Neutrophils prime a long-lived effector macrophage phenotype that mediates accelerated helminth expulsion

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We examined the role of innate cells in acquired resistance to the natural murine parasitic nematode, *Nippostrongylus brasiliensis*. Macrophages obtained from lungs as late as 45 d after *N. brasiliensis* inoculation were able to transfer accelerated parasite clearance to naive recipients. Primed macrophages adhered to larvae in vitro and triggered increased mortality of parasites. Neutrophil depletion in primed mice abrogated the protective effects of transferred macrophages and inhibited their in vitro binding to larvae. Neutrophils in parasite-infected mice showed a distinct transcriptional profile and promoted alternatively activated M2 macrophage polarization through secretory factors including IL-13. Differentially activated neutrophils in the context of a type 2 immune response therefore prime a long-lived effector macrophage phenotype that directly mediates rapid nematode damage and clearance.

Helminth infection can result in chronic malnutrition, significant morbidity and increased susceptibility to lethal pathogens1,2. A type 2 immune response is upregulated in humans and mice after helminth infection, and components of this response can contribute to both host tolerance and resistance3–5. At present, effective vaccines against helminth parasites have proved elusive, perhaps owing in part to an incomplete understanding of how components of type 2 immunity mediate host protection and acquired resistance. Infective larval stages of many intestinal nematode parasites typically enter through the skin and migrate to the lung and then to the enteric region. Studies have suggested that the lung may be an important organ for early parasite clearance in tissues and thus a potential target for vaccine-induced host resistance6.

Immune-mediated resistance to nematodes can include both innate and adaptive components of the type 2 immune response. The cytokines IL-4, IL-5, IL-9 and IL-13, produced by both type 2 helper T (Th2) cells and innate immune cell populations including eosinophils, basophils, mast cells and group 2 innate lymphoid cells, promote specific effector mechanisms that contribute to anti-parasite resistance with adaptive immune responses generally thought to drive acquired resistance4,7. Immune components mediating anti-parasite resistance may include increased luminal fluid flow and intestinal muscle contractility8, antibody-dependent mechanisms9, mast cell production of mediators9, secretion of mucus and resistin-like molecule-β11 and effector mechanisms triggered by eosinophils12.

Macrophages may also contribute to anti-parasite resistance13,14, although whether they mediate direct effects on helminths is unclear. Neutrophils, essential in resistance to microbial pathogens, have also been associated with helminth infection, in some cases surrounding the parasite in situ13,15. Other studies have suggested that neutrophils may cause damage after contact with helminths in vitro or in diffusion chambers implanted *in vivo*16. In infectious disease, neutrophils are thought to mediate end-stage effector functions with relatively little evidence as yet suggesting that they can promote the development of other immune effector cell populations. It should also be noted that these various anti-parasite resistance mechanisms have so far been described primarily in the context of intestinal resistance, with few studies identifying effector populations or mechanisms mediating protective immunity in the lung.

*N. brasiliensis*, an intestinal parasitic nematode that infects rodents, is a widely used experimental model where infective third-stage larvae (L3) migrate from the skin to the lung before entering the small intestine. The lung is an important site, at which both innate and adaptive components of the type 2 immune response interact with the migrating parasitic larval stages. Resistance mechanisms after primary inoculation lead to expulsion of the parasite from the intestine; in contrast, parasite migration to the intestine after secondary inoculation is impaired, and the lung is an important site of parasite clearance6,17. The immune mechanism of parasite damage in the lungs is poorly understood and little studied. Although CD4+ T cells are

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probably required for priming, as the recall response is blocked in mice deficient for major histocompatibility complex (MHC) class II, neither CD4+ T cells nor B cells are required for the protective immunity leading to an accelerated parasite clearance after secondary inoculation. This raises the possibility that the heightened immune protection (as compared to the primary response) is not mediated through adaptive immunity.

Here we examined the immune mechanism leading to accelerated worm clearance after secondary inoculation of mice with _N. brasiliensis_. Our findings suggest that neutrophils acquire an alternatively activated (N2) phenotype that is essential in providing helper functions that drive the development of a long-lived persistent macrophage phenotype, which subsequently mediates parasitic larval damage during recall responses in the lung.

**RESULTS**

**Larvae induce persistent changes in lung immune cells**

To examine whether accelerated parasitic larval clearance after a secondary inoculation was associated with changes in lung innate immune cell populations after primary inoculation, we performed a kinetics analysis of lung immune cell populations extending to three months after primary inoculation. Parasitic _N. brasiliensis_ larvae typically enter the lung between 19 and 32 h after subcutaneous inoculation, reside transiently in the lung for up to 50 h and then migrate to the small intestine, where they are expelled as early as day 8 (8 d after inoculation) 30. The parasitic larvae cause lung damage that results in extensive neutrophil inflammation and hemorrhaging by day 2, which is largely resolved by day 7. Macrophages and eosinophils infiltrate the lung through lung blood vessels by day 4 (ref. 21) (Supplementary Fig. 1a). To extend these observations, we isolated lung cells and identified them by flow cytometry. The numbers of macrophages with high expression of the marker CD4/80 (F4/80 hi), variable CD11c expression (CD11c var) and intermediate-high expression of MHC class II (MHCIInl-hi) (ref. 22), had increased by day 4 and further increased by day 7 after inoculation. These numbers remained high in the lung for as long as three months after _N. brasiliensis_ inoculation (Supplementary Fig. 1b). Similarly, the number of F4/80intMHCIIneg-loCD11cloSiglec-F hi eosinophils began to increase at day 4 and was further increased at day 7, but, in contrast to the macrophages, returned to baseline by 3 months after _N. brasiliensis_ inoculation. The number of neutrophils also increased transiently, peaking at day 2 and returning to baseline by 7 days. The number of CD4+ T cells was slightly higher than baseline at day 7 and had decreased by 3 months, and CD8+ T cells were unchanged from day 2 until 3 months after inoculation with L3 (Supplementary Fig. 1b). By day 2 after secondary inoculation, the number of macrophages, eosinophils and neutrophils had increased, although the number of neutrophils was higher after primary inoculation (Supplementary Fig. 1c). Thus, after primary inoculation, macrophages persist in the lung for prolonged periods after other innate immune cells have returned to near baseline.

**Immune cells surround larvae after secondary inoculation**

The persistently large number of macrophages in lung tissue after primary inoculation with _N. brasiliensis_ raised the possibility that these innate immune cells contribute to the acceleration of parasite clearance after a secondary inoculation. To directly examine possible interactions between macrophages and invading parasitic larvae, we examined cryosections of lung tissue at days 2 and 3 after _N. brasiliensis_ inoculation. Parasitic larvae in the lung were not surrounded by immune cell populations after primary inoculation; however, macrophages and eosinophils were immediately surrounding the parasitic larvae after secondary inoculation, as observed by immunofluorescence staining with antibodies to eosinophil cell major basic protein (MBP) and F4/80 at day 2 (Fig. 1a). This staining showed macrophages (MBP+F4/80+) and eosinophils (MBP-+F4/80var) surrounding individual larvae. This observation was corroborated by histological hematoxylin and eosin (H&E) staining, which showed accumulation of macrophages and eosinophils after secondary but not primary inoculation (Fig. 1a). We observed fewer macrophages...
in specific sections of the lung with both H&E and immunofluorescence staining after primary inoculation than after secondary inoculation, and these cells were not clustered around the larvae (Fig. 1a). To examine changes in gene expression after primary inoculation, we analyzed lung tissue by fluorogenetic quantitative real-time reverse-transcription PCR (qPCR). We observed marked increases in Il4 (which encodes IL-4), Il5, Il13, Il10, Arg1 (encoding arginase 1) and Chi3l3 (which encodes the secretory protein Ym1) mRNA, all of which are markers of type 2 immunity, as early as 2 d after secondary inoculation, a time point at which we had observed relatively little change in these markers after primary inoculation. Furthermore, Nos2 and Ifng (characteristic of type 1 responses) mRNA showed only modest changes after either primary or secondary inoculation (Fig. 1b), indicative of a highly polarized type 2 response. Thus, migrating larvae encounter macrophages more rapidly in the lung after secondary inoculation.

**Primed macrophages directly damage N. brasiliensis larvae**

The presence of macrophages immediately surrounding the invading parasite in the lung shortly after secondary, but not primary, inoculation raised the possibility that macrophages were damaging invading larval parasites in the lung. To test this possibility, we isolated parasitic larvae at days 2 or 3 after the primary and secondary inoculations with N. brasiliensis L3. The larval metabolic activity, a measure of morbidity determined by the abundance of parasite ATP\(^1\), was significantly lower for larvae from secondarily inoculated mice than for those from mice given primary inoculation only (Fig. 2a). To further test whether macrophages have a key role in damaging parasitic larvae after secondary inoculation, we electronically sorted (with >95% purity) ‘primed’ lung macrophages (F4/80\(^+\)CD11c\(^+\)MHC-II\(^{int−}\)hi) obtained at day 7 after primary N. brasiliensis inoculation (Supplementary Fig. 2a) and transferred to recipient mice, which were inoculated with N. brasiliensis 2 days later. At days 2 and 3 after primary inoculation of recipient mice with N. brasiliensis, larval ATP from lung tissue of the recipient mice was present at concentrations similar to that in secondarily inoculated mice (Fig. 2b). At day 4 after N. brasiliensis inoculation, the parasite numbers in the guts of recipient mice were lower than those in mice inoculated with N. brasiliensis that did not receive lung macrophages, indicating that protective mechanisms in the lung were preventing larval migration to the enteric region (Fig. 2c). Transfer of macrophages labeled with the cytosolic dye CFSE confirmed that donor macrophages are recruited to the lung (Supplementary Fig. 2b). Lungs of recipient mice showed higher numbers of eosinophils and lower numbers of neutrophils as early as day 2 after inoculation (Supplementary Fig. 2c). Concentrations of Il4, Il5, Il13 and Chi3l3 mRNA were higher, and no changes were detected in Il10, Arg1 or Nos2 mRNA concentrations (Supplementary Fig. 2d,e). Published studies have suggested that macrophages can inhibit parasite migration from the site of inoculation in the skin after N. brasiliensis secondary inoculation\(^2\). To test whether the transferred primed macrophages could also mediate this effect in naive mice, at day 7 after N. brasiliensis primary inoculation, we electronically sorted lung macrophages and transferred them to naive recipient wild-type mice, which were inoculated 2 d later with N. brasiliensis. At day 2 after inoculation, parasite numbers in the skin were significantly higher in mice that received macrophages from N. brasiliensis–primed wild-type mice than in mice that received lung macrophages from untreated donor mice (P < 0.05 (analysis of variance (ANOVA) followed by the Holm–Šidák test); Supplementary Fig. 2f). Thus, primed lung macrophages could mediate resistance in recipient mice through restricting larval migration from the skin at the site of subcutaneous inoculation, as well as in the lung.

To directly examine whether macrophages were capable of causing parasite damage *in vitro*, we isolated macrophages from the lungs of naive mice and of mice at day 7 after primary inoculation and cultured then *in vitro* with exsheathed larvae. Whereas macrophages from naive mice did not adhere to exsheathed larvae, those from mice inoculated with N. brasiliensis adhered in significant numbers to exsheathed larvae (Fig. 2d–f). After 5 d of culture, larval ATP concentrations were...
significantly lower (Fig. 2g) and the number of nonmotile straightened larvae with nonrefractive internal structures (indicative of dead larvae), was higher in cultures of larvae with macrophages from primed mice than in those with macrophages from naive mice or in cultures of larvae alone (Fig. 2h). The larval cultures with naive macrophages were no different with respect to ATP concentration or mortality from the cultures with larvae alone (Fig. 2h–j). To examine whether this anti-helminth effector macrophage phenotype persisted longer after primary inoculation, we isolated macrophages at day 45 after primary inoculation. Even at this much later time point, macrophages still adhered to parasitic larvae and mediated worm damage and killing to similar extents as macrophages isolated at day 7 after primary inoculation (Fig. 2i–k). These findings thus indicated that macrophages from N. brasiliensis–primed mice ‘preferentially’ adhere to N. brasiliensis larvae, lowering their metabolism and increasing their mortality. Taken together, these studies indicate that macrophages from primed donor mice can directly mediate worm killing, accelerate type 2 immune responses in the lung and ultimately enhance worm clearance in recipient mice after primary inoculation similar to that observed in mice after a secondary inoculation.

**Macrophage-mediated resistance requires IL-4R signaling**

Macrophages can polarize to classically activated (M1) macrophages by bacterial stimulation or to alternatively activated (M2) macrophages by helminth infection. We examined whether the type 2 immune environment typically associated with helminth infection was also an essential component for the development of the effector macrophages that could mediate enhanced worm expulsion. Wild-type mice were inoculated with N. brasiliensis or administered lipopolysaccharide (LPS), a bacterial product that promotes M1 macrophage differentiation. Seven days later, we electronically sorted macrophages and transferred them into naive wild-type mice, which we inoculated with N. brasiliensis 2 d later. At day 5 after inoculation, recipient mice with donor macrophages from mice inoculated with N. brasiliensis, but not from mice administered LPS, showed significantly reduced worm numbers in the gut (Fig. 3a). Transfer of macrophages from naive donor mice to recipient mice had no effect on worm burden, excluding the possibility that transfer of undifferentiated macrophages may be sufficient to promote immunity (Supplementary Fig. 2g). We next investigated signaling pathways that may drive the differentiation of the effector anti-helminth macrophages after N. brasiliensis inoculation. Published studies have shown that after N. brasiliensis inoculation M2 macrophage polarization in the lung is impaired by blocking signaling of the α-chain of the receptor for IL-4 (IL-4Rα, encoded by Il4ra) but not IL-10 signaling. We inoculated donor wild-type, Il4ra−/− and Il10−/− mice with N. brasiliensis, electronically sorted macrophages from their lungs at day 7 and transferred them into naive recipient mice, which were then inoculated with N. brasiliensis 2 days later. At day 5 after inoculation of recipient mice, parasite numbers in the gut were lower in recipient mice given macrophages from N. brasiliensis–primed wild-type and Il10−/− donor mice, but not in those given macrophages from N. brasiliensis–primed Il4ra−/− donor mice, than in control mice that did not receive macrophages (Fig. 3b,c). To directly examine whether macrophages from Il4ra−/− mice inoculated with N. brasiliensis showed impaired anti-parasite effects, we isolated macrophages from wild-type and Il4ra−/− mice inoculated with N. brasiliensis by electronic cell sorting on day 7 after inoculation and cultured them with exsheathed larvae, as described above. Five days later, macrophages from Il4ra−/− mice showed marked reductions in effector function, including decreases in macrophage-parasite binding (Fig. 3d) and a lower capacity to impair parasite metabolism as measured by ATP concentration (Fig. 3e) and larval mortality (Fig. 3f). Blockade of Arg1 can impair effective parasite expulsion. To examine this possibility in this experimental model, we administered S-(2-bromoethyl)-l-cysteine (BEC), a specific competitive inhibitor of Arg1 with limited off-target effects, to recipient mice after transfer of macrophages from N. brasiliensis–primed wild-type donor mice. Arg1 blockade inhibited reduction in parasite numbers in the gut after primary N. brasiliensis inoculation of recipient mice (Fig. 3g), and addition of BEC in cultures of larvae with macrophages (cell viability was 90%) from primed mice resulted in less macrophage adherence (Fig. 3h), higher larval ATP concentrations (Fig. 3i) and lower mortality (Fig. 3j). Thus, the development of activated anti-helminth effector macrophages requires IL4Rα signaling in the context of N. brasiliensis infection.
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**Protection in secondary infections requires neutrophils**

Neutrophils are key players in innate immune responses. They provide a first line of defense against invading microorganisms, but few studies have examined their role in resistance against metazoan parasites such as helminths. Neutrophils infiltrate the lung at day 2 after primary *N. brasiliensis* inoculation. To investigate whether neutrophils contribute to host protection against parasitic larvae, neutrophils were depleted in mice inoculated with *N. brasiliensis* by in vivo administration of antibodies specific to lymphocyte antigen 6 complex, locus G (Ly6G). Administration of anti-Ly6G 1 d before primary inoculation and on days 3 and 7 afterward and 1 d before secondary inoculation and on day 3 afterward resulted in significantly impaired parasite expulsion on day 5 after secondary inoculation (Fig. 4a). Administration of anti-Ly6G 1 d before and on day 3 after secondary inoculation did not affect the ability of the host to rapidly expel the parasite from the intestine (Fig. 4b). Neutrophil depletion was confirmed by flow cytometry in blood and whole lung (Supplementary Fig. 3a,b). To determine whether the effects of neutrophil depletion altered T12 cytokine production after primary inoculation, lung tissue was collected and T12-related cytokine gene expression was determined by qPCR at day 7 after inoculation. *Il4*, *Il13* and *Il10* mRNA were upregulated and not affected after neutrophil depletion during primary or secondary infection (Supplementary Fig. 3c). However, at day 1 after inoculation, elevations in *Il13* expression were significantly inhibited after neutrophil depletion (*P < 0.01* (ANOVA followed by the Holm-Šídák test); Supplementary Fig. 3d). These observations indicate that neutrophil depletion during priming impairs helminth expulsion after secondary inoculation, and results in a reduction in IL-13 abundance shortly after *N. brasiliensis* inoculation, but by day 7 T12 cytokine mRNA expression is largely restored.

**Figure 4** Neutrophils are required for differentiation of effector M2 lung macrophages after primary inoculation with *N. brasiliensis*. (a,b) Parasite numbers on day 5 after inoculation in mice administered neutrophil-depleting anti-Ly6G or isotype control (IgG) 1 d before and 3 d after primary inoculation or 1 d before and 3 and 7 d after primary inoculation followed by 1 d before and 3 d after secondary (2°) inoculation (a), or only 1 d before and 3 d after secondary inoculation (b). (c,d) Parasite expulsion from the intestine in mice inoculated with *N. brasiliensis* 2 d after receiving macrophages from donor mice treated with anti-Ly6G or IgG 1 d before and 3 d after (c) or 1 d before and 3 and 7 d after (d) inoculation. Donor macrophages were collected on day 7 (c) or 41 (d) after inoculation, and parasite expulsion from the intestines of recipient mice was assessed on day 5 (c) or 4 d (d) after inoculation. (e) Parasite numbers in the gut at day 5 in recipient mice given macrophages isolated from *N. brasiliensis*-infected donor mice at day 7 after inoculation, followed by treatment of recipients with anti-Ly6G or IgG 1 d before and 3 d after inoculation. (f) Larval adherence (left), ATP concentrations (middle) and percentage mortality (right) in macrophages isolated from *N. brasiliensis*-infected mice treated with anti-Ly6G or IgG after 5 d of culture with exsheathed larvae. *P < 0.05, **P < 0.01. Data are representative of two independent experiments with mean and s.e.m. of five mice per group (a–e) or three experiments with similar results (f; mean and s.e.m. of triplicate samples from pools of five mice per group).

**Figure 5** Depletion of neutrophils impairs effector lung macrophage polarization after primary inoculation. (a) qPCR analysis of expression of characteristic M1 and M2 macrophage markers and integrins in macrophages from lungs of naive mice or from inoculated mice at day 7, treated with anti-Ly6G or isotype control (IgG) 1 d before and 3 d after inoculation. Data shown are the mean from a pool of five mice per group (expressed as fold increase over mRNA from lungs of naive mice) and are representative of two independent experiments. (b) Larval adherence, ATP concentration and percentage mortality in macrophages isolated from wild-type mice on day 7 after inoculation and cultured for 5 d with exsheathed larvae, with or without anti-CD11b. *P < 0.01. Data are representative of three experiments with similar results (mean and s.e.m. of triplicate samples from five mice per group).
Neutrophils mediate development of effector macrophages

Unlike macrophages, numbers of neutrophils return to baseline in the lung within several days after \textit{N. brasiliensis} inoculation (Supplementary Fig. 1), which suggests that it is unlikely that they directly mediate worm clearance but raises the possibility that they contribute to parasite clearance indirectly by providing signals for effector macrophage development during the primary response. To test this possibility, we depleted neutrophils in donor mice by administering anti-Ly6G 1 d before and on day 3 after \textit{N. brasiliensis} primary inoculation. At day 7 after inoculation, we transferred electronically sorted macrophages from donor mice to naive recipient mice, which were inoculated with \textit{N. brasiliensis} 2 days later. At day 5 after primary inoculation, the parasite numbers in the gut were lower in recipient mice that had received macrophages from donor mice with intact neutrophil populations than in control mice that received a ‘mock transfer’ of vehicle only. However, macrophage transfer from donor mice with depleted neutrophil populations did not result in enhanced parasite expulsion in the intestine (Fig. 4c). These studies thus indicated that neutrophils were required for priming of effector macrophages. To examine how persistent this effector macrophage phenotype was after priming, we transferred macrophages isolated from donor mice at day 41 after primary \textit{N. brasiliensis} inoculation to naive mice. Transferred macrophages still mediated effective parasite expulsion in recipient mice at day 4 after inoculation. However, when donor mice were depleted of neutrophils by administration of anti-Ly6G 1 d before and on days 3 and 7 after inoculation, macrophages transferred at day 41 after inoculation did not mediate accelerated parasite expulsion (Fig. 4d). It is possible that this persistent macrophage phenotype was dependent on B cell secretion either of factors that contribute to maintaining a type 2 microenvironment in the lung or of antibodies that would then arm macrophages through Fc receptor binding, thereby facilitating macrophage adhesion to larval parasites. To test this possibility, we inoculated B cell–deficient \textit{Jh}−/− mice with \textit{N. brasiliensis} and transferred macrophages sorted at day 45 into naive wild-type mice. Transferred lung macrophages from \textit{Jh}−/− mice inoculated with \textit{N. brasiliensis} enhanced worm expulsion similarly to primed lung macrophages from wild-type mice, indicating that B cells are not required for the persistent anti-helminth macrophage phenotype (Supplementary Fig. 2h).

To examine whether neutrophils also have a direct role in mediating parasite expulsion after secondary inoculation, we transferred macrophages from \textit{N. brasiliensis}–infected mice at day 7 after inoculation to naive recipients, which were then administered anti-Ly6G or the isotype control antibody (Fig. 4f). Neutrophil depletion accelerated parasite expulsion after secondary inoculation, we transferred macrophages from \textit{N. brasiliensis}–infected mice at day 7 after inoculation to naive recipients, which were then administered anti-Ly6G or the isotype control antibody (Fig. 4f). Neutrophil depletion accelerated parasite expulsion, even in recipient mice depleted of neutrophils (Fig. 4e). The requirement of neutrophils for the development of effector macrophages that could mediate accelerated parasite expulsion \textit{in vivo} suggested that neutrophils directly affect the capacity of these effector macrophages to cause parasite damage. To investigate this possibility, we cultured macrophages from \textit{N. brasiliensis}–primed mice administered anti-Ly6G or isotype control with exsheathed larvae for 5 d. Macrophages from primed mice administered anti-Ly6G showed marked reductions in larval adherent cells, significantly higher amounts of parasite ATP and lower larval mortality than macrophages from primed mice given the isotype control antibody (Fig. 4f).

To examine whether depletion of neutrophils altered the accumulation of immune cell populations, we assessed total lung macrophages and eosinophils in mice inoculated with \textit{N. brasiliensis} and administered either anti-Ly6G or isotype control. Although macrophages were not affected, we observed consistent decreases in lung eosinophil numbers at day 2 after inoculation in primed mice administered anti-Ly6G (Supplementary Fig. 3a,b). Neutrophil depletion
Primed neutrophils express a distinct transcriptome

To explore the mechanism of helminth-induced neutrophil interactions with macrophages, we inoculated mice with L3 or with LPS intratracheally to drive a predominantly type 1 response. Two days later, we electronically sorted lung neutrophils by selecting cells that were Ly6Ghi and CD11bhi. There were no detectable basophils, defined by expression of the FcεRI high-affinity receptor for IgE and the basophil surface marker DX5, in the sorted neutrophil population (Supplementary Fig. 4). Sorted cells were ‘cytospun’ and stained with Wright-Giemsa. Purity of the neutrophil cell population was confirmed by homogeneity of stained cells on cytospin preparations, with macrophage or eosinophil phenotypes not detected. Neutrophils from mice inoculated with N. brasiliensis had a characteristic phenotype with a ring-form nucleus. In contrast, neutrophils from LPS-treated mice showed a more typical multilobular nucleus, similar to that of sorted lung neutrophils from naive mice (Fig. 6A). To further characterize lung neutrophil populations in the context of a helminth-induced type 2 immune response, we isolated mRNA from sort-purified lung neutrophils from untreated, LPS-inoculated and N. brasiliensis–inoculated mice and analyzed whole-genome gene expression. Relative to untreated controls, neutrophils showed a distinct pattern of gene expression when activated with either LPS or N. brasiliensis (Fig. 6B).

Although 153 genes were similarly upregulated in neutrophils after N. brasiliensis and LPS inoculation, 218 genes were uniquely upregulated after N. brasiliensis inoculation, whereas 1,315 genes were upregulated only after LPS inoculation. Neutrophils from mice inoculated with N. brasiliensis showed unique upregulation of Il13, Il33, Igf1, Retnla and Chi3l3, whereas those from LPS-inoculated mice showed distinct upregulation of Il6 and Il12b (Fig. 6C). Tnf and Nos2 were similarly upregulated in neutrophils from both sets of mice. We generated a heat map of genes characteristically upregulated by immune cells during type 1 and type 2 immune responses. Neutrophils showed a distinctive pattern of gene expression, with numerous genes associated with type 2 immune responses upregulated after N. brasiliensis but not LPS inoculation (Fig. 6D). Additionally, functional enrichment analysis by the DAVID bioinformatics database further confirmed a distinctive transcriptional signature for neutrophils from mice inoculated with N. brasiliensis, which showed significant enrichment for genes encoding molecules that are part of known type 2–associated...
pathways and protein families, such as collagen proteins (e.g., Col4a1, Col6a3, Col12a1 and P4ha2), extracellular matrix components (e.g., Adam15, Timp1, Mmp19, Mmp8, Col6a1 and Sfdp1) and molecules associated with chitin metabolism (e.g., Chi3l4, Fbn1, Chi3l1, Chi3l7, Chi3l3) (Benjamini-Hochberg corrected $\alpha$-value, 0.05). In contrast, the transcriptional signature of neutrophils from LPS-inoculated mice showed enrichment for genes encoding molecules characteristic of the type 1 response (Fig. 6e). We used qPCR for genes associated with type 1 and type 2 immune responses to corroborate findings with global transcriptome analyses. Highly purified neutrophils from mice inoculated with N. brasiliensis, but not LPS-inoculated mice, showed upregulation of Il12b, Il13 and Il33 but not Il4, as well as increases in M2 markers, whereas lung neutrophils from LPS-inoculated mice showed upregulation of Il12b (Fig. 7a). We also detected Il-13 and IL-33 cytoplasmic protein in neutrophils, but not in macrophages or CD4+ T cells, at day 2 after primary inoculation after stimulating lung cells with phorbol ester and ionomycin and staining with specific antibody, followed by flow cytometry analysis (Supplementary Fig. 5).

The known effect of IL-13 on M2 cell differentiation raised the possibility that neutrophils enhance M2 cell differentiation by their secretion of IL-13. To test this possibility, we cultured bone marrow–derived macrophages with neutrophils from lungs of mice inoculated with N. brasiliensis or LPS at day 2 after treatment. We observed marked increases in macrophage expression of Arg1, Chi3l3, Retnla and Igam in cultures of neutrophils from mice inoculated with N. brasiliensis (Fig. 7b). To investigate whether neutrophil-derived IL-13 might be contributing to M2 cell differentiation, we isolated neutrophils from N. brasiliensis–inoculated mice and cultured those cells with bone marrow–derived macrophages in separate chambers in transwell plates. Although the wells were separated by a 0.4-μm semipermeable membrane, N. brasiliensis–activated neutrophils still stimulated differentiation of M2 markers when cultured with an isotype control antibody. However, administration of anti–IL-13 effectively inhibited upregulation of M2 marker and Igam mRNA in bone marrow–derived macrophages (Fig. 7b). These results indicated that soluble factors, including IL-13, provide essential neutrophil–derived signals for M2 cell polarization. Taken together, our findings suggest that shortly after priming, neutrophils polarize to a distinctive phenotype and provide essential signals to macrophages, causing them to differentiate to effector macrophages with anti-helminth functions (Supplementary Fig. 6).

DISCUSSION

Both plants36 and invertebrates37 show acquired resistance, and published studies indicate that vertebrate innate immune compartments show recall responses (‘trained immunity’)38. Our studies indicate that macrophages primed during a type 2 immune response and under the influence of neutrophils maintain a long-lived phenotype that mediates accelerated helminth resistance in the absence of memory T cells or B cells. This is consistent with published studies suggesting that lung macrophages can maintain a long-lived state of desensitization after lung influenza infection39. The long-lived lung macrophage phenotype may be an important target for development of vaccines against helminths, as primed effector macrophages alone were sufficient to trigger accelerated parasite expulsion in naive recipient mice. Identification of this persistent effector macrophage phenotype may explain published studies in which CD4+ T cell depletion after secondary inoculation with N. brasiliensis did not impair resistance18. The persistence of M2 macrophages is associated with the development of fibrosis and emphysema-like impairment of lung function40,41. It is possible that the persistence of the M2 macrophages requires a type 2 immune microenvironment. Alternatively, a more fixed phenotype that is less dependent on extrinsic signals may develop in M2 macrophages, perhaps in association with stimulus-responsive regulatory molecules or epigenetic changes, as described in innate immune cells42,43. Studies suggest that basophil-primed macrophages in the skin inhibit migration of N. brasiliensis larvae from the subcutaneous site of inoculation23. We now show that transfer of primed lung macrophages to naive recipients inhibits parasite migration from the skin after primary inoculation, indicating that these primed anti-helminth macrophages also affect migrating larvae in skin, as well as the lung, shortly after inoculation.

Neutrophils are often studied during microbial infections in the context of type 1 immune responses44; however, neutrophils are also recruited to sites of helminth infection15,21 and can, when combined with macrophages in vitro, damage helminths16,45. Different subsets of neutrophils were identified in a model of Staphylococcus aureus infection, with one subset promoting M2 macrophage differentiation and also showing a ring-form nucleus46. Our studies now demonstrate that helminth infection triggers the development of an alternatively activated neutrophil (‘N2’) population that shows a characteristic global transcriptional profile distinct from LPS-activated (‘N1’) neutrophils. Although there was overlap between the two differentially activated neutrophil subsets, a number of genes were uniquely upregulated, including genes characteristic of type 2 responses. Our studies further showed that N2 neutrophils specifically activated in the context of helminth infection and IL-4R signaling mediated development of effector anti-helminth macrophages. Furthermore, neutrophil-mediated M2 macrophage differentiation was dependent on IL-13 production by neutrophils, although other soluble factors may also be involved. Adherence of macrophages primed in vivo with N. brasiliensis to parasitic larvae was blocked when neutrophils were depleted during priming. Indeed, neutrophil granule proteins can enhance monocyte adhesion mediated by β3 integrins47. Elevations in expression of M2 markers and integrins were accordingly reduced in macrophages from neutrophil-depleted mice primed with N. brasiliensis. Our findings thus suggest that a specific N2 population develops during helminth infection and can interact with macrophages to upregulate both M2 markers and adhesion molecules.

We observed close interactions of macrophages with parasitic larvae in vivo after secondary but not primary inoculation, consistent with primed macrophages surrounding and adhering to invasive larvae. Large numbers of macrophages adhering to migrating larvae may impair chemosensory function and access to nutrients, thereby contributing to parasite damage. Our findings that upregulated Igam expression on macrophages during N. brasiliensis infection was dependent on neutrophils and that blocking CD11b interactions inhibited macrophage adhesion to parasites indicates one mechanism through which neutrophils may promote anti-helminth macrophage effector function. Indeed, complement–opsonized adhesion of Leishmania promastigotes to macrophages is dependent on CD11b48. It will be of interest to examine whether complement has a role in opsonizing N. brasiliensis larval parasites for macrophage adhesion. Our studies also indicated an important role for Arg1 in parasite damage and killing, which is consistent with studies suggesting that Arg1 blockade can impair host resistance to tissue-dwelling metazoan parasites13,23.

Eosinophils also contribute to protective immunity to helminth infection, as transgenic mice with increased blood eosinophils show higher helminth resistance49. Our immunofluorescence analyses of infiltrates surrounding invading larvae in the lung indicate rapid accumulation of both macrophages and eosinophils, suggesting that eosinophils might also contribute to larval killing in vivo. It should
be noted, however, that highly purified macrophages from helminth-infected mice were sufficient to kill *N. brasiliensis* larvae in vitro. Basophilic studies have also been reported to mediate accelerated protective responses after secondary *N. brasiliensis* inoculation, although other studies have suggested that they may not be essential50. Further studies are needed to examine the role of basophilics, eosinophil, group 2 innate lymphoid cells and now neutrophils interacting with macrophages to mediate acquired resistance.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Microarray data are available in GEO under accession GSE46437.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

W.C.G., C.C.K. and F.J. Urban Jr. designed the experiments; FCW; W.C.G., A.M., J.F. Urban Jr. and N.P. did the experiments; J.L. prepared and provided the BEC; and W.C.G., C.C.K. and F.J. Urban Jr. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Female BALB/c mice 5–8 weeks old were purchased from NCI. B6.H-2Kd−/−BALB/c mice and B6.129−/−BALB/c mice were purchased from the Jackson Laboratory. H-2Kd−/−BALB/c mice were from TACONIC Laboratory. Colonies of genetically deficient mice were maintained and used with wild-type (WT) controls at ages 6–20 weeks. All mice were maintained in a specific pathogen-free, barrier facility excluding all common adventitious pathogens during the experiments. Healthy mice were selected for treatment groups from purchased or bred colonies without specific randomization methods or blinding methods.

Parasite inoculation, antibody, LPS and BEC administration. Nippostrongylus brasiliensis L3 larvae were incubated with 400 U penicillin, 400 µg/ml streptomycin and 400 µg/ml neomycin (GIBCO) for 2 h at room temperature and then washed with sterile PBS as described. For neutrophil depletion, Ly6G-specific antibody (BioXcell) or isotype control IgG was administered to mice both intraperitoneally (i.p.) (0.5 mg in 0.2 ml) and intratracheally (i.t.) (0.2 mg in 0.05 ml) 1 d before and 3 or 7 d after parasite inoculation, as described. To deplete the CD4+ T cell function in vivo, 800 µg of anti-CD4 mAb (GK1.5) was given i.p. 2 d before N. brasiliensis infection. LPS (lipopolysaccharides from Escherichia coli, Sigma) was administered to mice i.t. (50 µg in 50 µl of PBS). BEC was delivered orally to mice (10 mg per mouse per day from days 2 d after parasite inoculation), as described. The procedure to exsheath larvae included washing L3 larvae six times with sterile PBS containing 400 U penicillin and 400 µg/ml streptomycin. The parasitic L3 larvae were then incubated with 6.7 mM sodium hypochlorite in PBS for 15 min at room temperature, and exsheathed parasites were removed for subsequent culture. For the macrophage-larvae adherence assay, 20 larval parasites were removed from cultures with a p1000 Pipetman, washed in PBS, incubated in PBS with 0.5 mM EDTA for 1 h to remove adherent macrophages and counted by hemocytometer. The ATP concentrations for the L3 larvae were measured according to the manufacturer’s instructions. Briefly, 10 N. brasiliensis L3 larvae were collected in 100 µl of RPMI 1640 medium, to which 100 µl of the Celltiter-Glo Luminescent reagent (Promega) was then added. The larvae were homogenized and incubated for 5 min at room temperature to stabilize the luminescence signal. After the homogenate was centrifuged at 1,000 × g for 2 min, 100 µl of superantigen was applied to the luminescence to measure luminescence. As a negative control, larvae in RPMI 1640 medium were treated in boiling water for 5 min and homogenized with reagent after cooling. Dead larvae after incubation with macrophages were defined by nonmotile, outstretched bodies with nonrefractive internal structures. In other studies, bone marrow–derived macrophages (BMDMs) (1 × 106 cells per well) were cultured in 24-well plates with sorted lung neutrophils (1:1 ratio) obtained from mice at 2 d after inoculation with N. brasiliensis or after intratracheal administration of LPS. Neutrophils from each treatment were cultured with macrophages in separate chambers of Costar transwell (0.4-µm plates (Corning Incorporated). In some cases, anti–IL-13 or its isotype control (R&D Systems) was added to the cultures at a final concentration of 10 µg/ml. At day 3, the wells were washed with cold PBS and BMDMs were harvested and their RNA analyzed for gene expression by qPCR. Microscopic imaging of larvae was performed in the New Jersey Medical School Imaging Core Facility with a Nikon Diaphot inverted phase-contrast microscope.

Gene expression by microarray and qPCR. For microarray, lung macrophages (10,000 cells) were directly sorted into RNA lysis buffer (Ambion RNAqueous Micro for RNA isolation) and frozen on dry ice. RNA was isolated, amplified using the AminoAlyl MessageAmp II kit (Life Technologies), coupled to Cy3 dye and hybridized to SurePrint Mouse 8 × 60k gene expression microarrays (Agilent Technologies) per the manufacturer’s recommendations. Microarrays were scanned on an Agilent scanner, and spot intensities extracted with Feature Extraction software (Agilent Technologies). Data were quantile normalized, and statistical analysis was performed using significance analysis of microarrays. All microarray data are available from the NCBI Gene Expression Omnibus under accession number GSE46437. For qPCR, RNA was extracted from lung tissue or sorted macrophages and reverse transcribed to cDNA, as described. For qPCR, DNA was sequenced using the Applied Biosystems 7500 Real-Time PCR System. All data were normalized to 18S ribosomal RNA, and the quantification of differences between treatment groups was calculated according to the manufacturer’s instructions. Gene expression is presented as the fold increase over naive WT controls or other groups, as indicated in legends.

Histology. Lungs were formalin fixed and embedded in paraffin, and 5-µm sections were cut and stained with H&E to assess the inflammatory infiltrate, as described. Digital images were obtained with a Zeiss Axioskop 2 microscope and Zeiss Axiovision software.

Immunohistological staining. Lungs were perfused with 200 µl PBS and Tissue-Tek O.C.T. compound (Sakura), excised and frozen in chilled acetone. Tissue sections (5 µm) were obtained with an HM505E cryostat (Microm International GmbH) and stored at −80 °C. The tissue sections were allowed to dry at room temperature for 1 h, fixed in cold acetone for 10 min and stained with 24-well plates (2 × 106 cells per well) in 2 ml of RPMI 1640 medium with 10% FBS, 400 U penicillin, 400 µg/ml streptomycin and 100 µg/ml gentamicin. 100 µl of serum from the donor mice was also added to medium. 200 exsheathed L3 larvae were added to each well. In some experiments, anti-CD11b (eBioScience) (1–12 µg/ml) was also added to the macrophage-larvae culture. In other experiments, BEC was added, using published doses (0.1, 1 and 5 mM), to each well of the in vitro macrophage-larvae co-culture. Cells and larvae were cultured together for 5 d at 37 °C. The procedure to exsheath larvae included washing L3 larvae six times with sterile PBS containing 400 U penicillin and 400 µg/ml streptomycin. Macrophages were sorted using a BD FACSAria II (BD Biosciences). The stained with anti–mouse IL-13 (eBio13A; eBioscience) or IL-33 (396118, R&D Systems). Cells were fixed and permeabilized with saponin buffers and then stained with anti–CD4 mAb (GK1.5) were given i.p. 2 d before N. brasiliensis infection. LPS (lipopolysaccharides from Escherichia coli, Sigma) was administered to mice i.t. (50 µg in 50 µl of PBS). BEC was delivered orally to mice (10 mg per mouse per day from days 1–7 after inoculation with Nippostrongylus brasiliensis, at doses shown to effectively block arginase activity in the lung.

Flow cytometry and cell sorting. Lung tissue was minced and incubated with stirring at 37 °C for 30 min in HBSS with 1.3 mM EDTA (GIBCO), followed by treatment at 37 °C for 1 h with collagenase (1 mg/ml; Sigma) in RPMI 1640 (Mediatech) with 5% FCS (BioWest) and with 100 µg/ml of DNase (Sigma) for 10 min. Cells were lysed with ACK Lysing Buffer (Lonza) to remove erythrocytes, blocked with Fc Block (BD Biosciences), directly stained with fluorochrome-conjugated antibodies to CD3, CD4, CD8, Siglec-F, Ly6G (1A8), MHC class II, CD11c, CD11b, or F4/80 (BD Biosciences), and analyzed by flow cytometry with an LSRII flow cytometer (BD Biosciences Immunocytometry Systems). For intracellular staining, lung cells were stimulated with phorbol myristate acetate-ionomycin (PMA/Ion) for 5 h in the presence of Brefeldin A (Sigma). Cells were fixed and permeabilized with saponin buffers and then stained with anti–mouse IL-13 (eBio13A; eBioscience) or IL-33 (396118, R&D Systems). In some experiments, macrophages or neutrophils were sorted for adoptive transfer or cell culture using a BD FACSAria II (BD Biosciences). The sorted macrophages were confirmed by Giemsa–Wright (CAMCO) staining of cytospin slides with more than 95% purity. To track the position of transferred cells, sorted macrophages were incubated for 30 min at 37 °C in RPMI containing 5 µM carbonylfluorescein diacetate succinimidy1 ester (CFSE). After incubation, cells were washed before intravenous (i.v.) transfer into naive mice. Mice were killed 3 d later, and the presence of fluorescence-labeled cells in the lung was assessed by flow cytometry.

Adoptive transfer of macrophages. Cell suspensions were prepared from whole lungs of donor mice as described above, and macrophages were electronically sorted with antibodies to F4/80, MHC class II and CD11c (F4/80hiMHCIIint–hiCD11c+), as described. 2.5–5 × 106 cells (in 100 µl of PBS) were transferred into WT naive recipient mice through i.v. injection. After 2 d, mice were inoculated with infective N. brasiliensis L3 parasites. In some experiments, donor cells were collected from mice administered anti-Ly6G or isotype control IgG. In another experiments, donor cells were collected from mice administered LPS, as described above.

Macrophage cultures and larval ATP assay. Macrophages were electronically sorted and placed on 24-well plates (2 × 106 cells per well) in 2 ml of
with antibodies specific to F4/80 and MBP (rat mAb 14.7.4) supplied by J.J. Lee, Mayo Clinic, Scottsdale, AZ. Coverslips were applied to the slides using Fluoromount-G mounting medium (Southern Biotechnology Associates, Inc.). Images were taken using a Leica DM6000B and were tiled together using Image-Pro Plus 7.0 software (Media Cybernetics). Fluorescent channels were photographed separately, merged and overlaid with corresponding Nomarski images. Exposure times and fluorescence intensities were normalized to appropriate control images.

**Statistical analysis.** Data were analyzed using SigmaPlot 12 (Systat Software) and are reported as means ± s.e.m. Differences between multiple groups were assessed by one-way ANOVA, and individual comparisons were analyzed by the Holm-Šidák test. Tests were reported only where data met assumptions of tests. Differences of $P < 0.05$ were considered statistically significant. On the basis of preliminary experimental data, a power analysis of 0.8 with $P < 0.05$ indicates a minimum number of 3–4 inbred mice per group, but in most cases five mice per group were used.

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