mice (Fig. 6A, *bottom panel*). Basophils were not required for the switch to a type 2 immune response, as a comparable increase of Th2 cells and eosinophils was observed in both Mcpt8Cre and WT mice (Fig. 6A, *top and middle panels*). To study the immune response in different tissues, mice were analyzed at 9 and 12 wk postinfection. Th2 cells and eosinophils accumulated in spleens, mLN, and livers of infected mice independently of basophils (Fig. 6B). Furthermore, restimulated T cells from mLN of infected mice of both groups produced

strain. The *right panel* shows the mean percentage $\pm$ SE of eosinophils among total cells (excluding hepatocytes) in six random granulomas per section from each mouse. **(C)** H&E staining (*top panel*) and immunofluorescence staining (*bottom panel*) of liver granulomas stained with anti-Ym1 (green) and DAPI (blue). Inner circles indicate location of the eggs, outer circles the border of the granulomas. Original magnification $\times 200$. Scale bars, 100 μm . **(D)** Quantitative RT-PCR analysis for the expression of the indicated genes in the liver of naive and week 9 infected WT (white), 4-13Tko (gray), and 4-13ko (black) mice. PBGD served as housekeeping gene. Bar graphs depict the mean $\pm$ SE of seven to eight individual mice per group from two independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

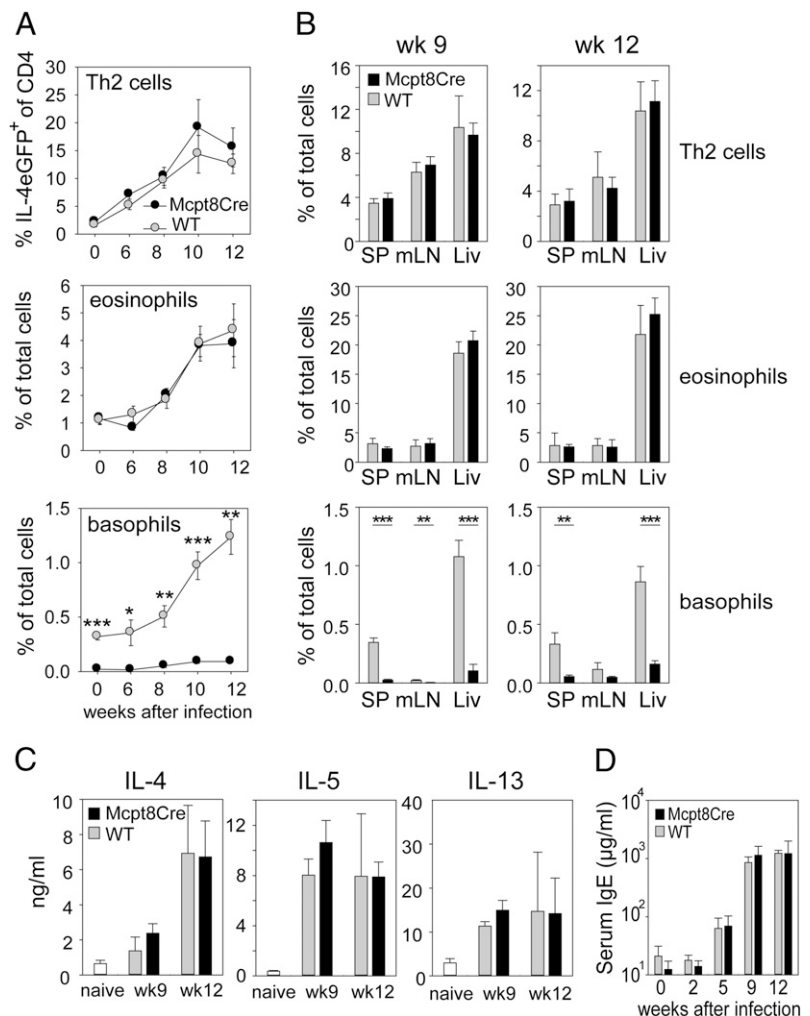
comparably high amounts of IL-4, IL-5, and IL-13 (Fig. 6C). In addition, serum IgE levels increased with the same kinetics and intensity in WT and Mcpt8Cre mice (Fig. 6D). These findings demonstrate that basophil-derived IL-4/IL-13 appears to be dispensable for the induction of Th2 differentiation and IgE production during *S. mansoni* infection. To investigate the effects of basophils on disease progression, we first measured egg burden in infected animals. The number of eggs in the liver and small intestine was comparable between WT and Mcpt8Cre mice (Fig. 7A), and liver granulomas had the same size in both groups of mice (Fig. 7B). To determine whether basophils are recruited into granulomas, cryosections from the liver of infected mice were stained with anti-Mcpt8. Mcpt8 is highly expressed in basophils but not in mast cells or other cell types. Basophils were readily detectable in granulomas of WT mice but not in Mcpt8Cre mice (Fig. 7C). Quantitative RT-PCR analysis of liver tissue revealed that the lack of basophils did not affect *S. mansoni*-induced expression of collagens and the differentiation or recruitment of AAMs and T_{reg} cells (Fig. 7D). Finally, no differences in survival were observed, indicating that basophils are not required for the protection against a fatal outcome of the infection (Fig. 7E). In conclusion, we could show that CD4⁺ T cell-derived IL-4/IL-13 are critical for granuloma formation, IgE production, alternative activation of macrophages, and survival. In contrast, IL-4/IL-13-producing basophils appeared to be dispensable for the development of protective type 2 immune responses and survival during patent infection. Thus, although we show that basophils are ac-

tively recruited into the granulomas and despite the finding by others that basophils can be efficiently activated by the schistosome-derived components, their functional role during *S. mansoni* infection still remains unclear (35).

Discussion

The cytokines IL-4 and IL-13 play an important protective role during the early stages of the immune response against *S. mansoni* eggs. Genetic deletion of IL-4 leads to a Th1-biased immune response, severe cachexia, hepatotoxicity, and ultimately death at ~9 wk postinfection probably caused by TNF- α -induced production of NO in the intestines (14). This phenomenon was even more pronounced in IL-4/IL-13-deficient mice and reflects the symptoms associated with acute human schistosomiasis (15). In a pulmonary model of *S. mansoni* egg-induced inflammation, granuloma formation and typical signs of type 2 immunity like eosinophilia and high IgE levels were abolished only in the combined absence of IL-4 and IL-13 (38). This illustrates that IL-4 and IL-13 play largely redundant roles because they share receptors, which induce similar signaling pathways. However, IL-13 also promotes liver fibrosis and fatal pathology during the chronic stage of infection as demonstrated by infection of IL-13-deficient mice or mice in which IL-13 had been neutralized by injection of the soluble form of the IL-13 decoy receptor IL-13R α 2 (15, 44). Using conditional IL-4/IL-13-deficient mice, we directly show that T cells are the critical source of these cytokines for granuloma formation and prevention of early lethal infection.

FIGURE 6. Immune response against *S. mansoni* in basophil-depleted Mcpt8Cre mice. **(A)** Frequency of Th2 cells (CD4⁺IL-4/eGFP⁺), eosinophils (IL-4/eGFP⁺SSC^{hi}Siglec-F⁺), and basophils (IL-4/eGFP⁺CD49b⁺IgE⁺) at the indicated time points postinfection of Mcpt8Cre (black) and WT (gray) mice. Symbols show the mean $\pm$ SD of three to four mice per group. **(B)** Th2 cells (*top panels*), eosinophils (*middle panels*), and basophils (*bottom panels*) in spleens (SP), mLN, and livers (Liv) at week 9 and 12 postinfection. Bars show the mean $\pm$ SD with three to four mice per group. **(C)** Total of 5×10^5 mLN cells were restimulated in vitro for 72 h with anti-CD3/anti-CD28. Thereafter, the resulting culture supernatant was analyzed for the presence of IL-4, IL-5, and IL-13 by ELISA. Bars show the mean $\pm$ SD with three to four mice per group. **(D)** Serum IgE levels were determined in samples obtained at the indicated time points by ELISA. Bars show the mean $\pm$ SD with three to four mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



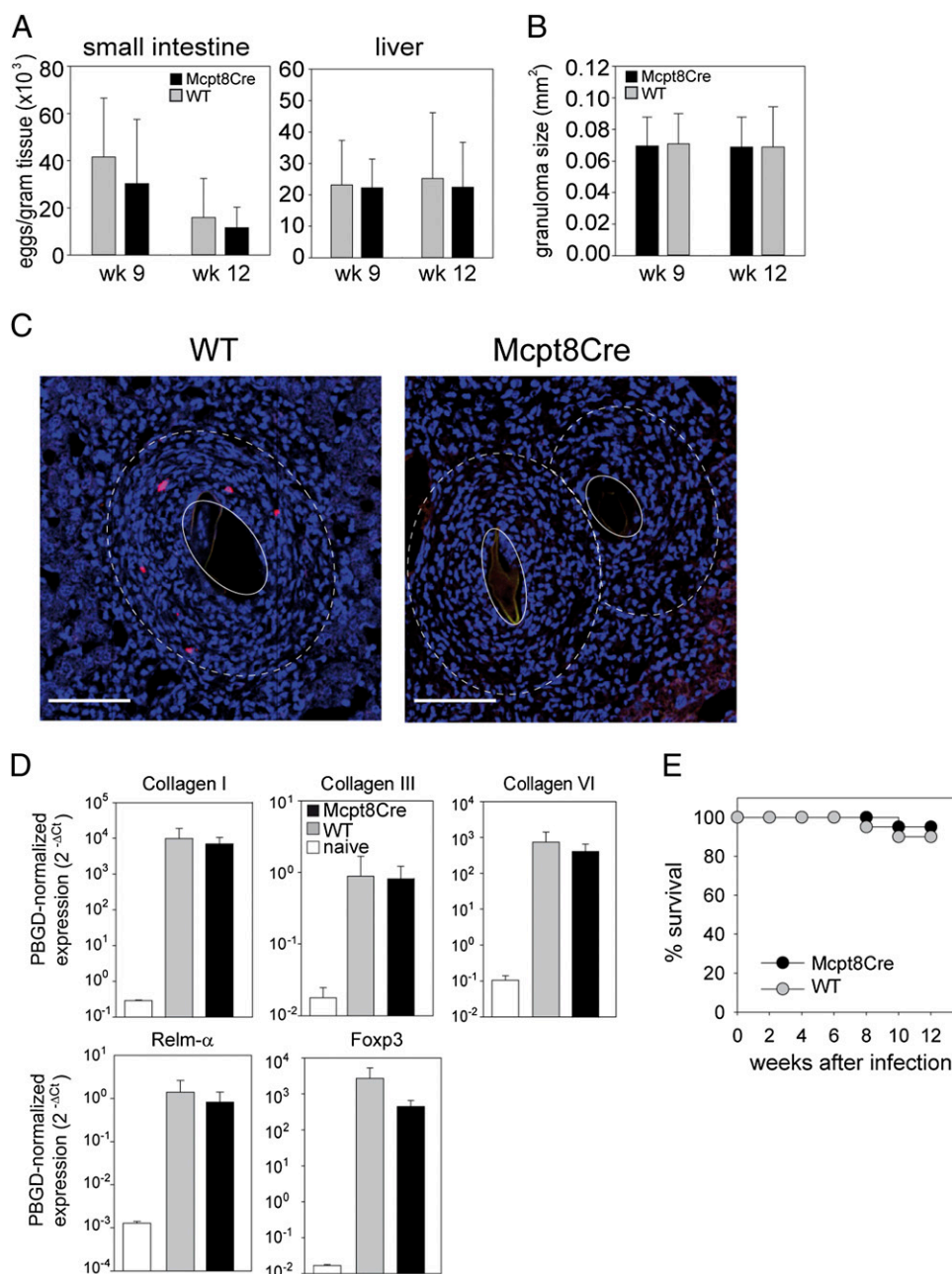


FIGURE 7. Normal granulomas and differentiation of AAMs in basophil-depleted Mcpt8Cre mice. **(A)** Number of eggs in small intestine and liver of WT (gray) and Mcpt8Cre (black) mice at weeks 9 and 12 postinfection. Bar graphs show the mean + SD of three to four mice per group. **(B)** Size of liver granulomas measured in DAPI-stained cryosections using LSM image browser software. At least 10 granulomas per mouse were analyzed. **(C)** Liver sections stained with anti-Mcpt8 (red) and DAPI (blue). Inner circles indicate location of the eggs, outer circles the border of the granulomas. Scale bars, 100 μm . **(D)** Quantitative RT-PCR for expression of indicated genes in the liver of naive (white) or week 9 or 12 infected WT (gray) and Mcpt8Cre (black) mice. PBGD served as housekeeping gene. Bars show the mean + SD with three to five mice per group. **(E)** Survival curve of WT (gray) and Mcpt8Cre (black) mice during the course of *S. mansoni* infection. Five mice were analyzed per group.

Because we used CD4Cre mice to delete the conditional allele, IL-4 and IL-13 were deleted in conventional Th2 cells but also in NKT cells. However, we consider it unlikely that NKT cells account for the phenotype that we report in this study; it was demonstrated that CD1d-deficient mice that lack NKT cells did not show a Th1-biased response during the chronic phase of *S. mansoni* infection and the size of the granulomas was comparable to WT mice (45). A previous study showed that CD40L-deficient mice develop a poor Th2 response to *S. mansoni*, generate smaller granulomas, and succumb to severe morbidity and mortality (28). However, in this study, pathology did not correlate with increased production of TNF- α or IFN- γ by restimulated T cells. This

suggests that pathology in 4-13Tko and 4-13ko mice may not simply be explained by a Th1-dominated immune response but rather highlights the critical role of Th2 cells to initiate mechanisms that protect against fatal pathology. 4-13Tko and 4-13ko mice also showed reduced basophilia in blood and liver. This phenomenon might be explained by enhanced apoptosis of basophils due to increased expression of Th1-associated cytokines as previously described (46). Alternatively, T cell-derived IL-4/IL-13 may promote the survival of basophils in vivo.

In addition, we found that basophils were dispensable for a normal Th2 response during *S. mansoni* infection as Th2 cells appeared with the same kinetics and magnitude in WT and

basophil-depleted Mcpt8Cre mice. We further observed that basophils were recruited into granulomas but they were not required for granuloma formation and protection against fatal infection. This result was unexpected because basophils can produce at least as much IL-4 as Th2 cells (47), and basophils were found to contribute to protection against other gastrointestinal helminths (30–32). Both human and murine basophils can be activated by *S. mansoni* egg Ags, in particular by the glycoprotein IPSE/α-1 that activates these cells in an IgE-dependent but Ag-independent manner (35, 48). Another study reported that Ab-mediated basophil depletion results in smaller granulomas after *S. mansoni* infection (49). The apparent discrepancy to our result might be explained by the fact that they used an Ab for basophil depletion (anti-CD200R3, clone Ba103), whereas we used genetically basophil-depleted mice. The anti-CD200R3 Ab has side effects as it engages the activating CD200R3 receptor that is expressed not only on basophils but also on mast cells, leading to systemic mast cell activation (50). Interestingly, eosinophil deficiency also had no impact on granuloma size and disease progression although eosinophils are abundant in granulomas and can kill schistosome migratory larvae (or schistosomulae) in vitro (43, 51–53). At present, we cannot exclude the possibility that eosinophils and basophils have redundant functions so that lack of basophils is compensated by the presence of eosinophils and vice versa.

Effector functions of macrophages can be directly regulated by IL-4/IL-13, and it has been demonstrated that deletion of the IL-4Rα-chain in macrophages prevents AAM differentiation (18). AAMs are generally involved in wound healing responses and tissue remodeling (54). They produce a variety of anti-inflammatory factors including arginase 1, programmed cell death ligand 2, and Relm-α (also named Fizz1) that inhibit the activity or proliferation of T cells (55–59) and dampen the fibrotic response to *S. mansoni* (55, 58). AAM-deficient mice (LysM^{Cre} × IL-4Rα^{flx}) succumb to a fatal course of *S. mansoni* infection, although the Th2 response and granuloma formation is not impaired (18). In this report, mortality was caused by sepsis, suggesting that AAMs were required for tissue repair in the small intestine to prevent bacterial invasion. However, other IL-4/IL-13-responsive cell types including collagen-producing fibroblasts and smooth muscle cells may also contribute to dampen pathology after *S. mansoni* infection (60). We show in this study that T cell-derived IL-4/IL-13 was critical for granuloma formation, AAM differentiation, and protection against fatal infection. However, in our model, pathology could not be explained by sepsis because endotoxin levels and neutrophils did not increase in 4-13ko and 4-13Tko mice. We rather believe that pathology results from a lack of Th2-mediated inhibitory mechanisms (IL-10 and AAMs) in combination with an increased Th1 response (IFN-γ and TNF-α) to *S. mansoni* egg Ags.

Taken together, we demonstrate that although innate IL-4/IL-13-producing cell types have been shown to contribute to protection against other gastrointestinal helminths, Th2 cells appear to be the biologically relevant cells for IgE switching, granuloma formation, AAM differentiation, and protection of mice against fatal infection with *S. mansoni*.

Acknowledgments

We thank A. Matthies and D. Döhler for technical assistance, M. Kirsch, J. Hein, and L. Gundel for animal husbandry, H. Parsch for AST/ALT determination, C. Bogdan for sharing equipment, and members of the Voehringer laboratory for comments.

Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figure 1

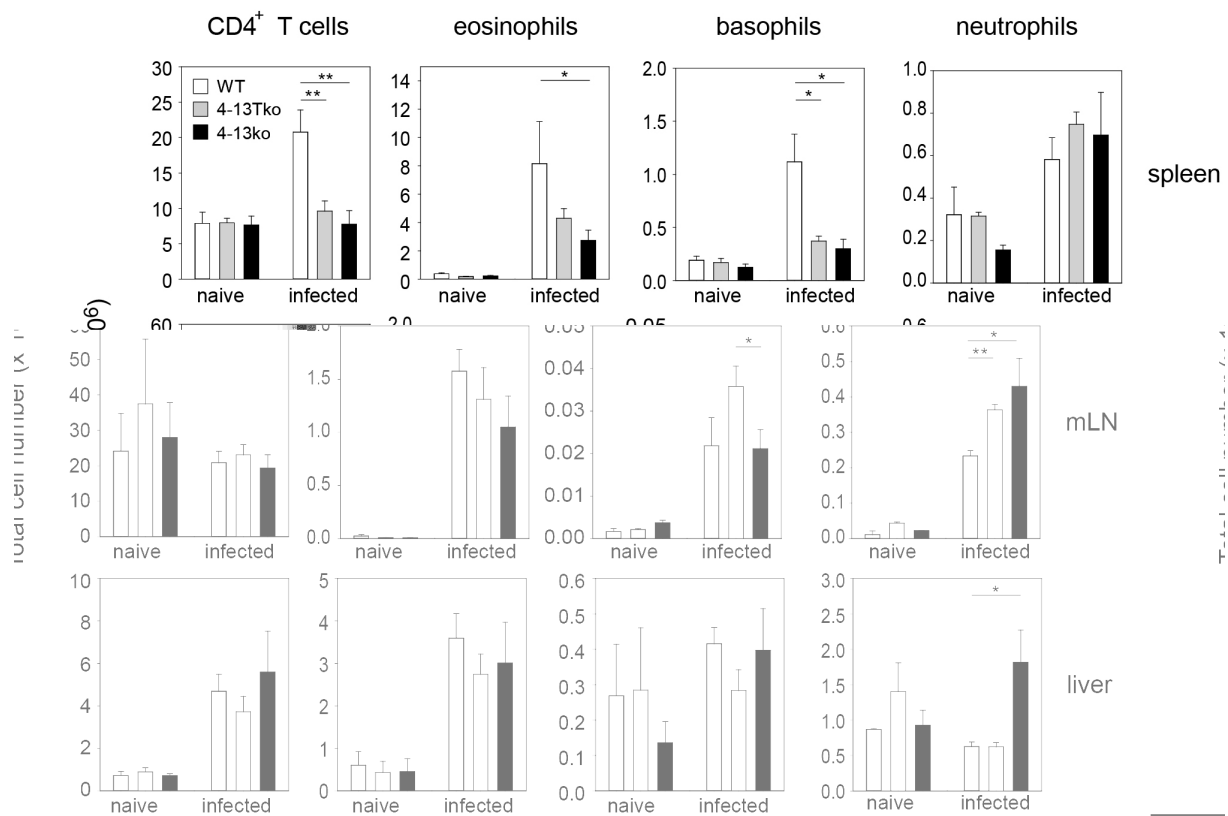


Figure S1 (related to Figure 1). Mobilization of eosinophils and basophils during *S. mansoni* infection in 4-13Tko and 4-13ko mice. 4-13ko, 4-13Tko and WT mice were infected with 140 *S. mansoni* cercariae and analyzed 9 weeks later by flow cytometry. Bar graphs show the total numbers of indicated cell types in spleens, mesenteric lymph nodes (mLN), and livers of naive and infected WT (white), 4-13Tko (gray) and 4-13ko (black) mice. Bar graphs show the mean+SE of 7-8 individual mice per group from two independent experiments. Statistical analysis was performed to determine significant differences between the groups of infected mice. *= $P<0.05$, **= $P<0.01$.

Supplemental Figure 2

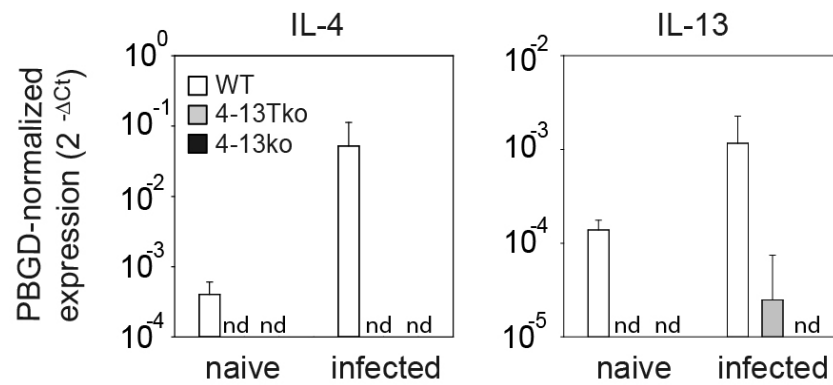


Figure S2. Relative quantification of cytokines in the livers of *S. mansoni* infected mice.

Quantitative RT-PCR analysis for expression of indicated genes in the liver of naive and week 9 infected WT (white), 4-13Tko (grey) and 4-13ko (black) mice. PBGD served as housekeeping gene. Bar graphs depict the mean+SE of 7-8 individual mice per group from two independent experiments. nd = not detectable.

Supplemental Figure 3

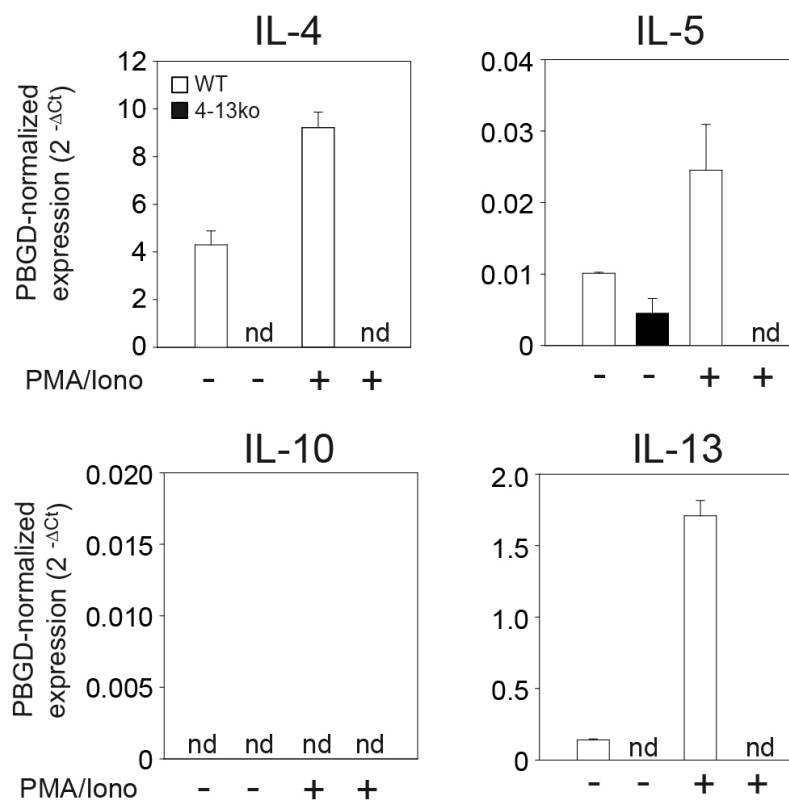


Figure S3. Relative quantification of cytokines produced by WT and 4-13ko basophils.

Basophils were sort-purified (>95%) from the spleens of naïve WT (white bars) and 4-13ko (black bars) mice. RNA was isolated from unstimulated cells and after 24h of stimulation with PMA/ionomycin and analyzed by quantitative RT-PCR. PBGD served as housekeeping gene. nd = not detectable.