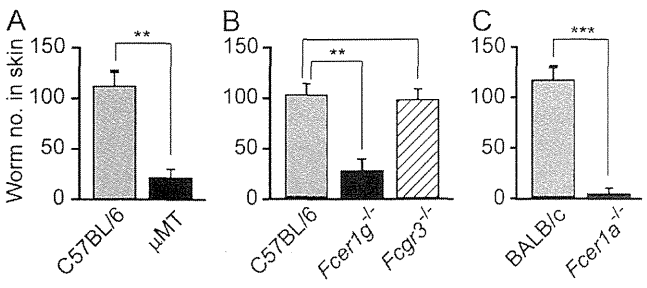

Figure 3. Antibodies and FcεRI but not FcγRIII are required for larval retention in the skin. (A–C) WT, μMT, *Fcer1g*$^{-/-}$, or *Fcgr3*$^{-/-}$ C57BL/6 mice (A and B) and WT or *Fcer1a*$^{-/-}$ BALB/c mice (C) were infected twice with Nb larvae. The skin of the larva inoculation site was isolated on 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

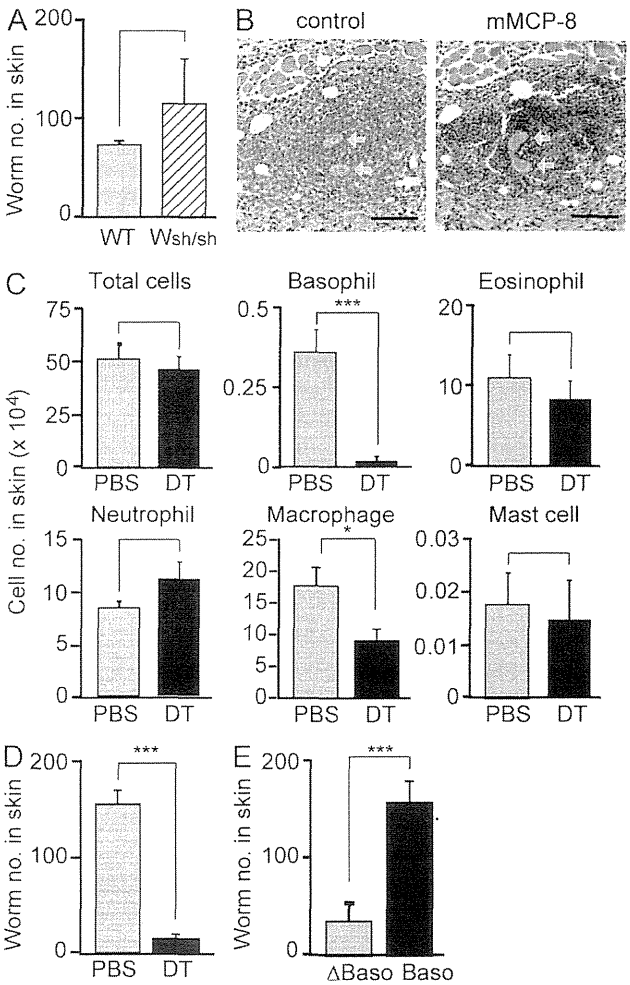

Figure 4. Ablation of basophils but not mast cells abolishes larval retention in the skin. (A) WT and mast cell–deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ C57BL/6 mice were infected twice with Nb, and larvae in the skin were enumerated on day 2 of the second infection. (B) The skin of the inoculation 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

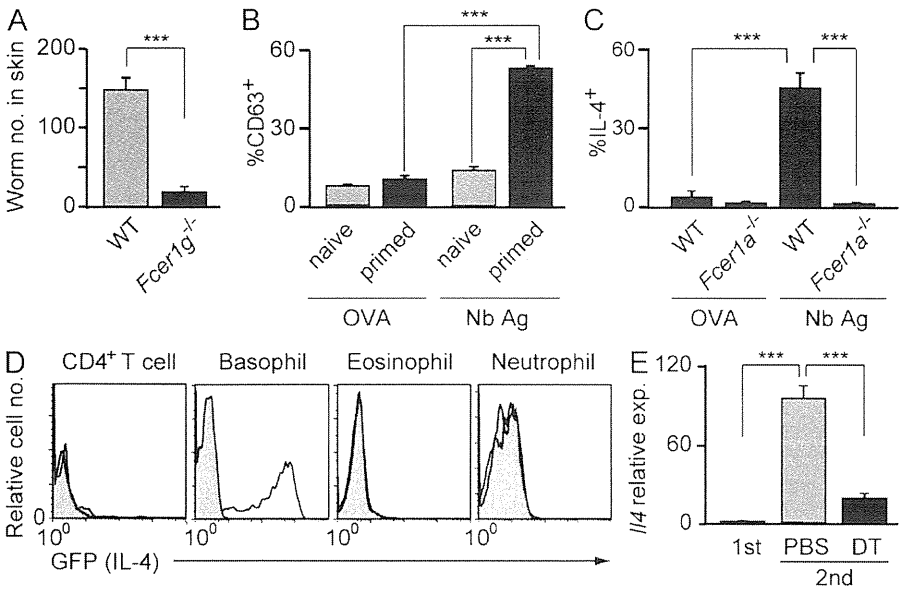

Figure 5. FcεRI on basophils is essential for larval retention in the skin and their activation in response to Nb antigens. (A) Basophils were isolated from the bone marrow and spleen of WT or *Fcer1g*$^{-/-}$ C57BL/6 mice 2 d after the second larval inoculation and adoptively transferred into *Mcpt8*^{DTR} 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

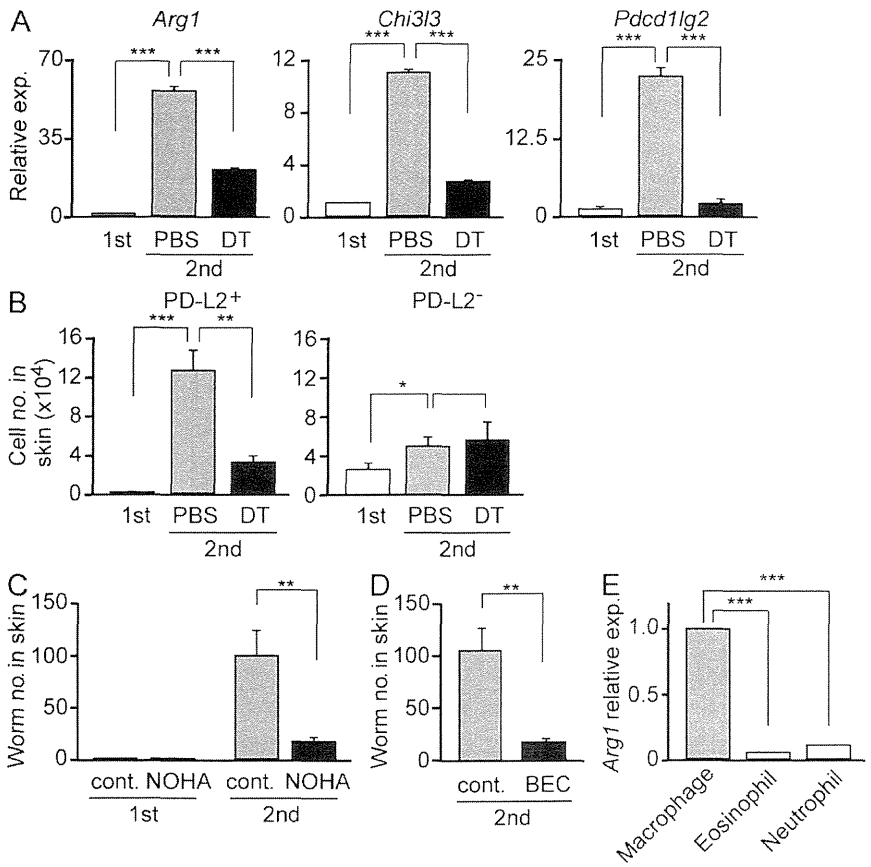

Figure 6. Basophils promote the generation of M2-type macrophages expressing Arg1, which contributes to the larval trapping in the skin. (A and B) *Mcpt8*^{DTR} BALB/c mice were infected with Nb once (white bars) or twice. Mice infected twice were treated with DT or control PBS 1 d before the second 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.

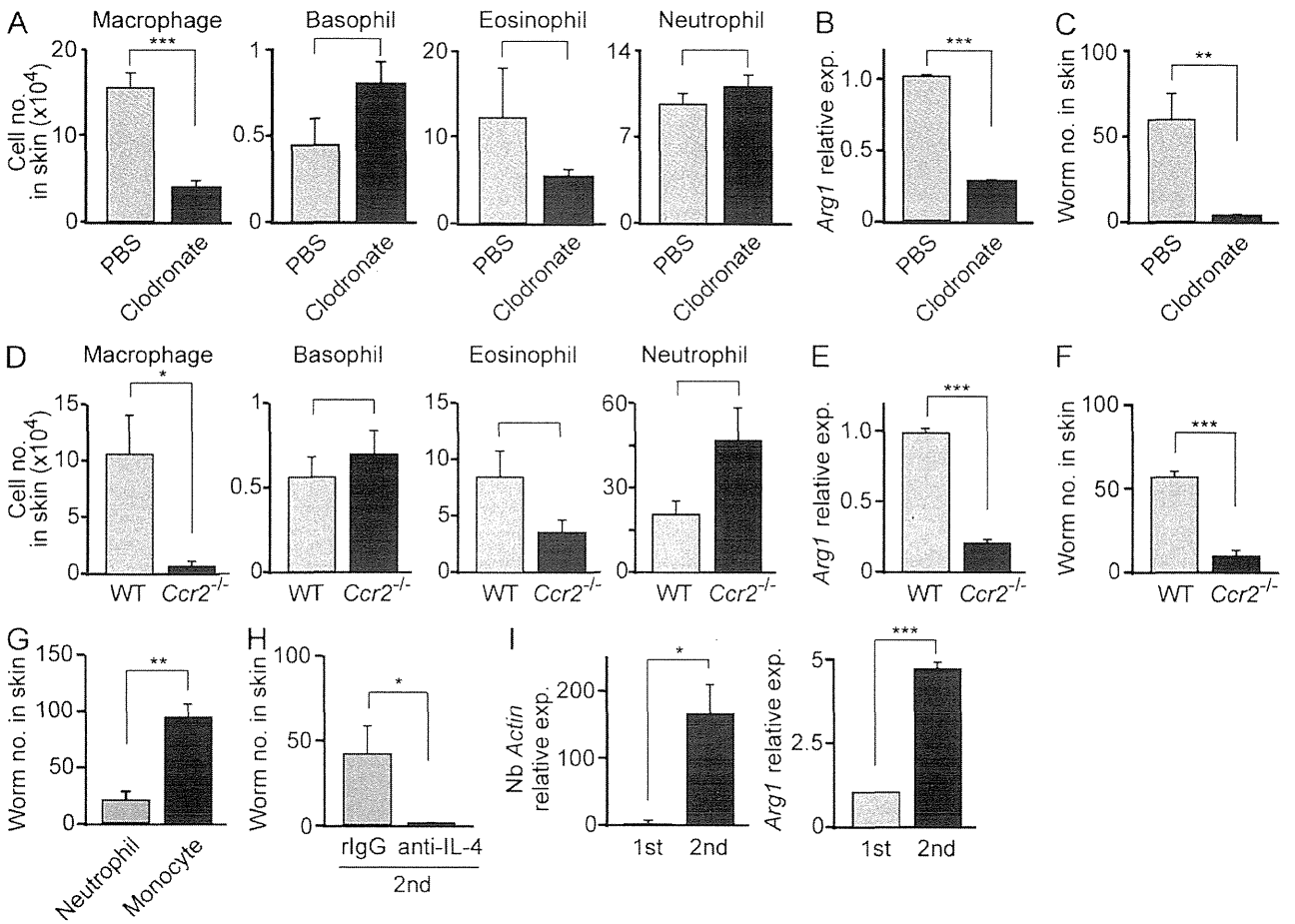

Figure 7. Ablation of macrophages in the infected skin results in reduced Arg1 expression and impaired larval trapping. (A–C) BALB/c mice were infected twice with Nb and treated with clodronate or control PBS liposomes before the second Nb infection. The skin of the larva inoculation site was isolated 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 ($Ly6G^+Siglec\text{-}F^-CD11c^-$) 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

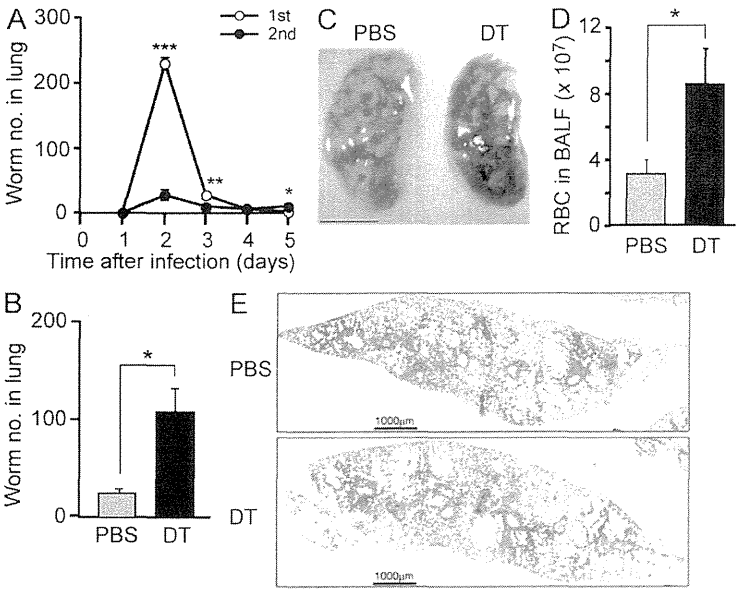

Figure 8. Basophil ablation in the second Nb infection increases the worm burden in the lung and exacerbates lung injury. (A) BALB/c mice were infected with Nb once or twice, and larvae were isolated from the lung and enumerated at the indicated time points after the first and second larva inoculation (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

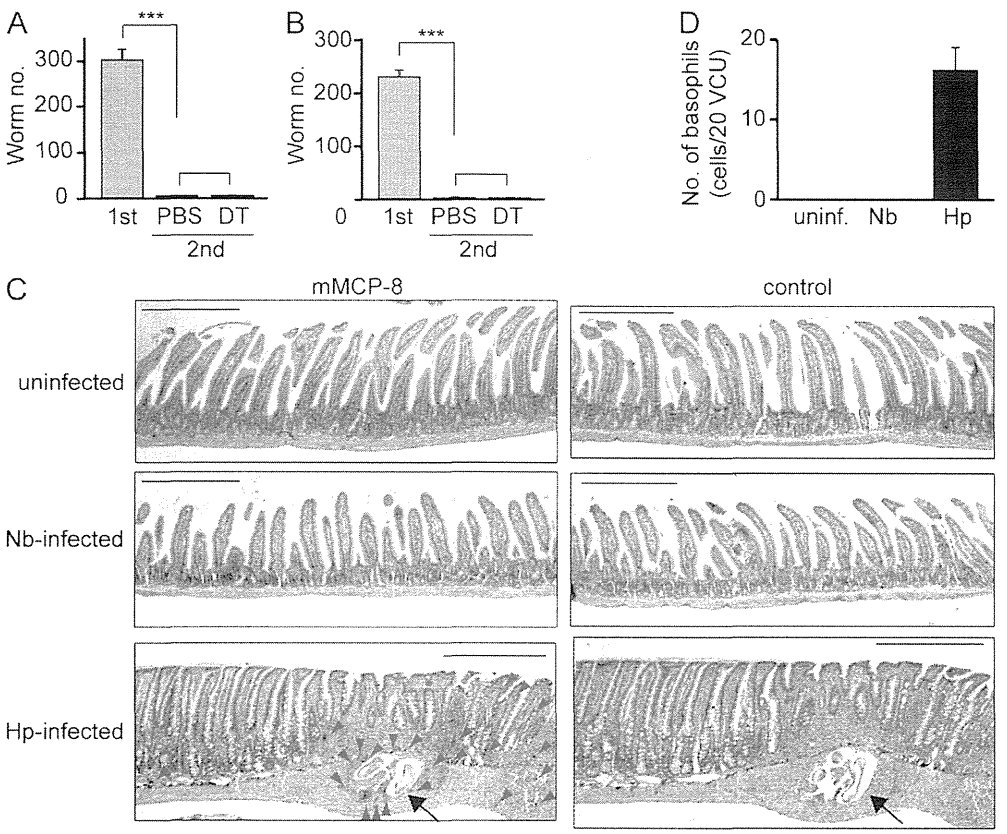

Figure 9. Little or no contribution of basophils to worm expulsion from the small intestine. (A and B) *Mcpt8*^{DTR} BALB/c mice were infected with Nb once or twice and treated with an intravenous injection of DT or control PBS 1 d before (A) or 2 d after (B) the second larva inoculation. The numbers 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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REFERENCES

Africa, C.M. 1931. Studies on the host relations of *Nippostrongylus muris* with special reference to age resistance and acquired immunity. *J. Parasitol.* 18:1–13. http://dx.doi.org/10.2307/3271737

Akuthota, P., and P.F. Weller. 2012. Eosinophilic pneumonias. *Clin. Microbiol. Rev.* 25:649–660. http://dx.doi.org/10.1128/CMR.00025-12

Allen, J.E., and R.M. Maizels. 2011. Diversity and dialogue in immunity to helminths. *Nat. Rev. Immunol.* 11:375–388. http://dx.doi.org/10.1038/nri2992

Anthony, R.M., J.F. Urban Jr., F. Alem, H.A. Hamed, C.T. Rozo, J.L. Boucher, N. Van Rooijen, and W.C. Gause. 2006. Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nat. Med.* 12:955–960. http://dx.doi.org/10.1038/nm1451

Anthony, R.M., L.I. Rutitzky, J.F. Urban Jr., M.J. Stadecker, and W.C. Gause. 2007. Protective immune mechanisms in helminth infection. *Nat. Rev. Immunol.* 7:975–987. http://dx.doi.org/10.1038/nri2199

Camberis, M., G. Le Gros, and J. Urban Jr. 2003. Animal model of *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*. *Curr. Protoc. Immunol.* Chapter 19:Unit 19.12.

Charles, N., D. Hardwick, E. Daugas, G.G. Illei, and J. Rivera. 2010. Basophils and the T helper 2 environment can promote the development of lupus nephritis. *Nat. Med.* 16:701–707. http://dx.doi.org/10.1038/nm.2159

Chen, F., Z. Liu, W. Wu, C. Rozo, S. Bowdridge, A. Millman, N. Van Rooijen, J.F. Urban Jr., T.A. Wynn, and W.C. Gause. 2012. An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection. *Nat. Med.* 18:260–266. http://dx.doi.org/10.1038/nm.2628

Chen, K., W. Xu, M. Wilson, B. He, N.W. Miller, E. Bengtén, E.S. Edholm, P.A. Santini, P. Rath, A. Chiu, et al. 2009. Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. *Nat. Immunol.* 10:889–898. http://dx.doi.org/10.1038/ni.1748

Denzel, A., U.A. Maus, M. Rodriguez Gomez, C. Moll, M. Niedermeier, C. Winter, R. Maus, S. Hollingshead, D.E. Briles, L.A. Kunz-Schughart, et al. 2008. Basophils enhance immunological memory responses. *Nat. Immunol.* 9:733–742. http://dx.doi.org/10.1038/ni.1621

Dombrowicz, D., V. Flamand, K.K. Brigman, B.H. Koller, and J.P. Kinet. 1993. Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor alpha chain gene. *Cell.* 75:969–976. http://dx.doi.org/10.1016/0092-8674(93)90540-7

Egawa, M., K. Mukai, S. Yoshikawa, M. Iki, N. Mukaida, Y. Kawano, Y. Minegishi, and H. Karasuyama. 2013. Inflammatory monocytes

recruited to allergic skin acquire an anti-inflammatory M2 phenotype via basophil-derived interleukin-4. *Immunity.* 38:570–580. http://dx.doi.org/10.1016/j.immuni.2012.11.014

Finkelman, F.D., T. Shea-Donohue, J. Goldhill, C.A. Sullivan, S.C. Morris, K.B. Madden, W.C. Gause, and J.F. Urban Jr. 1997. Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. *Annu. Rev. Immunol.* 15:505–533. http://dx.doi.org/10.1146/annurev.immunol.15.1.505

Finkelman, F.D., T. Shea-Donohue, S.C. Morris, L. Gildea, R. Strait, K.B. Madden, L. Schopf, and J.F. Urban Jr. 2004. Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunol. Rev.* 201:139–155. http://dx.doi.org/10.1111/j.0105-2896.2004.00192.x

Gause, W.C., J.F. Urban Jr., and M.J. Stadecker. 2003. The immune response to parasitic helminths: insights from murine models. *Trends Immunol.* 24:269–277. http://dx.doi.org/10.1016/S1471-4906(03)00101-7

Grimbaldeston, M.A., C.C. Chen, A.M. Piliponsky, M. Tsai, S.Y. Tam, and S.J. Galli. 2005. Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. *Am. J. Pathol.* 167:835–848. http://dx.doi.org/10.1016/S0002-9440(10)62055-X

Harris, N.L., R.J. Peach, and F. Ronchese. 1999. CTLA4-Ig inhibits optimal T helper 2 cell development but not protective immunity or memory response to *Nippostrongylus brasiliensis*. *Eur. J. Immunol.* 29:311–316. http://dx.doi.org/10.1002/(SICI)1521-4141(199901)29:01<311::AID-IMMU311>3.0.CO;2-B

Harvie, M., M. Camberis, S.C. Tang, B. Delahunt, W. Paul, and G. Le Gros. 2010. The lung is an important site for priming CD4 T-cell-mediated protective immunity against gastrointestinal helminth parasites. *Infect. Immun.* 78:3753–3762. http://dx.doi.org/10.1128/IAI.00502-09

Hazenbos, W.L., J.E. Gessner, F.M. Hofhuis, H. Kuipers, D. Meyer, I.A. Heijnen, R.E. Schmidt, M. Sandor, P.J. Capel, M. Daëron, et al. 1996. Impaired IgG-dependent anaphylaxis and Arthus reaction in Fc gamma RIII (CD16) deficient mice. *Immunity.* 5:181–188. http://dx.doi.org/10.1016/S1074-7613(00)80494-X

Herbst, T., J. Esser, M. Prati, M. Kulagin, R. Stettler, M.M. Zaiss, J.P. Hewitson, P. Merky, J.S. Verbeek, C. Bourquin, et al. 2012. Antibodies and IL-3 support helminth-induced basophil expansion. *Proc. Natl. Acad. Sci. USA.* 109:14954–14959. http://dx.doi.org/10.1073/pnas.1117584109

Holland, M.J., Y.M. Harcus, P.L. Riches, and R.M. Maizels. 2000. Proteins secreted by the parasitic nematode *Nippostrongylus brasiliensis* act as adjuvants for Th2 responses. *Eur. J. Immunol.* 30:1977–1987. http://dx.doi.org/10.1002/1521-4141(200007)30:7<1977::AID-IMMU1977>3.0.CO;2-3

Hotez, P.J., P.J. Brindley, J.M. Bethony, C.H. King, E.J. Pearce, and J. Jacobson. 2008. Helminth infections: the great neglected tropical diseases. *J. Clin. Invest.* 118:1311–1321. http://dx.doi.org/10.1172/JCI34261

Hotez, P.J., J.M. Bethony, D.J. Diemert, M. Pearson, and A. Loukas. 2010. Developing vaccines to combat hookworm infection and intestinal schistosomiasis. *Nat. Rev. Microbiol.* 8:814–826. http://dx.doi.org/10.1038/nrmicro2438

Hu-Li, J., C. Pannetier, L. Guo, M. Löhning, H. Gu, C. Watson, M. Assenmacher, A. Radbruch, and W.E. Paul. 2001. Regulation of expression of IL-4 alleles: analysis using a chimeric GFP/IL-4 gene. *Immunity.* 14:1–11. http://dx.doi.org/10.1016/S1074-7613(01)00084-X

Ishikawa, N., Y. Horii, and Y. Nawa. 1993. Immune-mediated alteration of the terminal sugars of goblet cell mucins in the small intestine of *Nippostrongylus brasiliensis*-infected rats. *Immunology.* 78:303–307.

Jacobson, R.H., N.D. Reed, and D.D. Manning. 1977. Expulsion of *Nippostrongylus brasiliensis* from mice lacking antibody production potential. *Immunology.* 32:867–874.

Jin, Z., N. Akao, and N. Ohta. 2008. Prolactin evokes lactational transmission of larvae in mice infected with *Toxocara canis*. *Parasitol. Int.* 57:495–498. http://dx.doi.org/10.1016/j.parint.2008.06.006

Khodoun, M.V., T. Orekhova, C. Potter, S. Morris, and F.D. Finkelman. 2004. Basophils initiate IL-4 production during a memory T-dependent response. *J. Exp. Med.* 200:857–870. http://dx.doi.org/10.1084/jem.20040598

Kim, S., M. Prout, H. Ramshaw, A.F. Lopez, G. LeGros, and B. Min. 2010. Cutting edge: basophils are transiently recruited into the draining lymph nodes during helminth infection via IL-3, but infection-induced Th2 immunity can develop without basophil lymph node recruitment or IL-3. *J. Immunol.* 184:1143–1147. http://dx.doi.org/10.4049/jimmunol.0902447

Kitamura, D., J. Roes, R. Kühn, and K. Rajewsky. 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature.* 350:423–426. http://dx.doi.org/10.1038/350423a0

Knol, E.F., F.P. Mul, H. Jansen, J. Calafat, and D. Roos. 1991. Monitoring human basophil activation via CD63 monoclonal antibody 435. *J. Allergy Clin. Immunol.* 88:328–338. http://dx.doi.org/10.1016/0091-6749(91)90094-5

Knott, M.L., K.I. Matthaei, P.R. Giacomin, H. Wang, P.S. Foster, and L.A. Dent. 2007. Impaired resistance in early secondary *Nippostrongylus brasiliensis* infections in mice with defective eosinophilopoeisis. *Int. J. Parasitol.* 37:1367–1378. http://dx.doi.org/10.1016/j.ijpara.2007.04.006

Kuziel, W.A., S.J. Morgan, T.C. Dawson, S. Griffin, O. Smithies, K. Ley, and N. Maeda. 1997. Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proc. Natl. Acad. Sci. USA.* 94:12053–12058. http://dx.doi.org/10.1073/pnas.94.22.12053

Lebrun, P., and H.L. Spiegelberg. 1987. Concomitant immunoglobulin E and immunoglobulin G1 formation in *Nippostrongylus brasiliensis*-infected mice. *J. Immunol.* 139:1459–1465.

Liu, Q., T. Kreider, S. Bowdridge, Z. Liu, Y. Song, A.G. Gaydo, J.F. Urban Jr., and W.C. Gause. 2010. B cells have distinct roles in host protection against different nematode parasites. *J. Immunol.* 184:5213–5223. http://dx.doi.org/10.4049/jimmunol.0902879

Loke, P., and J.P. Allison. 2003. PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. *Proc. Natl. Acad. Sci. USA.* 100:5336–5341. http://dx.doi.org/10.1073/pnas.0931259100

Love, R.J., J.D. Kelly, and J.K. Dineen. 1974. *Nippostrongylus brasiliensis*: effects of immunity on the pre-intestinal and intestinal larval stages of the parasite. *Int. J. Parasitol.* 4:183–191. http://dx.doi.org/10.1016/0020-7519(74)90103-9

Marsland, B.J., M. Kurrer, R. Reissmann, N.L. Harris, and M. Kopf. 2008. *Nippostrongylus brasiliensis* infection leads to the development of emphysema associated with the induction of alternatively activated macrophages. *Eur. J. Immunol.* 38:479–488. http://dx.doi.org/10.1002/eji.200737827

Moro, K., T. Yamada, M. Tanabe, T. Takeuchi, T. Ikawa, H. Kawamoto, J. Furusawa, M. Ohtani, H. Fujii, and S. Koyasu. 2010. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature.* 463:540–544. http://dx.doi.org/10.1038/nature08636

Neill, D.R., S.H. Wong, A. Bellosi, R.J. Flynn, M. Daly, T.K. Langford, C. Bucks, C.M. Kane, P.G. Fallon, R. Pannell, et al. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature.* 464:1367–1370. http://dx.doi.org/10.1038/nature08900

Ohnmacht, C., and D. Voehringer. 2010. Basophils protect against reinfection with hookworms independently of mast cells and memory Th2 cells. *J. Immunol.* 184:344–350. http://dx.doi.org/10.4049/jimmunol.0901841

Ohnmacht, C., C. Schwartz, M. Panzer, I. Schiedewitz, R. Naumann, and D. Voehringer. 2010. Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. *Immunity.* 33:364–374. http://dx.doi.org/10.1016/j.immuni.2010.08.011

Perrigoue, J.G., S.A. Saenz, M.C. Siracusa, E.J. Allenspach, B.C. Taylor, P.R. Giacomin, M.G. Nair, Y. Du, C. Zaph, N. van Rooijen, et al. 2009. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat. Immunol.* 10:697–705. http://dx.doi.org/10.1038/ni.1740

Piccinni, M.P., D. Macchia, P. Parronchi, M.G. Giudizi, D. Bani, R. Alterini, A. Grossi, M. Ricci, E. Maggi, and S. Romagnani. 1991. Human bone marrow non-B, non-T cells produce interleukin 4 in response to cross-linkage of Fc epsilon and Fc gamma receptors. *Proc. Natl. Acad. Sci. USA.* 88:8656–8660. http://dx.doi.org/10.1073/pnas.88.19.8656

Price, A.E., H.E. Liang, B.M. Sullivan, R.L. Reinhardt, C.J. Eisley, D.J. Erle, and R.M. Locksley. 2010. Systemically dispersed innate IL-13-expressing

cells in type 2 immunity. *Proc. Natl. Acad. Sci. USA.* 107:11489–11494. http://dx.doi.org/10.1073/pnas.1003988107

Pulendran, B., and D. Artis. 2012. New paradigms in type 2 immunity. *Science.* 337:431–435. http://dx.doi.org/10.1126/science.1221064

Sarinas, P.S., and R.K. Chitkara. 1997. Ascariasis and hookworm. *Semin. Respir. Infect.* 12:130–137.

Seder, R.A., W.E. Paul, A.M. Dvorak, S.J. Sharkis, A. Kagey-Sobotka, Y. Niv, F.D. Finkelman, S.A. Barbieri, S.J. Galli, and M. Plaut. 1991. Mouse splenic and bone marrow cell populations that express high-affinity Fc epsilon receptors and produce interleukin 4 are highly enriched in basophils. *Proc. Natl. Acad. Sci. USA.* 88:2835–2839. http://dx.doi.org/10.1073/pnas.88.7.2835

Sokol, C.L., N.Q. Chu, S. Yu, S.A. Nish, T.M. Laufer, and R. Medzhitov. 2009. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat. Immunol.* 10:713–720. http://dx.doi.org/10.1038/ni.1738

Sullivan, B.M., H.E. Liang, J.K. Bando, D. Wu, L.E. Cheng, J.K. McKerrow, C.D. Allen, and R.M. Locksley. 2011. Genetic analysis of basophil function in vivo. *Nat. Immunol.* 12:527–535. http://dx.doi.org/10.1038/ni.2036

Takai, T., M. Li, D. Sylvestre, R. Clynes, and J.V. Ravetch. 1994. FcR gamma chain deletion results in pleiotrophic effector cell defects. *Cell.* 76:519–529. http://dx.doi.org/10.1016/0092-8674(94)90115-5

Tetsutani, K., K. Ishiwata, H. Ishida, L. Tu, M. Torii, S. Hamano, K. Himeno, and H. Hisaeda. 2009. Concurrent infection with *Heligmosomoides polygyrus* suppresses anti-*Plasmodium yoelii* protection partially by induction of CD4(+)CD25(+)Foxp3(+) Treg in mice. *Eur. J. Immunol.* 39:2822–2830. http://dx.doi.org/10.1002/eji.200939433

Ugajin, T., T. Kojima, K. Mukai, K. Obata, Y. Kawano, Y. Minegishi, Y. Eishi, H. Yokozeki, and H. Karasuyama. 2009. Basophils preferentially express mouse Mast Cell Protease 11 among the mast cell tryptase family in contrast to mast cells. *J. Leukoc. Biol.* 86:1417–1425. http://dx.doi.org/10.1189/jlb.0609400

Valdivieso, D., and J.R. Tamsitt. 1969. A review of the immunology of parasitic nematodes. *Rev. Biol. Trop.* 17:1–25.

Van Rooijen, N., and A. Sanders. 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J. Immunol. Methods.* 174:83–93. http://dx.doi.org/10.1016/0022-1759(94)90012-4

Wada, T., K. Ishiwata, H. Koseki, T. Ishikura, T. Ugajin, N. Ohnuma, K. Obata, R. Ishikawa, S. Yoshikawa, K. Mukai, et al. 2010. Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. *J. Clin. Invest.* 120:2867–2875. http://dx.doi.org/10.1172/JCI42680

Watanabe, N., K. Katakura, A. Kobayashi, K. Okumura, and Z. Ovary. 1988. Protective immunity and eosinophilia in IgE-deficient SJA/9 mice infected with *Nippostrongylus brasiliensis* and *Trichinella spiralis*. *Proc. Natl. Acad. Sci. USA.* 85:4460–4462. http://dx.doi.org/10.1073/pnas.85.12.4460

Yoshimoto, T., K. Yasuda, H. Tanaka, M. Nakahira, Y. Imai, Y. Fujimori, and K. Nakanishi. 2009. Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat. Immunol.* 10:706–712. http://dx.doi.org/10.1038/ni.1737

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BRIEF COMMUNICATION

Augmentation of antitubercular therapy with IFNγ in a patient with dominant partial IFNγ receptor 1 deficiency

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Abstract Osteomyelitis due to *Mycobacterium bovis* Bacille Calmette–Guerin (BCG) often develops in patients with interferon-γ receptor 1 (IFNγR1) deficiency. In these patients, susceptibility appears to be caused by impaired interleukin-12- and IFNγ-mediated immunity. Here we report the case of a one-year-old girl with dominant partial IFNγR1 deficiency who suffered from lymphadenitis and multiple sites of osteomyelitis due to BCG infection. She was allergic to isoniazid and rifampicin – the prescribed standard treatment – and required prior desensitization therapy. She was subsequently treated with these drugs, but her symptoms did not improve. IFNγ therapy was added to the antitubercular therapy, increasing the serum level of IFNγ and leading to the resolution of the lymphadenitis and osteomyelitis. In conclusion, high dose IFNγ therapy in combination with antitubercular drugs led to resolution of BCG infection in a patient with dominant partial IFNγ deficiency.

1. Introduction

Mendelian susceptibility to mycobacterial disease (MSMD) is a rare primary immunodeficiency characterized by a deficiency in the interleukin (IL)-12/23–interferon-γ (IFNγ)

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axis. Type-1 cytokine response is crucial for human host defense against intracellular pathogens. Patients with MSMD demonstrate increased susceptibility to infections of environmental non-tuberculous mycobacteria, *Salmonella* and *Mycobacterium bovis* (*M. bovis*) Bacille Calmette–Guerin (BCG) [1,2]. Several genetic mutations have been identified in patients with MSMD. Mutations have been found in genes coding for IL-12β, IL-12 receptor β1, IFNγ receptor 1 (IFNγR1), IFNγ receptor 2 (IFNγR2), signal transducers and activator of transcription (STAT1), NF-κB essential modulator (NEMO), gp91phox, tyrosine kinase 2 (TYK2), interferon regulatory factor 8 (IRF8) and interferon-stimulated gene 15 protein (ISG15). The 818del4 mutation of *IFNGR1* gene results in a truncated protein that exerts a dominant negative effect on the wild-type IFNγR1 molecule. Accumulation of truncated IFNγR1 proteins impedes the function of normal IFNγR1 molecules encoded by the wild-type allele [3,4], leading to a diminished cellular response to ligand binding. Previous reports have shown that BCG causes recurrent and refractory osteomyelitis in patients with dominant partial IFNγR1 deficiency [5–8]. Among vaccinated children, almost 70% of those with dominant partial IFNγR1 deficiency develop osteomyelitis due to BCG [1].

Standard therapy caused by *M. bovis*, recommended by the Centers for Disease Control and Prevention (CDC) and the American Academy of Pediatrics (AAP), is a multidrug regimen that includes isoniazid, rifampin and ethambutol (*M. bovis* is inherently resistant to pyrazinamide). However, in patients with dominant partial IFNγR1 deficiency develop osteomyelitis, BCG infection is relatively resistant to this type of therapy [1,6]. Moreover, allergy against such drugs is also present in some cases. Therefore, an alternative approach is necessary for such patients.

Here, we describe a Japanese girl with dominant partial IFNγR1 deficiency who suffered from BCG multiple sites of osteomyelitis and lymphadenitis. She was allergic to isoniazid and rifampicin but was treated with the drugs following desensitization therapy. Unfortunately, standard therapy did not resolve the infection. In light of her genetic background, IFNγ was added to her antitubercular drug regimen. The combination of isoniazid, rifampicin, ethambutol and high dose IFNγ successfully cured her multi-site osteomyelitis and lymphadenitis.

2. Patient and method

The patient, a one-year-old girl (vaccinated with BCG at 2 months of age), suffered from axillary lymphadenitis at 10 months of age. Three months later, she was presented with osteomyelitis at multiple sites, including the skull, humerus, tibia and cervical vertebra (Fig. 1a). Axillary lymph node and skull tissue biopsies revealed the presence of *M. bovis* BCG (BCG Tokyo 172 strain). Immunological assessment was performed to evaluate the presence of primary phagocytic disorders, such as chronic granulomatous disease [9] or MSMD. Flow cytometry revealed a five-fold increase in the expression of IFNγR1 on $CD14^+$ monocytes isolated from the patient (Fig. 1c) (mean fluorescence intensity of IFNγR1: 10371, 51350, and 50583 for a healthy subject, the patient, and the patient's mother, respectively). The diagnosis of dominant partial IFNγR1 deficiency was confirmed by a genetic analysis that revealed one four-nucleotide deletion in exon 6 of *IFNGR1* (818del4) and one wild-type *IFNGR1* allele (Fig. 1d). Interestingly, the patient's mother carried the same *IFNGR1* deletion and suffered from one episode of osteomyelitis and multiple subcutaneous abscesses due to *Mycobacterium* spp. at one year of age, though she did not receive BCG vaccination. The patient and her mother were thus diagnosed with inherited dominant partial IFNγR1 deficiency.

3. Results

3.1. Addition of high dose IFNγ to antitubercular regimen

The patient was initially treated with isoniazid and rifampicin, based on the guidelines of the CDC and AAP. However, she developed generalized erythema multiform exudativum two weeks after commencement of treatment. Consequently, her antitubercular drug regimen was changed to ethambutol and levofloxacin due to the antimicrobial sensitivity to *M. bovis* isolated from her osteomyelitis lesion. This regimen proved ineffective in treating her lymphadenitis and osteomyelitis. We decided to put her on a two-month course of desensitization to isoniazid and rifampicin according to the Guidelines of Japanese Society of Tuberculosis [10]. Desensitization of each drug started at 0.2 mg/kg/day and the dose was doubled every 7–10 days to reach 10 mg/kg/day (Fig. 1e). Although desensitization therapy was successful, the patient failed to improve with this medical regimen.

Upon obtaining informed consent from the family, IFNγ was added to the regimen. IFNγ was initially administered subcutaneously at 250 000 JRU/m^2 per week, a sufficient dose for infection prophylaxis in chronic granulomatous disease [11], and up-titrated thereafter. Treatment with IFNγ resulted in an increase in serum IFNγ level, and a decrease in serum IL-6 level (Fig. 1e) with no change in serum TNFα and IL-12 levels (although the patient's serum TNFα level was higher than that of healthy subjects at baseline: 16.5 ± 4.0 pg/ml vs. 1.1 ± 0.9 pg/ml, respectively, $p < 0.001$). A final dose of 1250 000 JRU/m^2 IFNγ per week led to resolution of her axillary lymphadenitis and ossification of the multiple osteomyelitic lesions (Fig. 1b).

3.2. Assessment of the effect of high dose IFNγ on the patient's immune function

Peripheral blood mononuclear cells (PBMCs) were isolated from the patient, the patient's mother and healthy subjects (n = 3), and stimulated with lipopolysaccharide (LPS) with or without IFNγ. The amount of TNFα produced by the stimulated PBMCs was measured by quantitative multiplex detection using Milliplex (Millipore, Billerica, MA). When PBMCs were stimulated with LPS alone, similar amounts of TNFα were produced (Fig. 2). In PBMCs isolated from healthy donors, the addition of IFNγ led to an increase in TNFα production in a dose-dependent manner; whereas in PBMCs isolated from the patient and her mother, TNFα production increased only when IFNγ was added at the maximum concentration (10^5 JRU/mL) (Fig. 2).

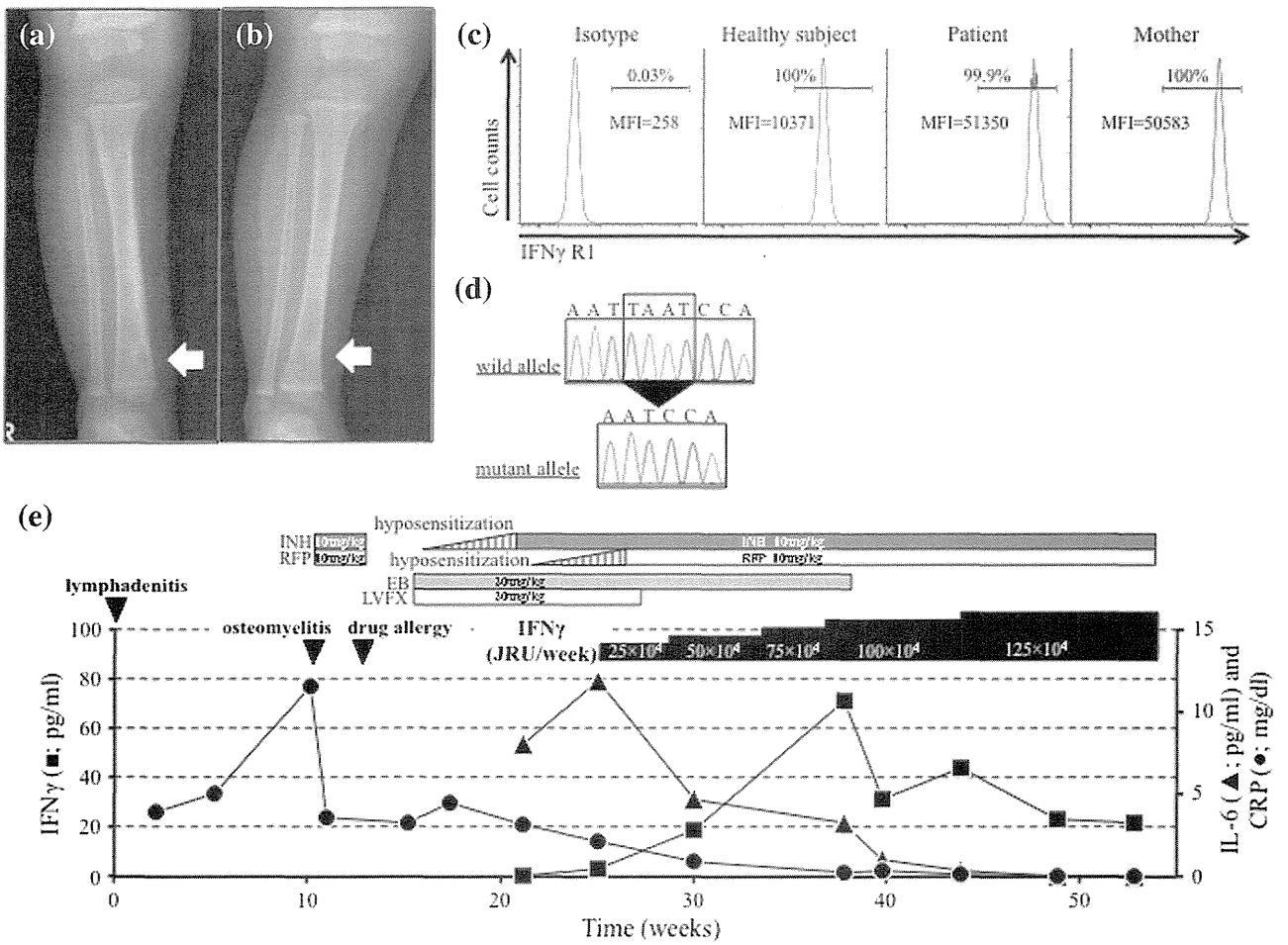

Figure. 1 Clinical course of BCG osteomyelitis in a patient with dominant partial IFNγ receptor 1 deficiency. (a)–(b) X-rays demonstrating osteomyelitis of the right tibia before IFNγ administration (a) and ossification of the lesion following IFNγ therapy (b). (c) Representative flow cytometry data. PBMCs were identified with FACSAria IIIu using anti-human CD14 and IFNγR1 monoclonal antibodies conjugated with allophycocyanin and phycoerythrin, respectively (BioLegend, San Diego, CA). MFI, mean fluorescence intensity. (d) Genetic analysis of the patient's genomic DNA revealed a 4-nucleotide deletion in exon 6 of *IFNGR1* (818del4) and a wild-type *IFNGR1* allele. (e) Clinical time course of treatment with IFNγ and multidrug antitubercular therapy. Serum levels of IFNγ (squares), IL-6 (triangles), and CRP (circles) were assessed during treatment.

4. Discussion

In the patients with dominant partial IFNγR1 deficiency, BCG infection is relatively resistant to conventional antitubercular therapy, although the clinical features of dominant partial IFNγR1 deficiency are less severe than those of complete deficiency [1,6]. Our data indicated that a high dose of IFNγ was capable of restoring the patient's impaired immune response to BCG infection. Superphysiologic IFNγ can overcome the dominant negative effect of truncated IFNγ by binding to the dimerized residual wild-type receptor, leading to the production of STAT1 associated-cytokines such as IFNγ, TNFα, IL-12 and IL-6 [7,12]. We found that in PBMCs from the patient and her mother, high dose IFNγ enhanced LPS-induced TNFα production.

Interestingly, PBMCs from the patient and her mother produced as much TNFα as healthy PBMCs in response to LPS alone. This suggests that the TLR4 signaling axis remains intact in PBMCs from patients with dominant partial IFNγR1 deficiency. It should be noted that serum TNFα levels were elevated in the patient but not in her mother, indicating residual BCG infection even in the context of normal serum IL-6 and C-reactive protein (CRP) levels. Collectively, these findings show that TNFα levels may be a useful measure of infection severity in patients with dominant partial IFNγR1 deficiency.

5. Conclusion

We have demonstrated that high dose IFNγ, when added to standard antitubercular regimen, is effective in the treatment of multi-site osteomyelitis and lymphadenitis in patients with dominant partial IFNγ deficiency.

Conflict of interest

The authors declare that they have no conflicts of interest.

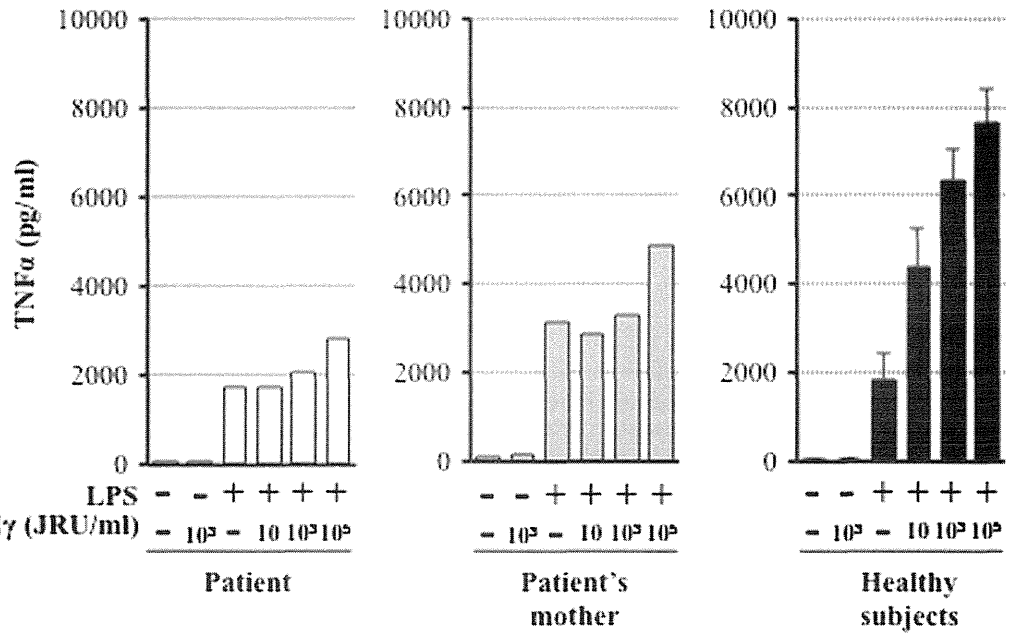

Figure. 2 TNFα production in response to LPS plus IFNγ in PBMCs. PBMCs isolated from the patient (white bars), patient's mother (gray bars), and healthy subjects (black bars) were stimulated with LPS alone or LPS plus serial concentration of IFNγ for 20 h. The level of TNFα in culture supernatant was measured by quantitative multiplex detection using Milliplex.

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References

[1] S.E. Dorman, C. Picard, D. Lammas, K. Heyne, J.T. van Dissel, R. Baretto, S.D. Rosenzweig, M. Newport, M. Levin, J. Roesler, D. Kumararatne, J.L. Casanova, S.M. Holland, Clinical features of dominant and recessive interferon gamma receptor 1 deficiencies, Lancet 364 (2004) 2113–2121.

[2] M.H. Haverkamp, E. van de Vosse, J.T. van Dissel, Nontuberculous mycobacterial infections in children with inborn errors of the immune system, J. Infect. 68 (2014) S134–S150.

[3] E. Jouanguy, S. Lamhamedi-Cherradi, D. Lammas, S.E. Dorman, M.C. Fondaneche, S. Dupuis, R. Doffinger, F. Altare, J. Girdlestone, J.F. Emile, H. Ducoulombier, D. Edgar, J. Clarke, V.A. Oxelius, M. Brai, V. Novelli, K. Heyne, A. Fischer, S.M. Holland, D.S. Kumararatne, R.D. Schreiber, J.L. Casanova, A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection, Nat. Genet. 21 (1999) 370–378.

[4] S.M. Arend, R. Janssen, J.J. Gosen, H. Waanders, T. de Boer, T.H. Ottenhoff, J.T. van Dissel, Multifocal osteomyelitis caused by nontuberculous mycobacteria in patients with a genetic defect of the interferon-gamma receptor, Neth. J. Med. 59 (2001) 140–151.

[5] K. Obinata, T. Lee, T. Niizuma, K. Kinoshita, T. Shimizu, T. Hoshina, Y. Sasaki, T. Hara, Two cases of partial dominant interferon-gamma receptor 1 deficiency that presented with different clinical courses of bacille Calmette–Guerin multiple osteomyelitis, J. Infect. Chemother. 19 (2013) 757–760.

[6] T. Hoshina, H. Takada, Y. Sasaki-Mihara, K. Kusuhara, K. Ohshima, S. Okada, M. Kobayashi, O. Ohara, T. Hara, Clinical and host genetic characteristics of Mendelian susceptibility to mycobacterial diseases in Japan, J. Clin. Immunol. 31 (2011) 309–314.

[7] E. Jouanguy, F. Altare, S. Lamhamedi, P. Revy, J.F. Emile, M. Newport, M. Levin, S. Blanche, E. Seboun, A. Fischer, J.L. Casanova, Interferon-gamma-receptor deficiency in an infant with fatal bacille Calmette–Guerin infection, N. Engl. J. Med. 335 (1996) 1956–1961.

[8] J.L. Casanova, E. Jouanguy, S. Lamhamedi, S. Blanche, A. Fischer, Immunological conditions of children with BCG disseminated infection, Lancet 346 (1995) 581.

[9] T. Kawai, H. Kusakabe, A. Seki, S. Kobayashi, M. Onodera, Osteomyelitis due to trimethoprim/sulfamethoxazole-resistant *Edwardsiella tarda* infection in a patient with X-linked chronic granulomatous disease, Infection 39 (2011) 171–173.

[10] Y. Kobashi, T. Abe, E. Shigeto, S. Yano, T. Kuraoka, M. Oka, Desensitization therapy for allergic reactions to antituberculous drugs, Intern. Med. 49 (2010) 2297–2301.

[11] B. Martire, R. Rondelli, A. Soresina, C. Pignata, T. Broccoletti, A. Finocchi, P. Rossi, M. Gattorno, M. Rabusin, C. Azzari, R.M. Dellepiane, M.C. Pietrogrande, A. Trizzino, P. Di Bartolomeo, S. Martino, L. Carpino, F. Cossu, F. Locatelli, R. Maccario, P. Pierani, M.C. Putti, A. Stabile, L.D. Notarangelo, A.G. Ugazio, A. Plebani, D. De Mattia, Ipinet, Clinical features, long-term follow-up and outcome of a large cohort of patients with chronic granulomatous disease: an Italian multicenter study, Clin. Immunol. 126 (2008) 155–164.

[12] M.J. Newport, C.M. Huxley, S. Huston, C.M. Hawrylowicz, B.A. Oostra, R. Williamson, M. Levin, A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection, N. Engl. J. Med. 335 (1996) 1941–1949.

A Case of Macrophage Activation Syndrome Developing in a Patient With Chronic Granulomatous Disease-associated Colitis

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Summary: Although macrophage activation syndrome (MAS) develops in some patients with chronic granulomatous disease (CGD), all of the reported cases have been associated with pathogenic microbial infections. We report a 2-year-old boy with CGD-associated colitis who suffered from MAS without any clinical signs of a microbial infection. He was treated with 1 course of methylprednisolone pulse therapy and the clinical symptoms improved; however, the colitis was difficult to control even with immunosuppressive drugs, and he eventually required hematopoietic stem cell transplantation 1 year after the onset of MAS. It is likely that MAS develops in patients with CGD colitis independent of microbial infections.

Key Words: chronic granulomatous disease, macrophage activation syndrome, CGD-associated colitis, inflammation, immunodeficiency

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Chronic granulomatous disease (CGD) is an inherited disorder characterized by an inability of phagocytes to produce reactive oxygen species that are generated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Patients with CGD have recurrent and life-threatening bacterial and fungal infections.[1] In addition, approximately one-half of CGD patients develop chronic bowel inflammation (known as CGD-associated colitis) through dysregulated inflammatory cytokine production. The clinical features of CGD-associated colitis are very similar to other inflammatory bowel diseases (IBDs), such as Crohn disease or ulcerative colitis.[2] Because of no demonstrable infection in the lesion, CGD-associated colitis is considered to be a sterile inflammatory response. Macrophage activation syndrome (MAS), which is characterized by excessive activation and proliferation of macrophages and T lymphocytes, is a serious complication that is often observed in patients with systemic juvenile idiopathic arthritis (sJIA) and systemic lupus erythematosus (SLE).[3] Diagnostic criteria for MAS in sJIA considered as an autoinflammatory syndrome[4,5] and in autoimmune diseases such as SLE[6] are different. Although the criteria for sJIA-associated MAS by Ravelli et al[4] are excellent for patients with SLE in terms of sensitivity (100%), they are poor in terms of specificity, because 71% of patients with SLE without MAS met these criteria.[6] Because the clinical features of MAS are similar to hemophagocytic lymphohistiocytosis (HLH), some have advocated the possibility of MAS as a secondary form of HLH.[7,8] One of the arguments for MAS to be included as secondary HLH is the demonstrated association of some cases of MAS with heterozygous changes in the genes known to cause familial HLH.[5] MAS develops in CGD patients, which may reflect the prolonged hyperinflammation accompanying increased susceptibility to bacterial and fungal infections in patients with CGD. Indeed, all of the published cases of MAS developing in CGD patients have been associated with microbial infections such as *Burkholderia cepacia*, *Staphyloccocus epidemidis*, *Leishmania*, and fungi at the onset of MAS.[9–11] In contrast, there have been no reports of MAS developing in patients with clinically significant CGD-associated bowel inflammation, although the patients also have persistent hyperinflammation and are at risk for MAS during the clinical course of the disease, similar to patients with sJIA.

Herein, we introduce a patient with CGD-associated colitis who developed MAS without a microbial infection. Although the clinical symptoms of MAS were mitigated by 1 course of methylprednisolone pulse therapy, the CGD-associated colitis was exacerbated, resulting in the need for stem cell transplantation 1 year after the onset of MAS.

CASE

The patient was a 2-year-old boy who was diagnosed with X-linked CGD (X-CGD) at 11 months of age. The diagnosis was confirmed by genetic analysis showing a mutated H111R in the *CYBB* gene. He received prophylactic treatment with oral trimethoprim/sulfamethoxazole and itraconazole daily together with subcutaneous interferon gamma weekly. He developed severe bloody diarrhea 2 months before admission. On admission, a colonoscopy revealed granular and edematous colonic mucosa with erosions and ulcers (Fig. 1A). The pathologic evaluation showed infiltration of inflammatory cells without cryptitis, crypt distortion, or granulomas (Fig. 1B). He was treated with oral 5-aminosalicylate, which did not result in improvement of his clinical symptoms.

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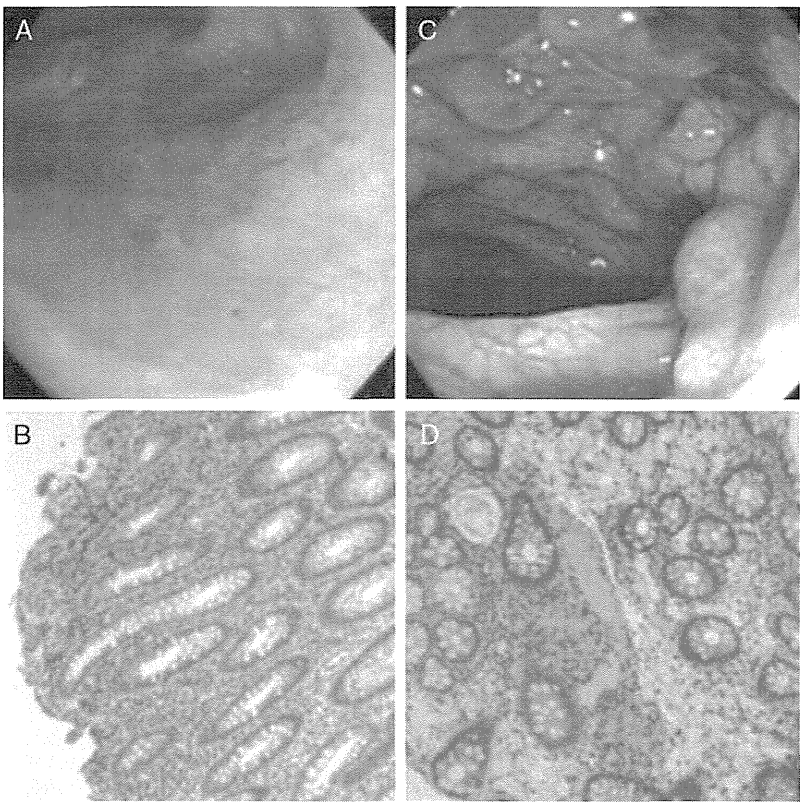

FIGURE 1. Gross and microscopic appearance of colon showing severe chronic granulomatous disease-associated colitis at the time of the macrophage activation syndrome (MAS) episode. A, Colonoscopy revealed granular and edematous colonic mucosa with erosions and ulcers on admission, which was before the development of MAS. B, The pathologic evaluation showed infiltration of inflammatory cells without cryptitis, crypt distortion, or granuloma on admission. C, Colonoscopy revealed edematous colonic mucosa with multiple pseudopolyps and ulcers 3 months after the onset of MAS. D, The pathologic evaluation showed granuloma formation and pigment-laden foamy macrophages apparent 3 months after the onset of MAS.

On day 28 after admission, he suddenly developed a high fever with pancytopenia (Fig. 2). A hematologic assessment revealed a ferritin level of 14838 ng/mL [normal range (NR), 39.4 to 340 ng/mL], an aspartate transaminase level of 172 IU/L (NR, 10 to 40 IU/L), a lactate dehydrogenase level of 1917 IU/L (NR, 115 to 245 IU/L), a leukocyte count of 3.5×10^9/L (NR, 3.9 to 9.8×10^9/L), and a platelet count of 42×10^9/L (NR, 131 to 369×10^9/L) on day 38 after admission. No pathogenic microorganisms were isolated from numerous blood and stool cultures during his episode. His clinical condition of pancytopenia, hepatosplenomegaly, and sharply increased levels of ferritin indicated the rapid progress of systemic hyperinflammation such as for example in sJIA-associated MAS. According to the preliminary criteria for JIA-associated MAS published by Ravelli et al,[4] a diagnosis of MAS could be made and he was treated with methylprednisolone pulse therapy [intravenous high-dose methylprednisolone (20 mg/kg) for 3 d followed by prednisolone (1.2 mg/kg)]. After 1 course of pulse therapy, he was afebrile with a decreased serum level of ferritin and an increased leukocyte count (Fig. 2).

The bloody diarrhea improved with pulse therapy; however, bloody diarrhea frequently recurred as the maintenance dose of corticosteroids was tapered. He remained hyperalimentation-dependent and could not be advanced to enteral feeding. A colonoscopy 3 months after the onset of MAS revealed edematous colonic mucosa with multiple pseudopolyps and ulcers (Fig. 1C). The pathologic evaluation showed granuloma formation and pigment-laden foamy macrophages were apparent (Fig. 1D). Although the patient was treated with a rectal preparation of dexamethasone, oral prednisolone, and azathioprine, together with intravenous hyperalimentation, the colitis did not improve and he developed multiple complications of immunosuppressive therapy, including a central-line infection, glaucoma, hypertension, and failure to thrive. As a result, he eventually underwent hematopoietic stem cell transplantation 1 year after development of MAS.

DISCUSSION

MAS is triggered by various factors, such as microbial or viral infections and hematologic malignancies. Although the patients with sJIA, SLE, and Kawasaki disease are highly prone to development of MAS,[3,12] CGD is also known as a disease that is associated with MAS. Notably, MAS that develops in patients with CGD has been associated with bacterial, fungal, or parasitic infections, such *Burkholderia cepacia*, *Aspergillus*, or *Leishmania*,[9,10] and there are no reports of MAS developing in patients with CGD-associated colitis independent of microbial infections, like the patient described herein.

CGD is a profoundly disconcerting disorder, because the genetic defect that causes immunodeficiency enhances inflammatory response per contra; thus, CGD patients who have bacterial or fungal infections present with inflammatory, but noninfectious conditions, such as granuloma formation (referred to as "complications not obviously caused by infection"[13]). Although the mechanism remains poorly understood, one of the most plausible explanations is imperfect degradation of inflammatory cytokines due to

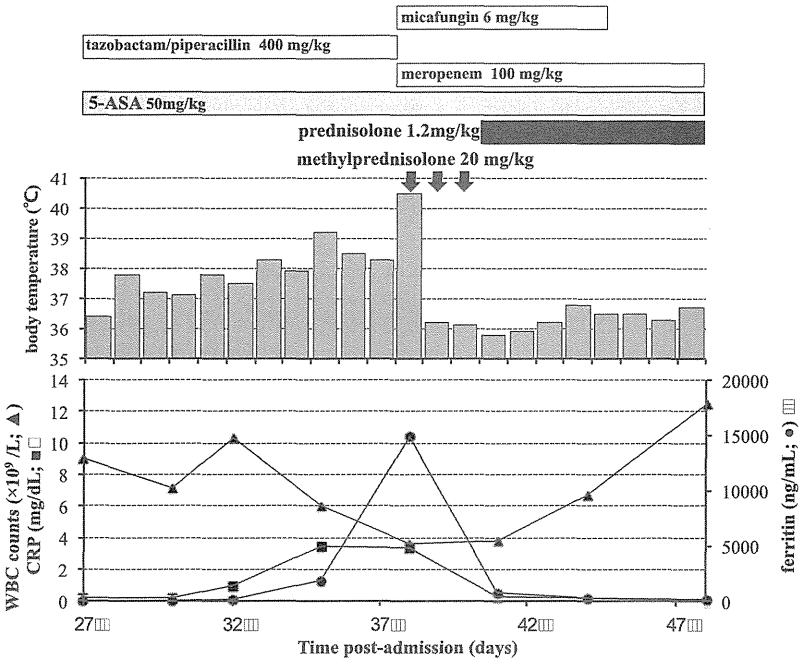

FIGURE 2. Clinical course of macrophage activation syndrome in a patient with chronic granulomatous disease-associated colitis. Shown are changes in body temperature (bars), leukocyte count (triangles), CRP (squares), and ferritin (circles), as well as medication (dose per day). 5-ASA indicates 5-aminosalicylate; CRP, C-reactive protein; WBC, white blood cell.

an inability to produce reactive oxygen species that function as a pleiotropic signal molecule in various inflammatory aspects in phagocytes, which leads to persistent cell activation of CGD macrophages by continuous phagocytosis of the apoptotic cells.[14] This situation is reminiscent of that seen in the primary (genetic) forms of HLH in which decreased function of natural killer cells and cytotoxic T cells (CTLs) results in a defect in clearing of activated immune cells (immune homeostasis) and defective clearing of infected cells. This results in a vicious cycle of continued antigen stimulation, production of excess proinflammatory cytokines, and activation of macrophages. Therefore, it is easy to imagine that patients with CGD-associated colitis would also be prone to development of MAS, because the macrophages within the intestinal tract might be continuously activated; however, such CGD patients have not been reported in the literature. Indeed among 14 patients with CGD-associated bowel inflammation treated at our hospital, only the patient described here developed MAS, suggesting that the level of activation of macrophage in CGD-associated colitis may be insufficient for the development of MAS.

A relationship between IBD and MAS was reported by James et al[15] who described the clinical features of 7 patients with IBD who developed MAS. Interestingly, all of the patients were taking >1 immunosuppressive medication, and all but 1 had coexisting infections with mycobacteria, histoplasma, cytomegalovirus, or Epstein-Barr virus. The cases with MAS that develop after infections related to immunosuppression are quite rare among the millions of patients with IBD treated with immunosuppressive therapy; such patients ordinarily are at increased risk of opportunistic infections. Therefore, the possibility exists that these patients have a genetic predisposition for progression to MAS. If this is the case for CGD-associated colitis, the patients have much higher risk of MAS than patients with IBD, because they are predisposed to serious infections and the macrophages are continuously activated by dysregulated inflammatory cytokines,[16] and MAS could develop if some inducing factors related to macrophage activation were present, even if lacking potency. Our patient did not exhibit any pathogenic infections, including pathogenic microorganisms, cytomegalovirus, or Epstein-Barr virus, and did not take any immunosuppressive medications, except for 5-aminosalicylate, at the onset of MAS. Considering the clinical symptoms (severe bloody diarrhea before the development of MAS), an incident worsening the colitis (eg, a viral infection) could have been the factor that triggered MAS.

Fortunately, the patient recovered from MAS with 1 course of methylprednisolone pulse therapy, suggesting the possibility that MAS in CGD-associated colitis may be milder than that observed in other clinical situations,[17] as well as the importance of early diagnosis of MAS permitting early alleviation of the symptoms. This patient eventually underwent stem cell transplantation for refractory colitis. Although the factors that trigged MAS in our patient remain unidentified, it is important that caregivers be aware that in addition to the previously described infectious triggers, activation of macrophages resulting in life-threatening MAS may occur in CGD-associated colitis without a demonstrable infection, likely induced by severe sterile inflammation.

ACKNOWLEDGMENT

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REFERENCES

1. Kawai T, Kusakabe H, Seki A, et al. Osteomyelitis due to trimethoprim/sulfamethoxazole-resistant Edwardsiella tarda infection in a patient with X-linked chronic granulomatous disease. *Infection*. 2011;39:171–173.
2. Marks DJ, Miyagi K, Rahman FZ, et al. Inflammatory bowel disease in CGD reproduces the clinicopathological features of Crohn's disease. *Am J Gastroenterol*. 2009;104:117–124.
3. Deane S, Selmi C, Teuber SS, et al. Macrophage activation syndrome in autoimmune disease. *Int Arch Allergy Immunol*. 2010;153:109–120.
4. Ravelli A, Magni-Manzoni S, Pistorio A, et al. Preliminary diagnostic guidelines for macrophage activation syndrome complicating systemic juvenile idiopathic arthritis. *J Pediatr*. 2005;146:598–604.
5. Vastert SJ, Kuis W, Grom AA. Systemic JIA: new developments in the understanding of the pathophysiology and therapy. *Best Pract Res Clin Rheumatol*. 2009;23:655–664.
6. Parodi A, Davi S, Pringe AB, et al. Macrophage activation syndrome in juvenile systemic lupus erythematosus: a multinational multicenter study of thirty-eight patients. *Arthritis Rheum*. 2009;60:3388–3399.
7. Grom AA. Macrophage activation syndrome and reactive hemophagocytic lymphohistiocytosis: the same entities? *Curr Opin Rheumatol*. 2003;15:587–590.
8. Gupta AA, Tyrrell P, Valani R, et al. Experience with hemophagocytic lymphohistiocytosis/macrophage activation syndrome at a single institution. *J Pediatr Hematol/Oncol*. 2009;31:81–84.
9. Martin A, Marques L, Soler-Palacin P, et al. Visceral leishmaniasis associated hemophagocytic syndrome in patients with chronic granulomatous disease. *Pediatr Infect Dis J*. 2009;28:753–754.
10. Sirinavin S, Techasaensiri C, Pakakasama S, et al. Hemophagocytic syndrome and *Burkholderia cepacia* splenic microabscesses in a child with chronic granulomatous disease. *Pediatr Infect Dis J*. 2004;23:882–884.
11. Alvarez-Cardona A, Rodriguez-Lozano AL, Blancas-Galicia L, et al. Intravenous immunoglobulin treatment for macrophage activation syndrome complicating chronic granulomatous disease. *J Clin Immunol*. 2012;32:207–211.
12. Latino GA, Manlhiot C, Yeung RS, et al. Macrophage activation syndrome in the acute phase of Kawasaki disease. *J Pediatr Hematol/Oncol*. 2010;32:527–531.
13. Johnston RB Jr. Clinical aspects of chronic granulomatous disease. *Curr Opin Hematol*. 2001;8:17–22.
14. Fernandez-Boyanapalli RF, Frasch SC, McPhillips K, et al. Impaired apoptotic cell clearance in CGD due to altered macrophage programming is reversed by phosphatidylserine-dependent production of IL-4. *Blood*. 2009;113:2047–2055.
15. James DG, Stone CD, Wang HL, et al. Reactive hemophagocytic syndrome complicating the treatment of inflammatory bowel disease. *Inflamm Bowel Dis*. 2006;12:573–580.
16. Meissner F, Seger RA, Moshous D, et al. Inflammasome activation in NADPH oxidase defective mononuclear phagocytes from patients with chronic granulomatous disease. *Blood*. 2010;116:1570–1573.
17. Miettunen PM, Narendran A, Jayanthan A, et al. Successful treatment of severe paediatric rheumatic disease-associated macrophage activation syndrome with interleukin-1 inhibition following conventional immunosuppressive therapy: case series with 12 patients. *Rheumatology*. 2011;50:417–419.

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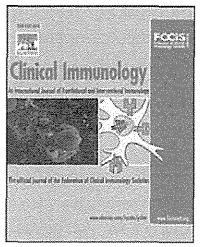

Thalidomide attenuates excessive inflammation without interrupting lipopolysaccharide-driven inflammatory cytokine production in chronic granulomatous disease☆

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Abstract Chronic granulomatous disease (CGD) is a rare inherited disorder characterized by an inability to produce reactive oxygen species, resulting in recurrent life-threatening infections. Curiously, half of the patients with CGD suffer from aseptic bowel inflammation (CGD colitis) due to dysregulated inflammation induced by TNF-α and IL-1β. Thus, developing therapies that regulate excessive inflammatory responses without interrupting antimicrobial immunity would benefit CGD colitis patients. Here, we show that thalidomide suppressed TNF-α-induced NF-κB activation and ATP-induced IL-1β secretion, but did not interrupt the production of IL-1β, IL-6, IL-8, and TNF-α in response to lipopolysaccharide in CGD monocytes. We report on a CGD colitis patient that showed decreased bowel inflammation characterized by reduced serum levels of inflammatory cytokines without evidence of progression of fungal and bacterial infections present at initiation of thalidomide therapy. Our results suggest that thalidomide could be an efficacious therapeutic option for patients with CGD colitis suffering from serious infections.

☆ Thalidomide regulates TNF-α-induced NF-κB activation, but does not interrupt lipopolysaccharide-driven inflammatory cytokine production in monocytes isolated from patients with chronic granulomatous disease.

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1. Introduction

Reactive oxygen species (ROS) generated by cellular nicotinamide adenine dinucleotide phosphate (NADPH) oxidase play an important role in killing microorganisms ingested by phagocytes. In addition, ROS function as pleiotropic signals in various inflammatory contexts. For example, ROS activate nuclear factor-κB (NF-κB), which produces inflammatory cytokines and facilitates formation of the inflammasome consisting of Nod-like receptor family, pyrin domain containing 3 (NLRP3), ASC, cardinal, and pro-caspase-1, which cleaves into caspase-1 and converts pro-interleukin-1β (IL-1β) to IL-1β [1,2]. Chronic granulomatous disease (CGD) is a primary immunodeficiency resulting from the inability to produce ROS due to defects in NADPH oxidase activity [3]. CGD patients suffer from recurrent infection and frequently develop CGD-associated bowel inflammation (CGD colitis) in the absence of any demonstrable infection [4]. The underlying mechanism of CGD colitis is not fully understood; however, dysregulated inflammatory cytokine production, such as IL-1β and tumor necrosis factor-α (TNF-α), by monocytes and macrophages is thought to contribute to the disease [1,5]. This concept is supported by many clinical reports that neutralizing anti-TNF-α or immunosuppressive drugs, such as steroids or azathioprine, effectively mitigate CGD colitis clinical symptoms. Notably, the use of these drugs results in an increased susceptibility to severe infections [4,5].

Thalidomide, originally developed in the 1950s as a tranquilizer, was discontinued when its potent teratogenic effects resulted in catastrophic birth defects in the 1960s. It is recently being reconsidered for its unique and pleiotropic medical effects, such as immunomodulation and anti-inflammatory properties. Thalidomide potentially reduces inflammation by blocking nuclear localization of NF-κB and inhibiting inflammatory cytokine production in patients with chronic inflammatory diseases such as Behcet's disease, rheumatoid arthritis, and Crohn disease [4,6–8]. So far, there are case reports regarding three patients with CGD colitis who were treated with thalidomide [4,9], although the immunomodulatory effect of thalidomide on CGD monocytes has not been described. In this report we show that thalidomide suppressed TNF-α-induced NF-κB activation and adenosine triphosphate (ATP)-induced IL-1β secretion while not interrupting lipopolysaccharide (LPS)-driven inflammatory cytokine production in CGD monocytes. We also show that thalidomide treatment improved bowel inflammation by reducing serum TNF-α levels without increasing susceptibility to infection in a CGD colitis patient.

2. Materials and methods

2.1. Monocyte isolation and stimulation

All experiments were performed following receipt of informed consent from patients with X-linked CGD or their parents as part of a protocol approved by the Institutional Review Board of the National Center for Child Health and Development. Monocytes isolated from peripheral blood by an antibody-mediated cell enrichment procedure from RossettSep (StemCell Technologies, Canada) were stimulated with serial concentrations of LPS and with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, St. Louis, MO) or thalidomide (Fujimoto Pharmaceutical Corporation, Japan).

2.2. Caspase-1 activation and cytokine production

Caspase-1 activity was assessed by flow cytometry on a FACSAria IIIu (Becton Dickinson Biosciences, San Diego, CA) using caspase-1 FLICA (Immunochemistry Technologies, Bloomington, MN), a fluorescent inhibitor of active caspase-1, according to the manufacturer's instructions [1]. Human IL-1β, IL-6, IL-8, and TNF-α levels were determined in 5×10^6 cells/ml monocyte cell cultures by quantitative multiplex detection using Milliplex (Millipore, Billerica, MA).

2.3. NF-κB activation

Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction kits (Thermo Scientific, Rockford, IL), and were used to assess NF-κB activation using a transcription factor kit for NF-κB (Thermo Scientific) according to the manufacturer's instructions. Experiments were done in duplicate.

2.4. IL-1β production

Monocytes were primed with 100 ng/ml LPS alone or LPS plus serial concentrations of thalidomide for 2 h before addition of 5 mM ATP. IL-1β levels in monocyte supernatants were measured by quantitative multiplex detection using Milliplex [10].

2.5. Immunologic assessment of a CGD patient treated with thalidomide

A three-year-old boy developed CGD colitis at age 11 months during treatment with isoniazid for *Bacille Calmette–Guérin (BCG)*-related lymphadenitis. Despite several trials with anti-inflammatory drugs, the patient suffered from persistent bowel inflammation and developed pulmonary aspergillosis 3 weeks prior to thalidomide therapy. After informed consent was received from his parents, he was treated with daily oral thalidomide for 8 weeks before undergoing conditioning for bone marrow transplantation. Peripheral blood lymphocyte subsets were determined with FACSAria IIIu using anti-human CD3, CD4, CD19, CD31, and CD45RA monoclonal antibodies conjugated with fluorescein isothiocyanate, phycoerythrin (PE), peridinin–chlorophyll proteins-Cy5.5, allophycocyanin, or PE-Cy7 (BioLegend, San Diego, CA).

2.6. Statistics

Experimental data were reported as the mean ± standard deviation (s.d.). Significance was determined by Student's *t*-test. Error bars indicate mean ± s.d.