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The skin is an important bulwark of acquired immunity against intestinal helminths

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Once animals have experienced a helminthic infection, they often show stronger protective immunity against subsequent infections. Although helminthic infections are well known to elicit Th2-type immune responses, it remains ill-defined where and how acquired protection is executed. Here we show that skin-invading larvae of the intestinal helminth *Nippostrongylus brasiliensis* are surrounded by skin-infiltrating cells and are prevented from migrating out of infected skin during the second but not the first infection. B cell– or IgE receptor FcεRI–deficient mice showed impaired larval trapping in the skin. Selective ablation of basophils, but not mast cells, abolished the larval trapping, leading to increased worm burden in the lung and hence severe lung injury. Skin-infiltrating basophils produced IL-4 that in turn promoted the generation of M2-type macrophages, leading to the larval trapping in the skin through arginase-1 production. Basophils had no apparent contribution to worm expulsion from the intestine. This study thus reveals a novel mode of acquired antihelminth immunity, in which IgE-armed basophils mediate skin trapping of larvae, thereby limiting lung injury caused by larval migration.

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Abbreviations used: BEC, S-(2-boronoethyl)-L-cysteine; DT, diphtheria toxin; DTR, DT receptor; Hp, *Heligmosomoides polygyrus*; ILC2, group 2 innate lymphoid cell; Nb, *Nippostrongylus brasiliensis*; nor-NOHA, Nω-hydroxy-nor-L-arginine.

Helminths are the most common infectious agents of humans in developing countries (Hotez et al., 2008). The major helminthiases are those caused by intestinal helminths including large roundworms, whipworms, and hookworms, followed by schistosomiasis and lymphatic filariasis. More than two billion people in worldwide populations are infected with intestinal helminths, suffering from deleterious outcomes such as malnutrition, growth stunting, and intellectual retardation. For the development of effective antihelminth vaccines, we need to understand both the helminth biology and the host immune response to helminthic infections (Anthony et al., 2007; Hotez et al., 2010; Allen and Maizels, 2011). Most helminths, unlike many other types of pathogens such as bacteria, protozoa, fungi, and viruses, do not replicate in the mammalian host, showing a complex multistage life cycle. Once host animals have experienced a helminthic infection, they often show a stronger protective immunity against subsequent infections with the same type of helminth (Africa, 1931; Valdivieso and Tamsitt, 1969; Love et al., 1974). This is the rationale for the development of antihelminth vaccines. However, it remains ill-defined how host animals manifest an acquired resistance to reinfection, even though it is well known that infection with intestinal helminths typically elicits a type 2 immune response that is characterized by high levels of serum IgE and increased numbers of type 2 helper T (Th2) cells, eosinophils, mast cells, and basophils (Finkelman et al., 2004; Anthony et al., 2007; Allen and Maizels, 2011; Pulendran and Artis, 2012).

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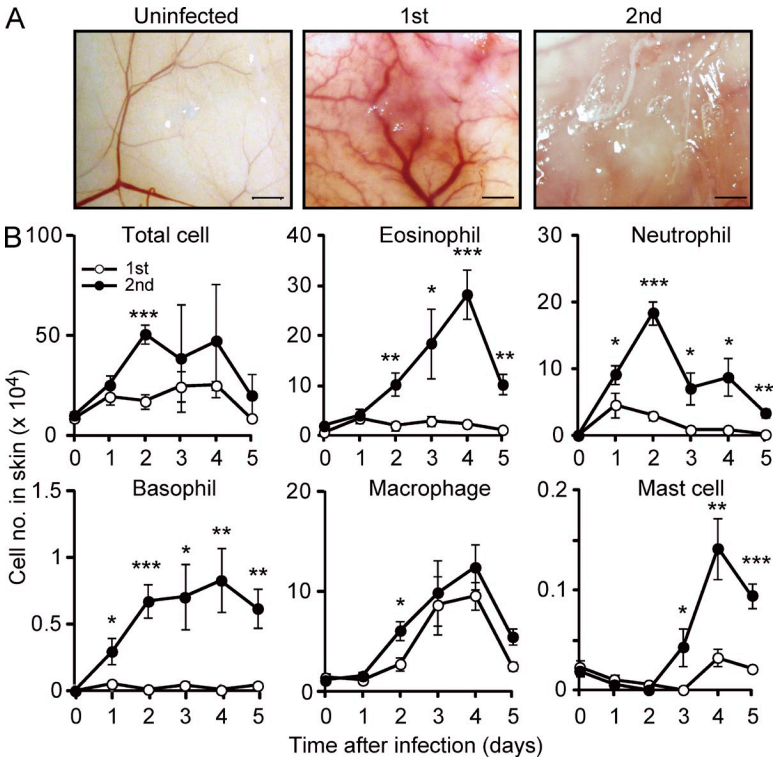

Figure 1. Prominent infiltration of proinflammatory cells in the skin of the larva inoculation site during the second but not first Nb infection. (A) BALB/c mice were left uninfected (left) or infected once (middle) or twice (right) with Nb larvae. Photographs of the subcutaneous tissue of the larva inoculation site were taken 2 d after the first or second inoculation. Bars, 8 mm. (B) Single-cell suspensions were prepared from the skin of the larva inoculation site at the indicated time points after the first and second inoculation and subjected to the flow cytometric analysis to identify the nature of skin-infiltrating cells. The numbers of total and individual cell lineages at each time point are plotted (mean ± SEM; $n = 3$ each). Data shown in A and B are representative of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Nippostrongylus brasiliensis (Nb) is a well-studied helminth in rodents and shows a life cycle similar to that of human hookworms *Necator americanus* and *Ancylostoma duodenale* (Finkelman et al., 1997; Gause et al., 2003). Infective larvae enter host animals through skin penetration and migrate to the lung within 2 d after invasion. They further migrate to the small intestine starting from day 3 and develop into mature worms to produce eggs. Adult worms are then expelled from the intestine by 10 d. Recent studies have illustrated that group 2 innate lymphoid cells (ILC2s) play an important role in worm expulsion from the intestine, through the production of IL-13 that in turn induces goblet cell hyperplasia in the intestine to increase mucus production for the "weep and sweep" response (Moro et al., 2010; Neill et al., 2010; Price et al., 2010). In contrast, it remains uncertain how host animals acquire and manifest the more efficient protective immunity against the subsequent infections. Of note, the number of worms recovered from the intestine on day 5 after infection is significantly lower in the second infection than in the first infection (Love et al., 1974; Knott et al., 2007). This suggests that worms may be efficiently expelled from the intestine in a shorter period of time during the second infection compared with the first one. Alternatively or in addition, the acquired anti-Nb immunity may exert its action at the preintestinal stage. Supporting this, the number of mobile larvae recovered from the lung on day 2 after infection was reported to be lower in the second infection than in the first (Knott et al., 2007; Harvie et al., 2010), implying that some larvae might be damaged within the lung or at the prelung stage. Previous studies failed to detect larval retention in the skin during the second infection, suggesting the lung rather than the skin as an important site for the acquired protection (Knott et al., 2007; Harvie et al., 2010). In contrast, leukocyte accumulation in the lung becomes prominent only on day 6 or later when larvae have already left the lung for the intestine (Knott et al., 2007; Harvie et al., 2010), making protection in the lung questionable. Although one study reported the contribution of basophils to worm clearance from the small intestine during the second Nb infection (Ohnmacht et al., 2010), the other failed to reproduce it (Sullivan et al., 2011). Thus, the site and cellular components of acquired protection remain elusive.

In the present study, we addressed these unsolved issues and found that, in the second but not first Nb infection, larvae are prevented from migrating out of infected skin, and are surrounded by skin-infiltrating proinflammatory cells. This larval trapping was dependent on high-affinity IgE receptor FcεRI and was abolished in mice depleted of basophils but not mast cells. Basophil-derived IL-4 promoted the generation of M2-type (alternatively activated) macrophages that in turn contributed to the larval trapping via their expression of arginase 1 (Arg1). Importantly, basophil-mediated larval trapping in the skin reduced the worm burden in the lung and hence protected mice from severe lung injury caused by the larval migration through the lung. Thus, basophils provide acquired protection against intestinal helminth Nb by means of restraining skin-invading larvae from migration toward the lung and intestine, rather than expelling adult worms from the small intestine.

RESULTS

Migratory larvae are retained within cellular infiltrates in the infected skin during the second but not first Nb infection

Multiple small hemorrhagic petechiae were detected in the skin of the larva inoculation site in the first Nb infection, reflecting that larvae penetrated and migrated into the blood vessels toward the lung (Fig. 1 A, middle). We noticed that an abscess-like white bump developed in the subcutaneous tissue of the larva inoculation site during the second but not first Nb infection (Fig. 1 A, right), suggesting that severe inflammation occurred in the skin lesion during the second infection. Indeed, flow cytometric analysis revealed that large numbers of eosinophils and neutrophils as well as a small number of basophils were recruited to and accumulated in the skin lesions during the second but not first infection (Fig. 1 B). The number of ILC2 cells in the skin lesions remained unaltered during the first and second infection (not depicted). Histopathological examination of skin sections prepared on day 2 after inoculation identified many nodule-like aggregates of infiltrating cells in dermis and subcutaneous tissue in the second but not first infection (Fig. 2 A). Notably, high magnification view of the tissue section revealed a cross section of larva-like structure within the cell aggregate (Fig. 2 B), suggesting that some larvae might be trapped by infiltrating cells within the skin of the inoculation site during the second Nb infection. To explore this possibility, fluorescent dye–labeled larvae were intradermally inoculated and subjected to multiphoton microscopic analysis to track them. 2 d after the inoculation, few larvae were detected within the skin around the inoculation site in the first infection, whereas a substantial number of larvae were visible within the skin in the second infection (Fig. 2 C). This did not seem to be an artifact because of the labeling of larvae. When mice were infected with unlabeled larvae, the retention of larvae within the skin was also suggested by significantly higher expression of Nb *Actin* mRNAs at the inoculation site in the second infection than in the first one (Fig. 2 D).

We then sought to isolate and enumerate larvae that were retained within the skin. We first tried to recover larvae from the infected skin by using the traditional method, in that infected tissues such as the lung are placed in PBS at 37°C to let viable larvae migrate out from the tissues (Camberis et al., 2003), but few or no larvae migrated out from the infected skin. We assumed that this could be a reason why previous studies failed to detect larvae retained within the skin in the second infection. To overcome this problem, infected skin was isolated and incubated with simulated gastric juice so that mouse tissues but not larvae were digested (Jin et al., 2008), and released larvae were enumerated under a microscope. This allowed us to examine the time course of larval migration out of the skin (Fig. 2 E). In the first infection, virtually all larvae left the skin of inoculation site by day 2 after inoculation. In contrast, in the second infection, as many as 100 larvae were retained in the skin on day 2 and even on day 5 (Fig. 2 E). The larval retention in the skin was also observed when larvae were inoculated for the second infection in ear skin in place of flank skin (not depicted), suggesting that the occurrence of larval retention may not be restricted to a certain anatomical district in the skin.

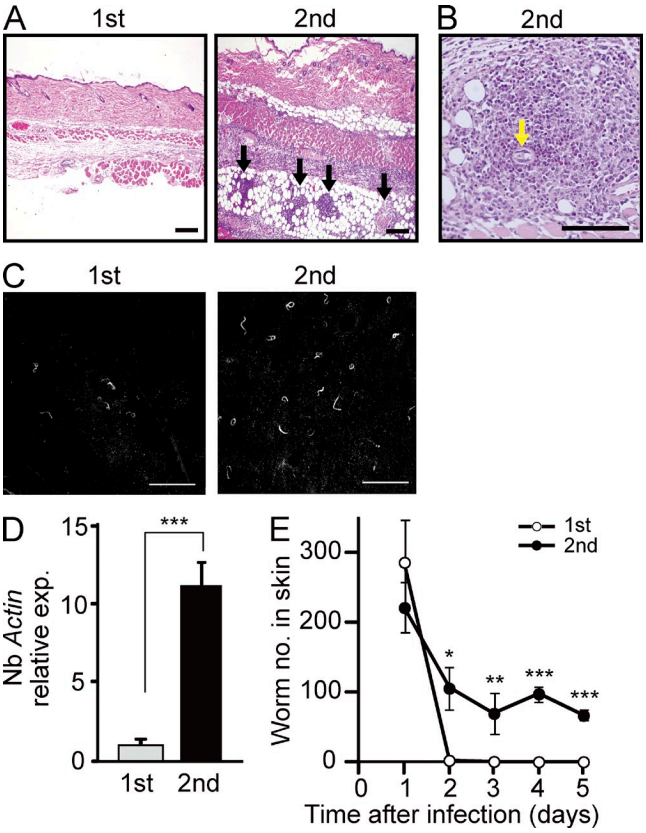

Figure 2. Retention of Nb larvae within cellular infiltrates in the infected skin during the second but not first infection. (A and B) The skin of the larva inoculation site was isolated 2 d after the first or second inoculation and subjected to histopathological examination by hematoxylin and eosin staining. Black arrows in A indicate clusters of cells surrounding larvae, and the yellow arrow in B indicates a section of larva surrounded by infiltrating cells. (C) PKH-labeled larvae were inoculated into the skin of naive (left) or previously infected (right) BALB/c mice and subjected to in vivo imaging analysis to examine their retention in the skin of the inoculation site. Photographs were taken 2 d after the larva inoculation. Bars: (A) 500 µm; (B) 200 µm; (C) 1 mm. (D) The skin of the inoculation site was isolated 2 d after the first and second inoculation and subjected to RT-PCR analysis. The relative expression of Nb *Actin* mRNA is shown (mean ± SEM; $n = 3$ each), and the level of expression in first infection was set as 1. (E) Larvae were isolated from the skin of the inoculation site at the indicated time points after the first and second inoculation. The number of isolated larvae at each time point is plotted (mean ± SEM; $n = 3$ each). Data shown in A–E are representative of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Antibodies and FcεRI are required for the larval retention in the skin

We next examined the possible mechanisms underlying larval retention in the skin during the second but not first Nb infections. A previous study has demonstrated that Nb infection elicits the production of anti-Nb antibodies (Lebrun and

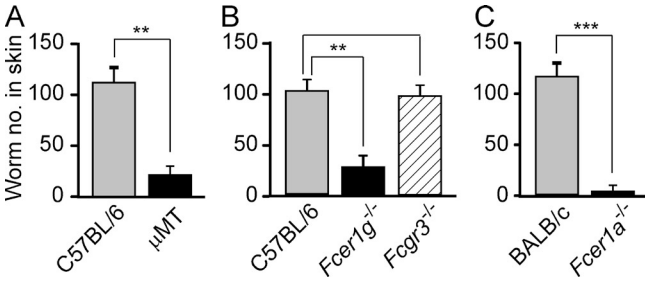

Figure 3. Antibodies and FcεRI but not FcγRIII are required for larval retention in the skin. (A–C) WT, μMT, *Fcer1g*$^{-/-}$, or *Fcgr3*$^{-/-}$ C57BL/6 mice (A and B) and WT or *Fcer1a*$^{-/-}$ BALB/c mice (C) were infected twice with Nb larvae. The skin of the larva inoculation site was isolated on day 2 of the second infection, and the number of larvae was enumerated (mean ± SEM; n = 3 each). Data shown are representative of three independent experiments. **, P < 0.01; ***, P < 0.001.

Spiegelberg, 1987), suggesting that the preexisting anti-Nb antibodies in the second infection may contribute to larval retention. Indeed, B cell–deficient μMT mice showed impaired larval retention (Fig. 3 A). In accordance with this, FcRγ-deficient mice lacking activating receptors for IgG and IgE also displayed impaired larval retention (Fig. 3 B). Importantly, mice deficient for FcεRIα but not FcγRIII showed the impairment in the larval retention (Fig. 3, B and C), suggesting that IgE rather than IgG raised against Nb after the first infection mediates the larval retention during the second Nb infection and therefore either mast cells or basophils or both may contribute to the larval retention.

Ablation of basophils but not mast cells abolishes larval retention in the skin

The number of tissue-resident mast cells in the larva-inoculated skin increased during the second Nb infection (Fig. 1 B), suggesting the possible involvement of mast cells in the larval retention. However, mast cell–deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice showed the larval retention as did mast cell–sufficient mice (Fig. 4 A). Thus, mast cells had little or no contribution to the larval retention. Immunohistochemical analysis readily identified clusters of mouse mast cell protease 8 (mMCP-8)$^{+}$ cells (Ugajin et al., 2009) within nodule-like structures composed of infiltrates surrounding larvae (Fig. 4 B), indicating the accumulation of basophils in close proximity to skin-trapped larvae. To examine the possible contribution of basophils to larval retention, we took advantage of *Mcpt8*^{DTR} mice, in which only basophils express human diphtheria toxin (DT) receptor (DTR), and hence they can be selectively and transiently ablated on demand by the treatment of mice with DT (Wada et al., 2010). DT treatment of *Mcpt8*^{DTR} mice before the second infection impaired the skin infiltration of basophils, as expected, whereas it had no significant impact on the accumulation of eosinophils, neutrophils, or mast cells at the inoculation site (Fig. 4 C). Intriguingly, the number of macrophages in the skin lesion was significantly reduced in DT-treated mice compared with control PBS-treated mice (Fig. 4 C). DT treatment showed no impact on the number of ILC2s in the skin lesions

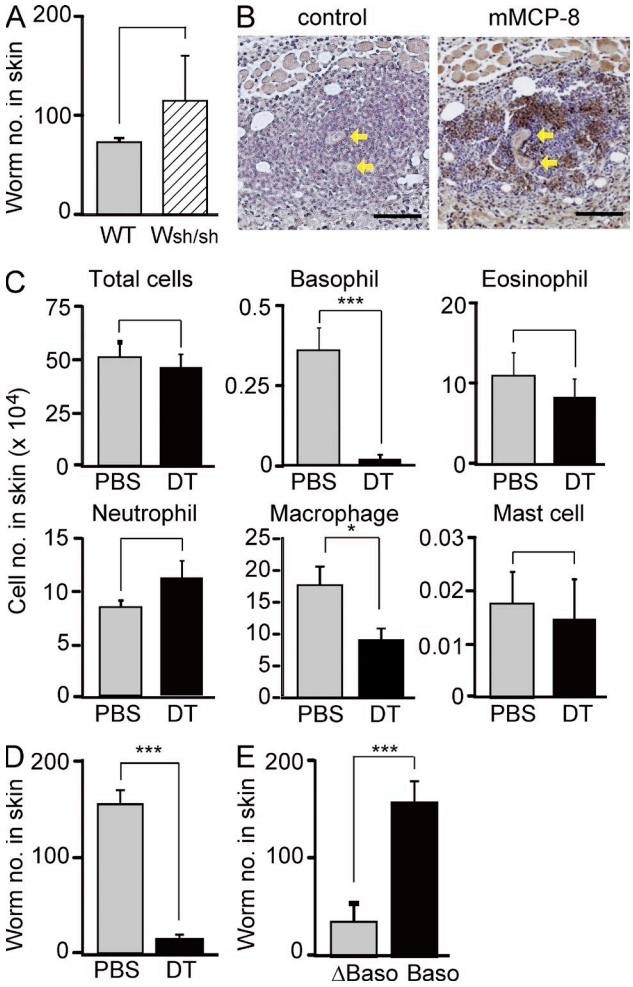

Figure 4. Ablation of basophils but not mast cells abolishes larval retention in the skin. (A) WT and mast cell–deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ C57BL/6 mice were infected twice with Nb, and larvae in the skin were enumerated on day 2 of the second infection. (B) The skin of the inoculation site in BALB/c mice was isolated on day 2 of the second infection and subjected to immunohistochemical analysis of tissue sections stained with anti–mMCP-8 (right, brown) or control (left) antibody. Yellow arrows indicate larvae trapped within clusters of cellular infiltrates. Data shown are representative of four independent experiments. Bars, 200 μm. (C and D) *Mcpt-8*^{DTR} BALB/c mice were treated with DT or vehicle (PBS) 1 d before the second inoculation. The skin of the larva inoculation site was isolated 2 d after the second inoculation, and infiltrating cells (C) and larvae (D) were enumerated. (E) Basophils and nonbasophil cells (gray bar) were separately isolated from the bone marrow and spleen of WT C57BL/6 mice 2 d after the second larval inoculation and intraperitoneally transferred into *Fcer1g*$^{-/-}$ C57BL/6 mice (4 × 10^{4} cells/mouse) that had been infected with larvae 18 d before. On the day of cell transfer, the recipient mice were subjected to the second Nb infection, and 2 d later the skin of the larva inoculation site was isolated and larvae were counted. Data shown in A and C–E are the mean ± SEM (n = 3 each) and are representative of at least three independent experiments. *, P < 0.05; ***, P < 0.001.

(not depicted). Of note, the larval retention in the skin was almost completely abolished by the treatment with DT but not control PBS (Fig. 4 D). Furthermore, the adoptive transfer of basophils (but not nonbasophil cells) isolated from Nb-infected

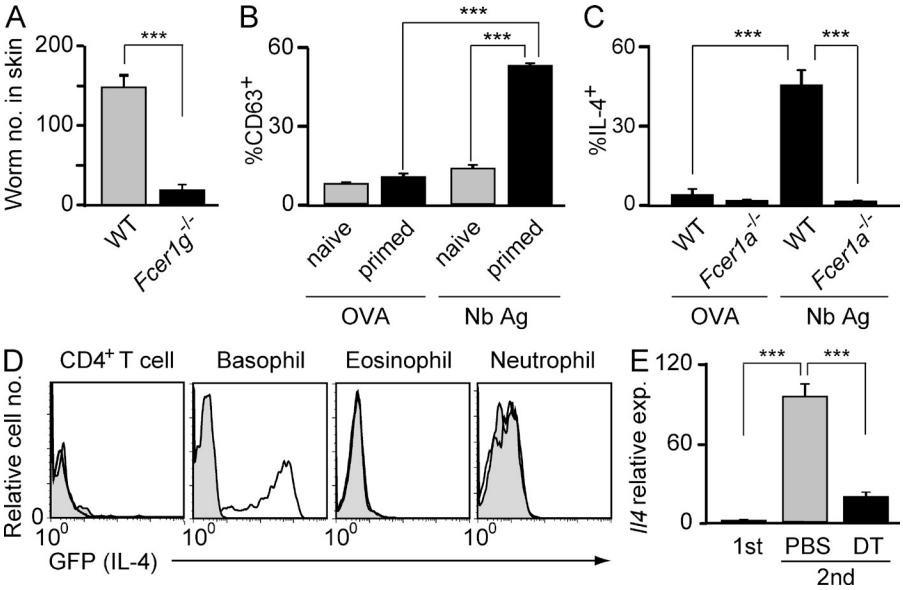

Figure 5. FcεRI on basophils is essential for larval retention in the skin and their activation in response to Nb antigens. (A) Basophils were isolated from the bone marrow and spleen of WT or *Fcer1g*$^{-/-}$ C57BL/6 mice 2 d after the second larval inoculation and adoptively transferred into *Mcpt8*^{DTR} C57BL/6 mice (4 × 10^4 cells/mouse) that had been infected with larvae 18 d before and treated with DT 1 d before. On the day of cell transfer, the recipient mice were subjected to the second Nb infection, and 2 d later the skin of the larva inoculation site was isolated and larvae were counted (mean ± SEM; *n* = 3 each). (B) Basophils were enriched from the spleen of uninfected (naive) BALB/c mice or mice infected once with Nb 18 d before (primed) and incubated ex vivo with Nb antigens or control OVA at 37°C for 20 min, followed by flow cytometric analysis for the CD63 expression on their surface. (C) Basophils were enriched from the spleen of primed WT or *Fcer1a*$^{-/-}$ BALB/c mice and incubated ex vivo with Nb antigens or control OVA at 37°C for 6 h, followed by flow cytometric analysis for intracellular IL-4. In B and C, the frequency (%) of cells positive for CD63 and IL-4 among the basophil population in each group is shown (mean ± SEM; *n* = 4 each). (D) G4 mice (open histograms) and C57BL/6 mice (shaded histograms) were infected twice with Nb larvae, the skin of the larva inoculation site was isolated on day 2 of the second infection, and GFP expression in the indicated cell types was analyzed by flow cytometry. (E) *Mcpt8*^{DTR} BALB/c mice were infected with Nb once or twice and treated with DT or vehicle (PBS) 1 d before the second inoculation. The skin of the larva inoculation site was isolated 2 d after the inoculation and subjected to RT-PCR analysis for the *Il4* expression (mean ± SEM; *n* = 4 each). The level of expression in the first infection was set as 1. Data shown in A–E are representative of at least two independent experiments. ***, P < 0.001.

WT mice conferred the ability of larval retention during the second infection on *Fcer1g*$^{-/-}$ mice (Fig. 4 E). These results demonstrated that basophils rather than eosinophils, neutrophils, or mast cells play a key role in larval trapping in the skin during the second infection.

FcεRI on basophils is essential for the larval retention in the skin and their activation in response to Nb antigens

The importance of both FcεRI (Fig. 3 C) and basophils (Fig. 4, D and E) in larval trapping during the second infection suggested that basophils exert their function via FcεRI-mediated activation in response to IgE plus Nb antigens. Indeed, the adoptive transfer of basophils isolated from Nb-infected WT but not *Fcer1g*$^{-/-}$ mice conferred the ability of larval retention on *Mcpt8*^{DTR} mice that had been treated with DT to deplete basophils (Fig. 5 A). Basophils from previously infected (primed) but not uninfected (naive) WT mice were activated ex vivo in response to Nb antigens but not control OVA, as assessed by up-regulation of CD63 expression on their surface (Fig. 5 B). Moreover, primed basophils produced IL-4 ex vivo in response to Nb antigens in an FcεRI-dependent manner (Fig. 5 C), suggesting that basophils recruited to the larva-inoculated skin might produce IL-4 during the second Nb infection. Indeed, flow cytometric analysis of IL-4 reporter G4 mice demonstrated that among cells accumulating in the larva-inoculated skin during the second infection, basophils but not CD4$^+$ T cells, eosinophils, or neutrophils expressed high levels of GFP (IL-4; Fig. 5 D). DT-mediated basophil ablation in *Mcpt8*^{DTR} mice before the second Nb inoculation greatly diminished IL-4 expression in the skin lesions during the second infection (Fig. 5 E). These results indicated that basophils were the major producer of IL-4 in the skin lesions. In contrast to IL-4 expression, IL-13 expression in the skin lesions was not up-regulated during the second infection and remained unaltered after DT treatment (not depicted).

Basophils promote the generation of M2-type macrophages in the skin lesions, leading to the larval trapping

Two observations, the IL-4 production by basophils (Fig. 5, C–E) and the accumulation of macrophages (Fig. 1 B) in the skin lesions, prompted us to examine the possibility that basophil-derived IL-4 might promote the generation of M2-type (alternatively activated) macrophages, as we recently found in IgE-mediated cutaneous allergic inflammation (Egawa et al., 2013). Indeed, macrophages isolated from the skin lesions 2 d after the second inoculation showed up-regulated M2 markers, such as *Arg1*, *Chi3l3*, and *Pdcd1lg2*, and the DT-mediated basophil ablation before the second inoculation in *Mcpt8*^{DTR} mice resulted in impaired up-regulation of the M2 markers (Fig. 6 A). Programmed death 1 ligand 2 (PD-L2) is a cell surface marker of M2-type macrophages (Loke and Allison, 2003; Egawa et al., 2013). Flow cytometric analysis revealed that PD-L2$^+$ M2-type macrophages accumulated in the larva inoculation site during the second but not first Nb infection and that the DT-mediated basophil ablation

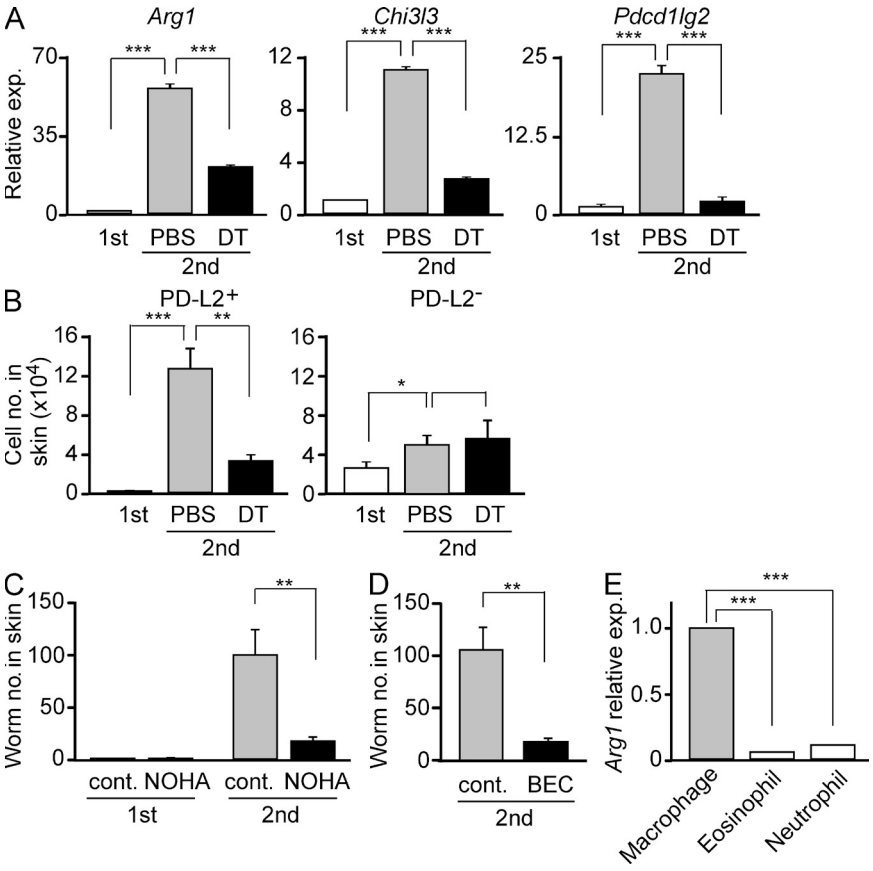

Figure 6. Basophils promote the generation of M2-type macrophages expressing Arg1, which contributes to the larval trapping in the skin. (A and B) *Mcpt8*^{DTR} BALB/c mice were infected with Nb once (white bars) or twice. Mice infected twice were treated with DT or control PBS 1 d before the second inoculation. (A) Gr-1^{lo}F4/80^{+}FSC^{hi}SSC^{lo} macrophages were isolated from the skin of the larva inoculation site 2 d after the final inoculation, and expression of the indicated genes was analyzed by RT-PCR (mean ± SEM; n = 4 each). The level of expression in the first infection was set as 1. (B) PD-L2 expression on Gr-1^{lo}F4/80^{+}FSC^{hi}SSC^{lo} macrophages in the skin lesion was analyzed by flow cytometry, and the numbers of PD-L2^{+} and PD-L2^{-} macrophages were calculated (mean ± SEM; n = 4 each). (C) BALB/c mice were infected with Nb once or twice and treated twice with nor-NOHA or control PBS (cont.) before and after the final larva inoculation. The skin of the larva inoculation site was isolated 2 d after the final inoculation, and larvae were counted (mean ± SEM; n = 4). (D) BALB/c mice were infected twice with Nb and received drinking water containing BEC or not (cont.) during the second infection. The number of larvae trapped in the skin was determined as in C. (E) BALB/c mice were infected twice with Nb larvae. On day 2 of the second infection, macrophages (Gr-1^{lo}F4/80^{+}FSC^{hi}SSC^{lo}), eosinophils (Gr-1^{lo}FSC^{lo}SSC^{hi}), and neutrophils (Gr-1^{hi}) were separately isolated from the skin of the second inoculation site, and *Arg1* expression was analyzed by RT-PCR (mean ± SEM; n = 4 each). The relative expression in macrophages was set as 1. Data shown in A–E are representative of two or three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

led to a strong reduction of PD-L2^{+} but not PD-L2^{-} macrophages (Fig. 6 B).

We next examined the functional significance of the basophil-elicited M2 generation in terms of the larval trapping in the skin. Treatment of mice with an inhibitor of Arg1, *N*ω-hydroxy-nor-L-arginine (nor-NOHA), in the first Nb infection showed no significant effect on the larval migration out of the skin during the first infection (Fig. 6 C). In contrast, nor-NOHA treatment in the second infection strongly inhibited larval trapping (Fig. 6 C), even though it had no significant effect on the number of basophils and macrophages accumulating in the skin lesions (not depicted). Another Arg1 inhibitor, S-(2-boronoethyl)-L-cysteine (BEC), showed a comparable inhibitory effect on the larval trapping (Fig. 6 D). Among three major cell types accumulating in the skin lesions, macrophages expressed a much higher level of *Arg1* than eosinophils and neutrophils (Fig. 6 E). In accordance with this, clodronate liposome–mediated depletion of macrophages before the second Nb inoculation (Fig. 7 A) resulted in the drastic reduction of *Arg1* expression in the skin lesion (Fig. 7 B) and impaired trapping of larvae in the skin (Fig. 7 C). Notably, mice deficient for chemokine receptor CCR2 showed impaired accumulation of macrophages in the skin lesions (Fig. 7 D) as we previously demonstrated in IgE-mediated cutaneous allergic inflammation (Egawa et al., 2013), concomitantly with reduced *Arg1* expression (Fig. 7 E) and impaired larval trapping in the skin lesions (Fig. 7 F). Adoptive transfer of CCR2^{+}Ly6C^{+} monocytes isolated from naive WT mice restored the larval trapping in CCR2-deficient mice (Fig. 7 G). Moreover, the treatment of BALB/c mice with an anti–IL-4 neutralizing antibody before the second infection abolished the larval trapping in the skin (Fig. 7 H). These observations suggest that CCR2^{+} inflammatory monocytes are recruited to the second inoculation site and differentiate into Arg1^{+} M2-type macrophages under the influence of basophil-derived IL-4, and those basophil-elicited M2-type macrophages contribute to the larval trapping in the skin, at least in part, through their expression of Arg1. When mice were reinfected through percutaneous exposure of larvae in place of intradermal inoculation, we observed larval trapping (assessed by the expression of Nb *Actin* mRNAs) and *Arg1* up-regulation in the skin (Fig. 7 I), suggesting that skin trapping of larvae mediated by M2-type macrophages also occurs in natural infection.

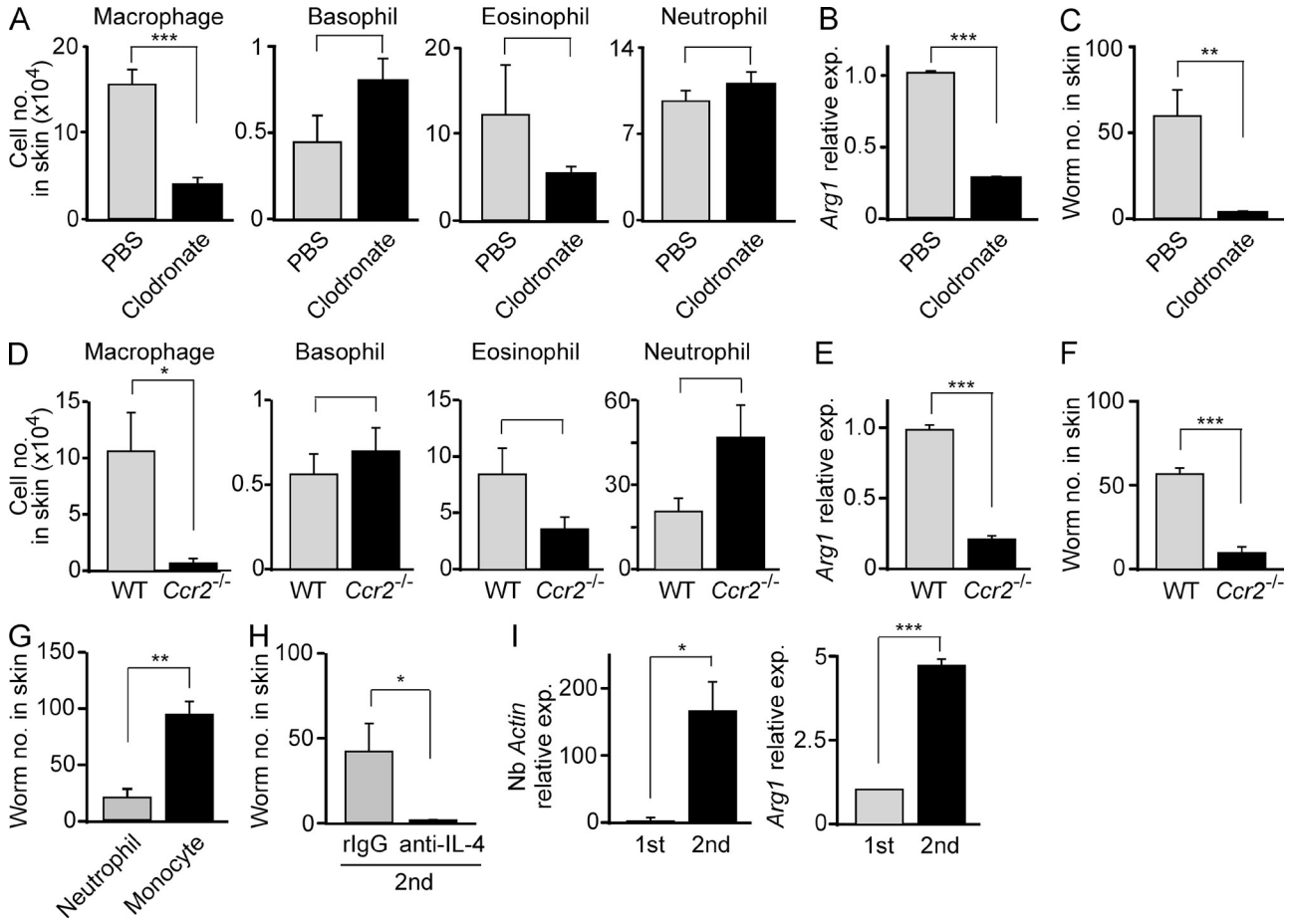

Figure 7. Ablation of macrophages in the infected skin results in reduced Arg1 expression and impaired larval trapping. (A–C) BALB/c mice were infected twice with Nb and treated with clodronate or control PBS liposomes before the second Nb infection. The skin of the larva inoculation site was isolated 2 d after the second inoculation, and infiltrating cells (A) and larvae (C) were enumerated. (B) Expression of *Arg1* was assessed by RT-PCR (mean ± SEM; $n = 4$). (D–F) WT and *Ccr2*$^{-/-}$ BALB/c mice were infected twice with Nb larvae. The skin of the larva inoculation site was isolated 2 d after the second inoculation, and infiltrating cells (D) and larvae (F) were enumerated. (E) *Arg1* expression was analyzed by RT-PCR (mean ± SEM; $n = 3$ each). (G) *Ccr2*$^{-/-}$ BALB/c mice were infected twice with Nb larvae. In the second infection, 3×10^5 monocytes (CD115$^+$Ly6C^{hi}Ly6G$^-$CD11c$^-$) or control neutrophils (Ly6G$^+$Siglec-F$^-$CD11c$^-$) sorted from bone marrow cells of naive BALB/c mice were adoptively transferred by intradermal injection together with larvae. On day 2 of the second infection, the number of larvae in the skin was counted (mean ± SEM; $n = 3$ each). (H) BALB/c mice were infected twice with Nb larvae and treated with anti–IL-4 or control rat IgG before the second inoculation. The skin of the larva inoculation site was isolated 2 d after the second inoculation, and larvae were counted (mean ± SEM; $n = 3$ each). (I) BALB/c mice were left uninfected or infected with Nb by subcutaneous injection of 500 larvae in the back and then percutaneously infected with Nb by laying 100 larvae on shaved abdominal skin for 1 h. The skin of the larva inoculation site was isolated 2 d after the final inoculation, and Nb *Actin* and mouse *Arg1* mRNA expression was analyzed by RT-PCR (mean ± SEM; $n = 3$ each). The relative expression in the first infection was set as 1. Data shown in A–I are representative of two or three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Larval trapping in the infected skin reduces worm burden and tissue injury in the lung

We next examined the functional consequence of the larval trapping in the skin during the second infection. While the number of larvae recovered from the lung peaked on day 2 after inoculation in both the first and second Nb infection, 10 times less the number of larvae were recovered in the second infection than in the first one (~20 versus ~200; Fig. 8 A). DT treatment of *Mcpt8*^{DTR} mice before the second infection significantly increased the number of larvae recovered from the lung (Fig. 8 B), concomitantly with the decreased larval retention in the skin (Fig. 4 D). Thus, the larval trapping in the infected skin greatly reduced the worm burden in the lung during the second infection.

After Nb larvae reach the lung via the circulation, they pierce the pulmonary capillaries and enter the alveolar spaces, leading to bleeding and damage of the lung (Marsland et al., 2008; Chen et al., 2012). Therefore, we next compared the extent of lung injury on day 2 of the second infection in *Mcpt8*^{DTR} mice treated with either DT or control PBS before the second inoculation. Macroscopic hemorrhage in the lung was more prominent in the DT-treated mice than in the

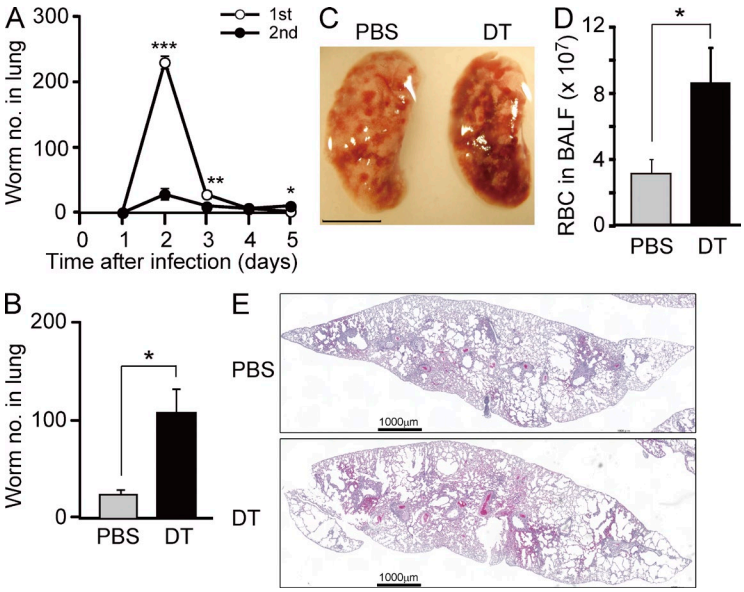

Figure 8. Basophil ablation in the second Nb infection increases the worm burden in the lung and exacerbates lung injury. (A) BALB/c mice were infected with Nb once or twice, and larvae were isolated from the lung and enumerated at the indicated time points after the first and second larva inoculation (mean ± SEM; $n = 3$ each). (B–E) *Mcpt8*^{DTR} BALB/c mice were treated with DT or control PBS 1 d before the second Nb inoculation. (B) The numbers of larvae isolated from the lung 2 d after the second inoculation (mean ± SEM; $n = 4$ each). (C) Photographs of the left lung lobe isolated 2 d after the second inoculation. Bar, 5 mm. (D) The number of RBCs in the bronchoalveolar lavage fluid (BALF) collected 2 d after the second inoculation (mean ± SEM; $n = 4$ each). (E) Tissue sections of lung isolated 2 d after the second inoculation and stained with hematoxylin and eosin. Data shown in A–E are representative of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

PBS-treated control mice (Fig. 8 C), and the number of red blood cells in bronchoalveolar lavage fluid was significantly higher in the former (Fig. 8 D). Moreover, histological analysis of the lung showed more severe bleeding and emphysema (dilation of distal airspaces caused by loss of alveolar septa) in the former (Fig. 8 E). Collectively, the larval trapping in the infected skin reduced the lung injury caused by migratory larvae during the second Nb infection.

Basophils have little or no contribution to worm expulsion from the small intestine

The protective immunity against Nb infection has been traditionally evaluated by the extent of worm expulsion from the intestine, and previous studies drew opposing conclusions in terms of the role for basophils in the second Nb infection on the basis of worm expulsion (Ohnmacht et al., 2010; Ohnmacht and Voehringer, 2010; Sullivan et al., 2011). We therefore revisited this issue and examined the effect of DT-mediated basophil ablation on the worm expulsion in *Mcpt8*^{DTR} mice. The number of worms recovered from the small intestine on day 5 of the first infection was 200–300 (Fig. 9 A). In contrast, the number of worms recovered on day 5 of the second infection was nearly zero, regardless of the treatment with DT or control PBS 1 d before the second Nb inoculation (Fig. 9 A), in spite of the fact that the DT treatment increased the number of worms recovered from the lung (Fig. 8 B). When *Mcpt8*^{DTR} mice were treated on day 2 of the second Nb infection, namely after the majority of larvae had left the skin, the number of worms recovered from the small intestine was again nearly zero, as observed in PBS-treated control mice (Fig. 9 B). Immunohistochemical analysis with a basophil-specific anti–mMCP-8 mAb revealed no apparent infiltration of basophils in the small intestine of Nb-infected mice on day 5 of the second infection as in that of naive mice, even though substantial numbers of basophils were readily detected in the small intestine of mice infected twice with another intestinal helminth *Heligmosomoides polygyrus* (Hp) that intrudes into the submucosa of the small intestine (Fig. 9, C and D). Collectively, basophils showed no apparent contribution locally or systemically to the Nb worm expulsion from the small intestine.

DISCUSSION

The elucidation of the mechanism by which host animals manifest the strong resistance to reinfection with helminths is essential for the development of effective antihelminth vaccines. The present study identified five key elements in the acquired protective immunity against reinfection with an intestinal helminth Nb: (1) the skin of the larval penetration site is an important place for the manifestation of acquired protection, (2) basophils recruited to the infected skin during the second infection play a critical role in the acquired protection by triggering the immobilization of migratory larvae within infected skin, (3) IgE raised against Nb antigens contributes to the manifestation of acquired protection by sensitizing basophils through binding to their FcεRI, (4) IL-4 produced by IgE- and Nb antigen–stimulated basophils promotes the generation of M2-type macrophages, which in turn express Arg1 and contribute to the larval trapping in the skin, and (5) larval trapping in the infected skin reduces the worm burden in the lung and hence protects host animals from severe lung injury caused by the larval migration through the lung.

No apparent trapping of Nb larvae in the skin during the second infection was demonstrated in previous studies (Knott et al., 2007; Harvie et al., 2010). One study examined the larval retention in the skin by creating air pouches in the skin, in that larvae were inoculated into and then recovered from air pouches (Knott et al., 2007). Based on our observations, we assume that inoculated larvae might have migrated from the air pouch into surrounding skin and been retained within the skin during the second infection, and hence no larvae could

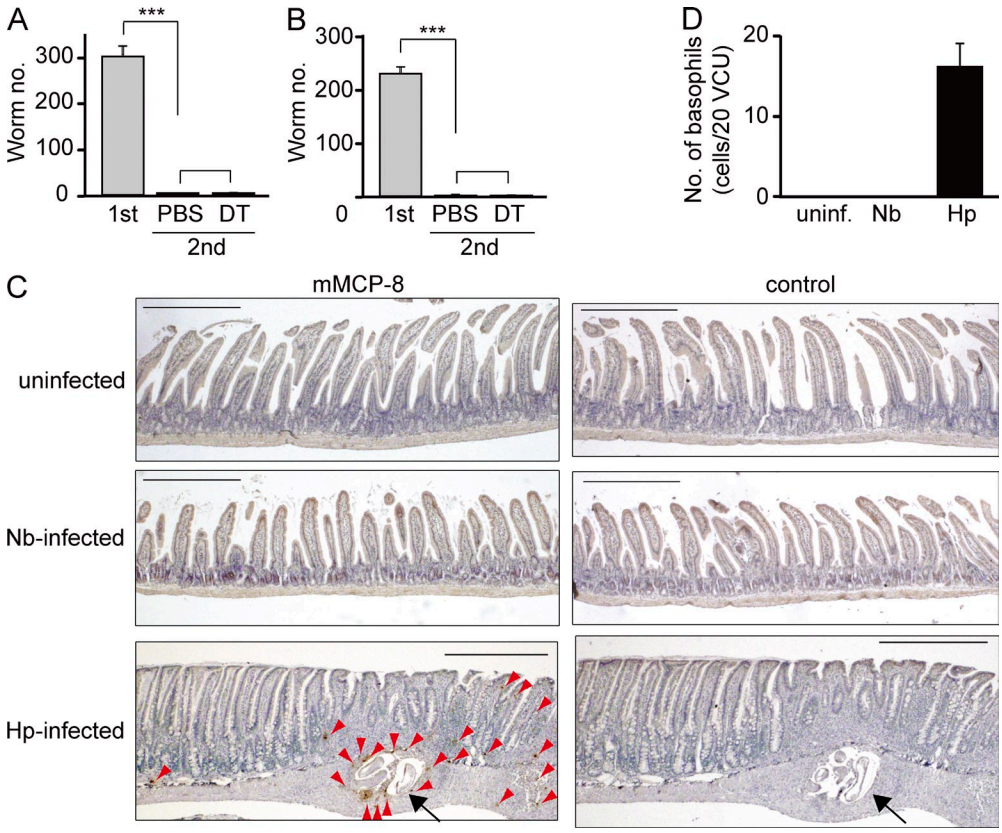

Figure 9. Little or no contribution of basophils to worm expulsion from the small intestine. (A and B) *Mcpt8*^{DTR} BALB/c mice were infected with Nb once or twice and treated with an intravenous injection of DT or control PBS 1 d before (A) or 2 d after (B) the second larva inoculation. The numbers of worms recovered from the small intestine 5 d after the final inoculation are shown (mean ± SEM; n = 3 each). (C and D) BALB/c mice were left uninfected or infected twice with Nb or Hp. On days 5 and 7 of the second infection, the small intestine was isolated and subjected to immunohistochemical analysis using basophil-specific anti–mMCP-8 mAb or isotype-matched control antibody. Representative images are shown in C. Red arrowheads indicate mMCP-8–expressing basophils (brown), and black arrows indicate Hp larvae in the submucosa. Bars, 500 µm. The number of basophils detected in epithelia, lamina propria, and submucosa per 20 villus crypt units is summarized in D (mean ± SEM; n = 3 each). Data shown are representative of three independent experiments. ***, $P < 0.001$.

be recovered from the air pouch. Another study determined the worm burden of infected tissues by counting mobile worms that could migrate out of infected tissues during in vitro culture at 37°C (Harvie et al., 2010). The histological examination in the present study identified many nodule-like aggregates of infiltrating cells surrounding larvae in the skin of reinfected mice. The digestion of skin tissues with simulated gastric juice was required for isolation of encapsulated larvae. Thus, the failure in the detection of skin-trapped larvae in previous studies could be attributed to the methods used for the recovery of larvae from the infected skin.

The role for basophils in acquired protective immunity against Nb reinfection remained controversial as long as it was assessed by the extent of worm expulsion from the intestine (Ohnmacht and Voehringer, 2010; Ohnmacht et al., 2010; Sullivan et al., 2011). Two different engineered mice with selective and constitutive defect of basophils, Basoph8 × Rosa-DTα mice and *Mcpt8Cre* mice, were independently used for the analysis of basophil function in Nb infection (Ohnmacht et al., 2010; Sullivan et al., 2011). Although both studies agreed that basophils have no apparent contribution to the worm expulsion in the first Nb infection, they drew conflicting conclusions regarding the role of basophils in the second infection (Ohnmacht et al., 2010; Sullivan et al., 2011). The number of worms recovered from the small intestine on day 5 of the second infection was nearly zero in Basoph8 × Rosa-DTα mice as observed in WT mice, concluding that basophils are dispensable for the acquired protective immunity against Nb infection (Sullivan et al., 2011). In contrast, in *Mcpt8Cre* mice, ~20–30 worms were recovered from the intestine on day 5 of the second infection, leading to the conclusion that basophils contribute to the acquired protective immunity (Ohnmacht et al., 2010). We found that the DT-mediated basophil ablation in *Mcpt8*^{DTR} mice before the second Nb inoculation resulted in no increase of worm recovery from the small intestine. In any case, considering the fact that >200 worms are usually recovered on day 5 in the first infection, the influence of basophil deficiency on worm expulsion from the small intestine during the second infection seems negligible or very subtle, regardless of the type of engineered mice examined.

When adult Nb worms were implanted directly into the duodenum of rats, worms were expelled from the intestine in a much shorter period in previously infected (primed) rats than in naive rats (Ishikawa et al., 1993). This suggests that the acquired protective immunity operates locally in the small intestine, regardless of the presence or absence of the protection at the preintestinal stage. Indeed, DT treatment of *Mcpt8*^{DTR} mice on day 2 after the second inoculation showed no significant impact on the worm expulsion. Previous studies demonstrated that antibody- or IgE-deficient mice can normally expel worms from the intestine during the second Nb infection (Jacobson et al., 1977; Watanabe et al., 1988; Harris et al., 1999; Liu et al., 2010). Collectively, basophils and antibodies appear to have little or no contribution to the acquired protective immunity in the small intestine. Instead, we demonstrated in the present study that IgE-armed basophils contribute to the earlier phase of the acquired protective immunity against Nb reinfection by means of immobilizing larvae in the skin and hence protect host animals from severe lung damage caused by the larval migration through the lung. Hookworm infections in human also induce pulmonary injury and inflammation (Sarinas and Chitkara, 1997; Akuthota and Weller, 2012), and therefore our findings may hold true for protection against hookworms in human as well.

Basophils readily generate large quantities of Th2 cytokines such as IL-4 (Piccinni et al., 1991; Seder et al., 1991), which contribute to the initiation of Th2 cell differentiation (Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009), the activation of B cells with the enhancement of humoral memory responses (Khodoun et al., 2004; Denzel et al., 2008; Chen et al., 2009; Charles et al., 2010), and the generation of antiinflammatory macrophages to dampen allergic inflammation (Egawa et al., 2013). However, the role for basophil-derived IL-4 in parasitic infections remained elusive. Basophils are dispensable for Th2 cell differentiation in Nb infection (Kim et al., 2010; Ohnmacht et al., 2010). A previous study demonstrated that IL-4 produced by memory CD4$^+$ T cells promoted the generation of M2-type macrophages in the small intestine of mice with the second Hp infection (Anthony et al., 2006). In contrast to Nb, Hp larvae enter host animals through the oral route as opposed to skin penetration and intrude into the submucosa of the small intestine. Basophils have been shown to provide some contribution to the acquired protection against Hp reinfection (Herbst et al., 2012), but the underlying mechanism remains to be investigated. We clearly demonstrated in the present study that basophils but not CD4$^+$ T cells accumulating in the Nb larva–infected skin are the major source of IL-4 that promotes M2 generation. Basophil-elicited M2-type macrophages appear to contribute to larval trapping in the infected skin, at least in part, through their expression of Arg1. An antiparasitic effect of Arg1 produced by M2-type macrophages in the intestine was demonstrated in the second Hp infection (Anthony et al., 2006). Intriguingly, M2-type macrophages generated in the lung during the late phase of the first Nb infection have been shown to contribute to the resolution of migratory larva-elicited lung injury, rather than elimination of larvae in the lung (Chen et al., 2012). Basophils are dispensable for this M2 generation, as DT-mediated basophil ablation in *Mcpt8*^{DTR} mice during the first Nb infection showed no impact on it (not depicted). Collectively, even though the underlying mechanisms are quite distinct, both M2-type macrophages generated in the lung during the late phase of the first infection and those generated in the skin during the early phase of the second infection appear to play crucial roles in limiting lung damage caused by the larval migration.

In summary, basophils play a key role in the acquired protection against Nb reinfection by restraining larvae from migrating out of their entry point in the skin, at least in part, through the IL-4–elicited generation of Arg1-expressing M2-type macrophages. Basophil-mediated larval trapping in the skin reduces the worm burden in the lung and hence protects host animals from severe lung damage caused by the migration of larvae through the lung. The present study, together with our previous study on the acquired protection against tick infestation (Wada et al., 2010), has highlighted the importance of basophil-mediated acquired immunity in the skin for the resistance to parasite reinfection and casts new light on the development of vaccines against skin-invading parasites.

METERIALS AND METHODS

Mice. BALB/c and C57BL/6J mice were purchased from CLEA Japan. *Fcer1a*$^{-/-}$ BALB/c (Dombrowicz et al., 1993) and *Fcgr3*$^{-/-}$ C57BL/6 mice (Hazenbos et al., 1996) were purchased from the Jackson Laboratory. *Kit*$^{W\text{-}sh/W\text{-}sh}$ (provided by S.J. Galli [Stanford University School of Medicine, Stanford, CA], S. Nakae [University of Tokyo, Bunkyo-ku, Tokyo, Japan], and K. Sudo [Tokyo Medical University, Shinjuku-ku, Tokyo, Japan]), μMT, *Fcer1g*$^{-/-}$, and G4 C57BL/6 mice and *Ccr2*$^{-/-}$ BALB/c mice (provided by N. Mukaida [Kanazawa University, Kanazawa, Japan] and W.A. Kuziel [Daiichi Sankyo Group, Edison, NJ]) were as described previously (Kitamura et al., 1991; Takai et al., 1994; Kuziel et al., 1997; Hu-Li et al., 2001; Grimbaldeston et al., 2005). *Mcpt8*^{DTR} BALB/c mice were generated by backcrossing the *Mcpt8*^{DTR} knockin locus in C57BL/6 mice (Wada et al., 2010) onto BALB/c mice. Mice were maintained under specific pathogen–free conditions in our animal facilities. All animal studies were approved by the Institutional Animal Care and Use Committee of the Tokyo Medical and Dental University and the Animal Care Committee of the Jikei University School of Medicine.

Antibodies. APC-conjugated anti-F4/80 (BM8), anti-Ly6G (1A8), and anti–IL-4 (11B11); APC-Cy7–conjugated anti–Gr-1 (RB6-8C5) and streptavidin; Pacific blue–conjugated anti-CD11b (M1/70); PE-conjugated anti-CD200R3 (Ba13), anti-CD63 (NVG-2), anti–PD-L2 (TY25), anti-CD11c (N418), and control rat IgG2a; biotinylated anti-CD115 (AFS98); and PE-Cy7–conjugated anti–c-kit (2B8) were purchased from BioLegend. APC-conjugated anti-CD49b (DX5), FITC-conjugated anti-Ly6C (AL-21), biotinylated anti-IgE (R35-72), and anti-CD49b (DX5) were purchased from BD. Anti–mMCP-8 mAb (TUG8) was established as reported previously (Ugajin et al., 2009).

Helminth infection and isolation of larvae from infected tissues. Mice were infected with Nb by intradermal injection of 500 third-stage larvae (L3) into the flank. For the repeated infections, mice were first injected subcutaneously with 500 L3 in the back (tail base), and 18 d later they were injected intradermally with 500 L3 in the flank. For isolation of larvae from the skin, flank skin around the inoculation site was excised. For isolation of larvae from the lung, the whole lung was surgically excised. Isolated skin and lung were finely minced with scissors and incubated with simulated gastric juice (0.24% of hydrochloric acid and 0.32% of pepsin) under constant agitation at

37°C for 2 h (Jin et al., 2008). For isolation of adult worms, the small intestine was excised and opened longitudinally, followed by incubation in PBS with a Baermann apparatus at 37°C for 2 h. The number of worms recovered from the infected tissues was counted under a microscope (SZX12; Olympus). No significant difference was observed between intradermal and subcutaneous inoculation of larvae in terms of the larval retention in the skin during the second infection. For repeated infection with Hp, mice were orally inoculated with 200 infective Hp larvae and 14 d later treated with pyrantel pamoate to expel worms, followed by reinoculation with 200 Hp larvae 5 wk later (Anthony et al., 2006; Tetsutani et al., 2009). For inhibition of arginase activity, mice were treated twice with intraperitoneal injection of 100 µg nor-NOHA (Cayman Chemical) in 0.2 ml PBS, 30 min before and 1 d after the larval inoculation. Alternatively, mice were given 0.2% BEC (Cayman Chemical) in their drinking water from 1 d before to 2 d after the final inoculation. For in vivo depletion of macrophages, mice were treated twice with intravenous injection of 0.2 ml clodronate liposomes (Van Rooijen and Sanders, 1994; or control PBS liposomes) 1 and 2 d before the second inoculation, followed by an intradermal injection of clodronate liposomes (or control PBS liposomes) simultaneously with larval inoculation. For in vivo neutralization of IL-4, mice were treated once with an intraperitoneal injection of 1 mg anti–IL-4 (11B11) or control rat IgG 1 h before the second inoculation.

In vivo imaging of larvae. Larvae were labeled with PKH26 (Sigma-Aldrich) under conditions that did not impair their viability and migratory activity and injected into the flank skin. The labeled larvae localized inside of the skin of inoculation site were detected under a multiphoton microscope (A1R MP; Nikon).

Quantitative RT-PCR. Total RNA was isolated from the skin or macrophages by using TRIzol reagent (Life Technologies). cDNAs were synthesized with ReverTra Ace-α (TOYOBO). Quantitative RT-PCR was performed on StepOne Plus (Life Technologies) using the following primer sets: for Nb *Actin*: forward, 5′-TTCAAGCAGTGTTGTCGCTGTA-3′; and reverse, 5′-CCAGTGTCGAGGACGATACCA-3′; for mouse *Hprt*: forward, 5′-GGCCAGACTTTGTTGGATTTG-3′; and reverse, 5′-CGCTCATCTTAGGCTTTGTATTTG-3′; for mouse *Il4*: forward, 5′-ACTTGAGAGAGATCATCGGCA-3′; and reverse, 5′-AGCTCCATGAGAACACTAGAGTT-3′; for mouse *Arg1*; forward, 5′-CTCCAAGCCAAAGTCCTTAGAG-3′; and reverse, 5′-AGGAGCTGTCATTAGGGACATC-3′; for mouse *Chi3l3*: forward, 5′-TCACTTACACACATGAGCAAGAC-3′; and reverse, 5′-CGGTTCTGAGGAGTAGAGACCA-3′; for mouse *Pdcd1lg2*: forward, 5′-CTGCCGATACTGAACCTGAGC-3′; and reverse, 5′-GCGGTCAAAATCGCACTCC-3′.

Flow cytometric and histological analyses. For flow cytometric analysis of skin-infiltrating cells, excised skin tissues were treated with 125 U/ml collagenase (Wako Chemicals USA) and 10 µg/ml DNase I (Roche) to obtain single-cell suspensions. Cells were preincubated with anti-CD16/32 mAb and normal rat serum on ice for 15 min to prevent the nonspecific binding of irrelevant antibodies. Cells were then stained with the indicated combination of antibodies and analyzed with FACSCanto II (BD). Each cell lineage was defined as follows: eosinophils (Gr-1^{lo}FSC^{lo}SSC^{hi}), neutrophils (Gr-1^{hi}), basophils (CD49b^{+}c-kit^{-}IgE^{+} or CD49b^{+}c-kit^{-}CD200R3^{+}), macrophages (Gr-1^{lo}F4/80^{+}FSC^{hi}SSC^{lo}), and mast cells (c-kit^{+}IgE^{+} or c-kit^{+}CD200R3^{+}). For histological analysis, isolated skin and lung tissues were fixed with 4% formalin and embedded in paraffin, followed by staining tissue sections with hematoxylin and eosin. To detect basophils infiltrating the skin, paraffin-embedded skin specimens were prepared and stained with anti–MCP-8 as described previously (Ugajin et al., 2009).

Manipulation of basophils. For basophil depletion in vivo, *Mcpt8*^{DTR} mice were treated with an intravenous injection of DT (300 ng/20g body weight; Sigma-Aldrich) or vehicle PBS as a control 1 d before the second larva inoculation. For basophil isolation, basophils were first enriched from the bone marrow and spleen by using biotin-conjugated anti-CD49b and streptavidin-IMag beads (BD), followed by sorting of CD49b^{+}CD45^{lo} cells with FACSAria II (BD). For stimulation ex vivo, basophils were incubated with L3 Nb antigens that were prepared as described previously (Holland et al., 2000) or OVA as a control. The activation of basophils was assessed by the up-regulation of surface expression of CD63 (Knol et al., 1991) and IL-4 production.

Statistical analysis. Statistical analysis was performed using an unpaired Student's *t* test. A p-value < 0.05 was considered statistically significant.

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