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# Alternative Activation Is an Innate Response to Injury That Requires CD4$^+$ T Cells to be Sustained during Chronic Infection[1]

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**Alternatively activated macrophages (AAMΦ) are found in abundance during chronic Th2 inflammatory responses to metazoan parasites. Important roles for these macrophages are being defined, particularly in the context of Th2-mediated pathology and fibrosis. However, a full understanding of the requirements for alternative activation, particularly at the innate level, is lacking. We present evidence that alternative activation by the Th2 cytokines IL-4 and IL-13 is an innate and rapid response to tissue injury that takes place even in the absence of an infectious agent. This early response does not require CD4$^+$ Th2 cells because it occurred in RAG-deficient mice. However, class II-restricted CD4$^+$ T cell help is essential to maintain AAMΦ in response to infection, because AAMΦ were absent in RAG-deficient and MHC class II-deficient, but not B cell-deficient mice after chronic exposure to the nematode parasite, *Brugia malayi*. The absence of AAMΦ was associated with increased neutrophilia and reduced eosinophilia, suggesting that AAMΦ are involved in the clearance of neutrophils as well as the recruitment of eosinophils. Consistent with this hypothesis, AAMΦ show enhanced phagocytosis of apoptotic neutrophils, but not latex beads. Our data demonstrate that alternative activation by type 2 cytokines is an innate response to injury that can occur in the absence of an adaptive response. However, analogous to classical activation by microbial pathogens, Th2 cells are required for maintenance and full activation during the ongoing response to metazoan parasites.** *The Journal of Immunology,* **2007, 179: 3926–3936.**

It is now well known that macrophages can be alternatively activated by the type 2 cytokines IL-4 and IL-13 (1). Although the in vivo properties of these alternatively activated macrophages (AAMΦ)[5] remain ill defined, their prevalence in chronic type 2 inflammatory conditions such as parasite infection (2–7) and allergy (8–10) strongly suggests an important function under type 2 conditions. The importance of AAMΦ during parasitic infection has been confirmed using mice in which the IL-4Rα is specifically deleted from macrophages, significantly altering the outcome of infection with both protozoan and metazoan parasites (11, 12). It has been suggested that these macrophages are involved in tissue remodeling and wound healing (13), and our own data strongly support this (3). Furthermore, there is increasing evidence that AAMΦ play a role in tumorigenesis (14, 15), which has sometimes been compared with wound healing gone wrong (16). However, the origin and life cycle of these cells, as well as the cells and mediators that are necessary for the induction and upkeep of AAMΦ, have not been well characterized beyond a requirement for type 2 cytokines.

This is in contrast to the type 1 proinflammatory pathway in which the interplay between innate and adaptive immunity has been a major focus of the immunology field for the past decade (17, 18). The importance of macrophage or neutrophil activation via the engagement of TLRs (19) or by the IFNs (αβ or γ) (20) during the early phase of an acute microbial infection is increasingly well understood. Subsequent Th1 cell development promotes more effective macrophage killing that is essential in the control of many intracellular pathogens (21). We wished to investigate the requirements for macrophage activation in the context of infection with nematode parasites, which typically induce potent type 2 responses.

Our previous studies have shown that infection of mice with the filarial nematode, *Brugia malayi*, leads to the recruitment of large numbers of AAMΦ to the peritoneal cavity (2, 22). These macrophages have an IL-4-dependent phenotype that is sustained throughout several weeks of infection. This includes the suppression of cellular proliferation in a contact-dependent manner (2) and the expression of the molecules Ym1/Chi3l3, Fizz1/Relm-α, and arginase 1 (3, 23), which are now considered reliable markers of alternative macrophage activation (1, 6, 7). We thus chose to use this in vivo system to investigate alternative activation during both the early and late stages of a type 2 immune response. Our findings reveal that type 2-dependent alternative activation can occur very early in the immune response, independent of the adaptive arm of the immune system and independent of infection. However, maintenance of the full alternative macrophage activation phenotype requires CD4$^+$ T cells. These studies suggest that analogous to type 1 proinflammatory processes, alternative type 2 activation needs to be considered both in terms of an early innate response

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[5] Abbreviations used in this paper: AAMΦ, alternatively activated macrophage; PEC, peritoneal exudate cell; WT, wild type.

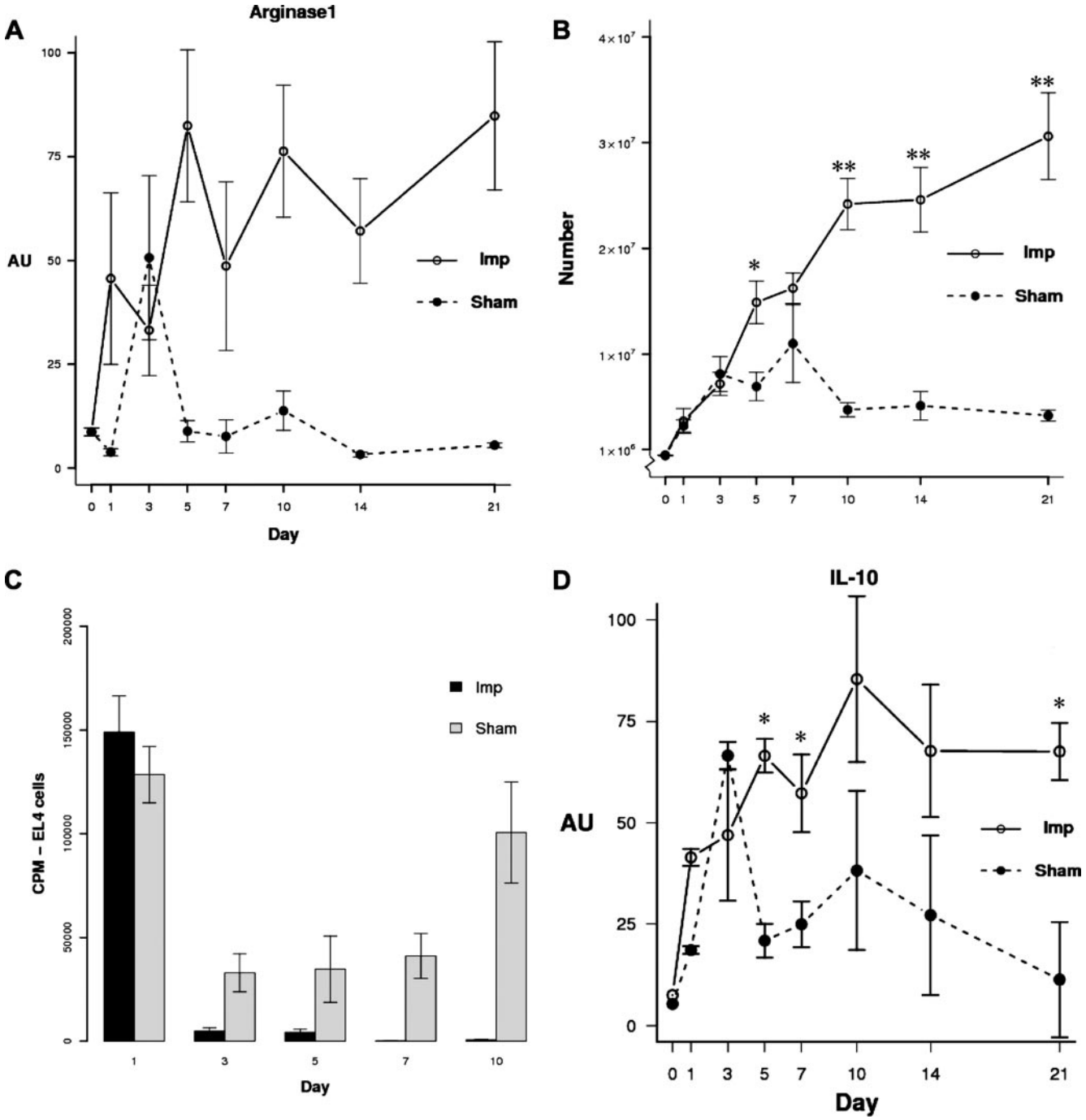


**FIGURE 1.** Kinetic differences between surgery only (Sham) and parasite implantation (Imp). *A*, Time course of arginase 1 expression. Real-time RT-PCR was used to determine arginase 1 expression in PEC over the course of implantation with *B. malayi* parasites as well as in control mice undergoing surgery only. *B*, Time course of cellular recruitment. Total cell numbers recruited to the peritoneal cavity were determined over the course of the experiment for mice implanted with *B. malayi* or those undergoing surgery alone. The *y*-axis changes from a log scale to linear scale at $1 \times 10^7$. *C*, Proliferative suppression by PEC. PEC from implanted or surgery-only mice were cocultured with the EL-4 thymoma cell line. Proliferation was monitored by the uptake of [$^3$H]thymidine. *D*, Time course of IL-10 expression by real-time RT-PCR, as described above for arginase. For each time point, $n = 5$ for the implant group and $n = 4$ for the surgery-only group. A significant difference between the two experimental groups at a particular time point is indicated by $*$ ($p < 0.05$) or $**$ ($p < 0.01$).

and later adaptive immune response, with distinct mediators and functions at each stage.

## Materials and Methods

### *Animals*

For all experiments, mice used were 6–12 wk old at the start of the experiment. All mice were bred and maintained in accordance with the animal care and use regulations of either University of California, University of Edinburgh, or Trudeau Institute. Breeding stocks of RAG1$^{-/-}$ and MHC class II$^{-/-}$ mice (on the C57BL/6 background) as well as wild-type (WT) C57BL/6 control mice were obtained from The Jackson Laboratory. C57BL/6 IL-4$^{-/-}$ were originally purchased from B&K Universal with permission of the Institute of Genetics, University of Cologne. BALB/c IL-4Rα-deficient mice (24) and C57BL/6 μMT (25) and WT control mice were bred in house. Both male and female mice were used with identical

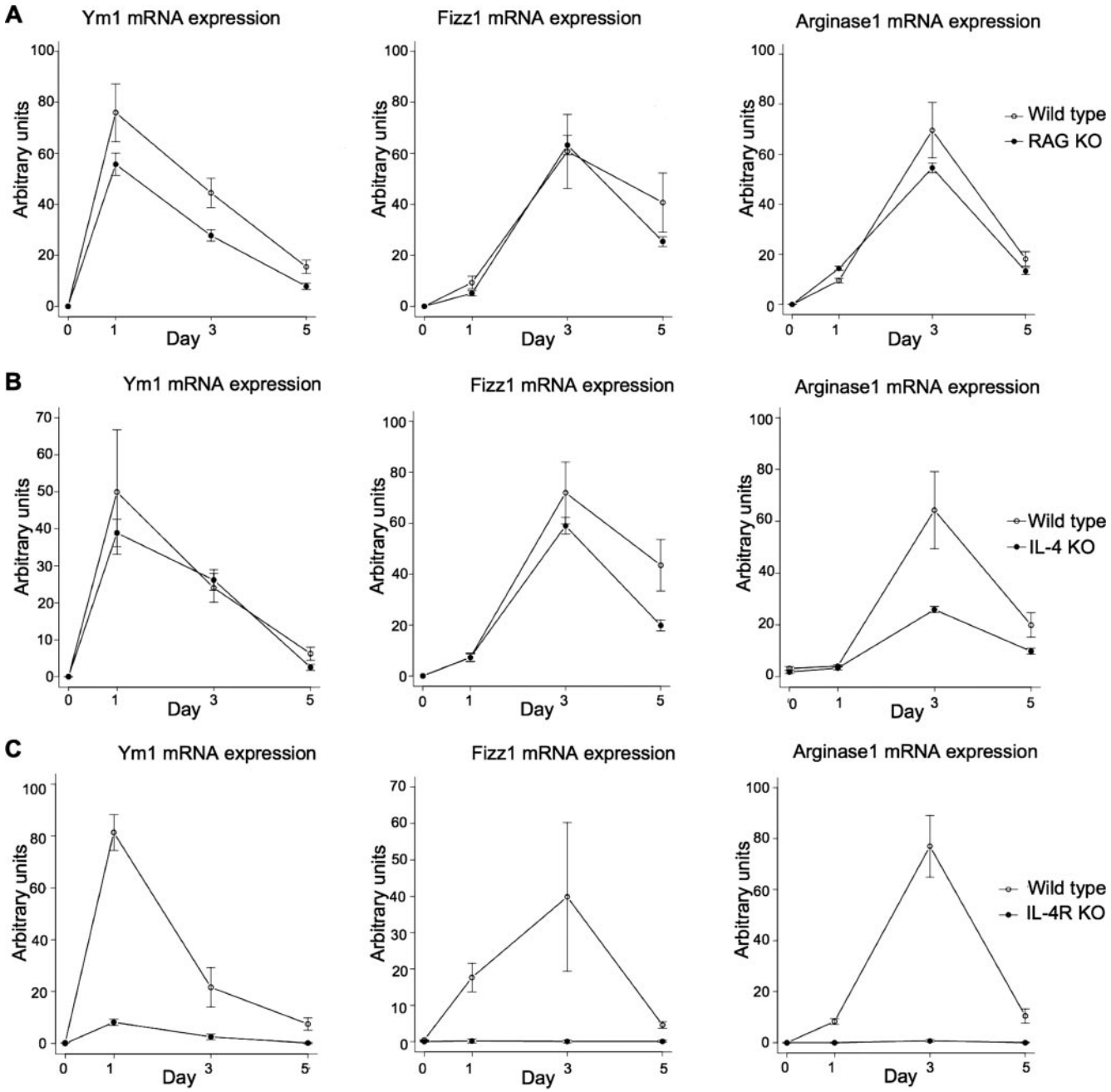


**FIGURE 2.** Requirements for early expression of alternative activation marker genes. *A*, Early alternative activation is independent of the adaptive immune response. Real-time RT-PCR analysis of PEC was performed on RAG-deficient mice or WT C57BL/6 control mice that have undergone a surgical incision and staple closure of the peritoneum. *B*, Role of IL-4 in early alternative activation. Real-time RT-PCR analysis of PEC was performed on IL-4-deficient mice or WT C57BL/6 control mice treated as above. *C*, IL-4R signaling is required for alternative activation in response to injury. Real-time RT-PCR analysis of PEC was performed on IL-4R-deficient mice or WT BALB/c control mice treated as above. Error bars represent SDs between 4 and 5 per group.

results. The 4get mice (C.129-*Il4*$^{tm1Lky}$/J) were generated, as previously described (26), and backcrossed to BALB/c for 10 generations.

### *Nematode implant and surgery*

*B. malayi* adult parasites were obtained from infected gerbils purchased from TRS Laboratories or maintained in house. Adult worms were removed from the peritoneal cavity of gerbils and washed in RPMI 1640. Under anesthesia, a small surgical incision is made through both the skin and the peritoneal membrane of the mouse, and five live adult *B. malayi* females are introduced in a volume of up to 1 ml. The incision is closed with the use of a medical stapler. Animals undergo sham surgery following the above steps, but with no parasites introduced. After 3–6 wk (or times indicated in the text), mice were euthanized by cardiac puncture and peritoneal exudate cells (PEC) were harvested by thorough washing of the peritoneal cavity with 15 ml of ice-cold RPMI 1640 medium. For thioglycolate-induced macrophages, mice were injected with 4% thioglycolate for 48–96 h before PEC were harvested.

### *Suppression assay*

Peritoneal lavage cells were plated at $1 \times 10^6$ cells/ml. Following 2- to 3-h adherence at 37°C, the nonadherent cells were removed, leaving a cell population highly enriched for macrophages (routine >85% F4/80$^+$). Adherent PEC were cocultured in 96-well flat-bottom plates with EL-4 cells ($1 \times 10^4$ cells/well) for 48 h. A total of 1 $\mu$Ci of [$^3$H]TdR in 10 $\mu$l of complete medium was then added to each well. Plates were incubated overnight before harvesting and counting using a liquid scintillation

counter (Micro$\beta$ 1450; Trilux). Triplicate or quadruplicate measurements per sample were performed. Results were plotted as cpm.

### *Real-time PCR*

Total RNA was isolated using either TRIzol (Invitrogen Life Technologies) or Tri reagent (Sigma-Aldrich), and cDNA was synthesized using either Moloney murine leukemia virus reverse transcriptase (Stratagene) or Superscript II (Invitrogen Life Technologies). Real-time PCR of the cDNA was conducted using either the Roche LightCycler or a GeneAmp 5700 (Applied Biosystems). Serial dilutions of a positive control sample of cDNA were used as a standard curve in each reaction. The level of each gene was expressed as a ratio to the level of either actin or GAPDH to control for differing levels of cDNA in each sample. The results are shown as arbitrary units. The primers have been described previously (3).

### *FACS, cytospin analysis, and ELISA*

For FACS analysis, cells ($2–5 \times 10^5$) were preincubated with unlabeled $\alpha$-CD16/32 (24G2), and then incubated with $\alpha$-F4/80 (Caltag Laboratories) and anti-Ly6G (eBioscience). Cytocentrifuge preparations of $1 \times 10^5$ cells were made using a Shandon Cytospin 3 (Thermo Shandon). Cytospins were air dried, fixed in methanol, stained with GIEMSA (Sigma-Aldrich) or Diff-Quik reagent (Dade Behring), and examined with a microscope for differential cell counting. For analysis of PEC from 4get mice, adherent cells were depleted by incubation in culture dishes in RPMI 1640 for 1 h at 37°C and flow cytometry was performed, as previously described (27). For splenocyte recall assays, $1 \times 10^6$ cells were stimulated with medium alone or *B. malayi* Ag (5 $\mu$g/ml). Following stimulation for 84 h, 100 $\mu$l of supernatant was removed for cytokine assays. The IL-13 concentration was determined by capture ELISA using the Quantikine mouse IL-13 immunoassay kit (R&D Systems), according to the manufacturer's instructions.

### *Ag presentation and phagocytosis assays*

Macrophages from the peritoneal cavity were purified by adherence in 24-well plates and incubated for 30 min at 37°C with FITC-labeled latex beads (Sigma-Aldrich) or CFSE-labeled human apoptotic neutrophils provided by the Centre for Inflammation Research (University of Edinburgh). The phagocytosis of beads/neutrophils was assessed by microscopic visualization of the wells or by FACS.

### *Statistical analysis*

Unless otherwise stated, data between groups were compared with two-tailed unpaired Student's *t* test using Prism 3.0 (GraphPad) with a normality test. Error bars always show variation between individual mice.

## Results

### *Expression of arginase 1 occurs early in response to surgery, but is sustained only in the parasite-implanted group*

Our experimental model involves the surgical implant of nematode parasites into the peritoneal cavity of mice through a small incision in the peritoneal wall. We had previously noted that some of the markers associated with alternative macrophage activation were seen in the first 1–3 days in both implanted mice and control mice that underwent the surgical procedure only (3). To further investigate this finding, we assessed whether arginase 1, a AAM$\Phi$ marker known to be associated with wound repair (28), displayed a similar pattern in parasite-implanted and surgery-only mice. We also compared cell recruitment in these two groups of mice to assess whether cell recruitment patterns differed at these early time points.

As seen previously with Ym1 and Fizz1 (3), arginase 1 mRNA was transiently elevated in PEC in response to surgical trauma and returned to baseline by 1 wk postsurgery. In contrast, animals with implanted parasites exhibited elevated arginase levels throughout the 3-wk course of the experiment (Fig. 1*A*). The total numbers of cells recruited into the peritoneal cavity were similar between surgery-only and parasite-implanted mice in the first 3 days, suggesting that the early cell recruitment is driven as much by surgical intervention as parasite presence (Fig. 1*B*). A breakdown of specific cell types indicated that the cell recruitment patterns were

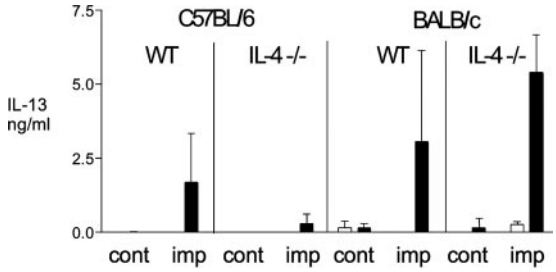


**FIGURE 3.** C57BL/6, but not BALB/c mice make reduced IL-13 in the absence of IL-4. WT and IL-4-deficient mice were implanted with *B. malayi* parasites for 3 wk. Splenocytes from naive (cont) or *B. malayi*-implanted mice (imp) were cultured in medium alone (□), or restimulated with *B. malayi* Ag (■) for 72 h, following which the supernatants were recovered for IL-13 cytokine ELISA. Error bars show SD from groups of four to five individual mice.

statistically indistinguishable in the first 3 days, with the one exception that parasite implant led to an immediate increase in eosinophils at 24 h ($6.7 \pm 3.0 \times 10^5$) that was less pronounced in the surgery-only mice ($0.7 \pm 0.3 \times 10^5$). The presence of the parasite, however, led to a further increase in total cell numbers that was still rising at 3 wk when the experiment was terminated with parasites still present (Fig. 1*B*). As previously published, the sustained cellular exudate was comprised of eosinophils, macrophages, and lymphocytes, with macrophages representing >70% of the total cell composition (29). In contrast, by day 10, the cell numbers in surgery-only mice had declined, but macrophages and lymphocytes remained somewhat elevated for the remainder of the experiment (data not shown). Thus, the drop in arginase seen above was not simply a reflection of the drop in macrophage numbers, suggesting that either alternative activation is short-lived in this setting or the alternatively activated cells are specifically taken up by the wound itself. As noted previously by ourselves and others (29, 30), parasite implant led to an early rise in neutrophil numbers that declined as eosinophil and macrophage numbers increased.

Another hallmark of our nematode-elicited macrophages has been the ability to suppress cellular proliferation in coculture, which is dependent on IL-4 in the C57BL/6 strain (2). We thus incubated the PEC from implanted or surgery-only mice with the EL-4 cell line, a rapidly dividing thymoma cell line (Fig. 1*C*). As described previously, PEC from nematode-implanted mice block proliferation completely by 1 wk postimplant, and this suppression remains for many weeks (31). We see a similar pattern in this study, but also demonstrate significant suppressive capacity in the PEC from surgery-only mice at days 3, 5, and 7 postsurgery, which is no longer evident 10 days after surgery. Our results are consistent with a role for AAM$\phi$ in wound healing after physical trauma. The proper progression of wound healing can only occur if the initial inflammatory response is controlled (32–35), and this would be consistent with the suppressive function of these macrophages. Analysis of nematode-elicited macrophage cytokine production by cytokine bead array has revealed that they produce abundant IL-10 and IL-6 (data not shown), cytokines consistent with both anti-inflammatory and wound healing functions. We therefore assessed mRNA profiles of PEC from nematode-implanted mice and sham surgery mice to determine whether the types and amounts of cytokines were similar. IL-10 (Fig. 1*D*) and IL-6 (data not shown) followed a similar profile as the alternative marker arginase (Fig. 1*A*).

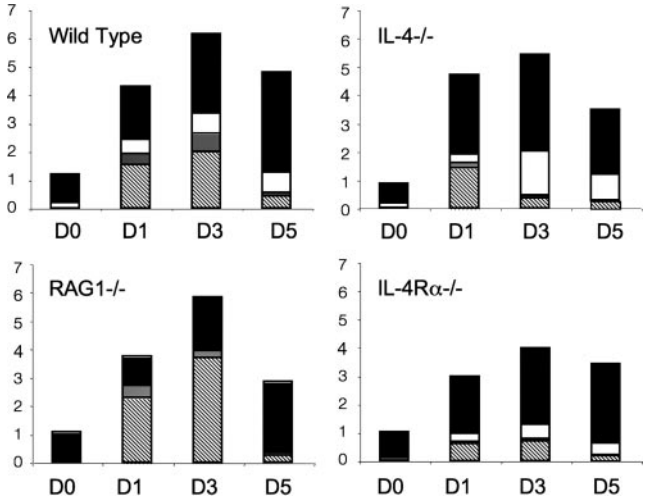


**FIGURE 4.** Cell recruitment in mice undergoing surgery alone. Differential cell counts $\times 10^6$ are shown for naive mice (D0) and 24 h (D1), 72 h (D3), and 5 days (D5) postsurgery. From *top* to *bottom*, cells are as follows: monocytes/macrophages, ■; lymphocytes, □; eosinophils, ▨; and neutrophils, ▧. There were four or five mice per group. The following differences were statistically significant ($p < 0.03$, Mann-Whitney *U* test with Bonferroni correction): neutrophils and eosinophils were reduced in the IL-$4^{-/-}$ vs WT at day 3, whereas lymphocytes were increased. Neutrophils and eosinophils were reduced in the IL-$4R^{-/-}$ vs WT at days 1, 3, and 5. Neutrophils were elevated in the $RAG1^{-/-}$ vs WT at day 3, and lymphocytes were reduced at all days in the $RAG1^{-/-}$ compared with the WT mice. The WT data from all three experiments are shown in a single combined graph, but statistics were calculated between gene-deficient and WT mice for each individual experiment.

### *Ym1, Fizz1, and arginase 1 up-regulation in response to surgery occurs independently of T or B cells, but requires IL-4 and/or IL-13*

The rapid elevation (1–3 days) and then decline (3–5 days) in Ym1, Fizz1 (3), and arginase 1 (Fig. 1*A*) suggested that these proteins are part of the innate response to surgical trauma. To ask whether these responses could occur in the absence of adaptive immunity, we performed the surgical procedure on $RAG^{-/-}$ mice that lack both B and T cells. Ym1, Fizz1, and arginase 1 mRNA were all elevated in response to the surgical procedure, and there was no significant difference between WT and $RAG^{-/-}$ mice (Fig. 2*A*). Thus, this early response is entirely innate with no requirement for B or T cells. These data also illustrated subtle differences in kinetics that we consistently observe. Ym1 expression peaks at 24 h, whereas Fizz1 and arginase 1 are slightly delayed, with expression peaking at 72 h.

In parasite-implanted C57BL/6 mice, the expression of Ym1, Fizz1, and arginase 1 is dependent on IL-4 (3, 23). So, we asked whether the early innate expression of these proteins was also IL-4 dependent. IL-4-deficient mice and the WT C57BL/6 controls underwent the surgical procedure, and Ym1, Fizz1, and arginase 1 mRNA levels were assessed in the PEC by real-time RT-PCR. Fizz1 and arginase 1 expression were reduced, but not absent in IL-4-deficient mice. In contrast, Ym1 expression did not require IL-4 at these early time points because mRNA levels were identical in WT and IL-4-deficient mice (Fig. 2*B*).

Ym1, Fizz1, and arginase 1 expression has previously been shown to be strictly dependent on IL-4 on the C57BL/6 background following a 3-wk implant with *B. malayi* (3, 23). However, on the BALB/c background, an absence of IL-4 was insufficient to prevent alternative activation, and the absence of both IL-4 and

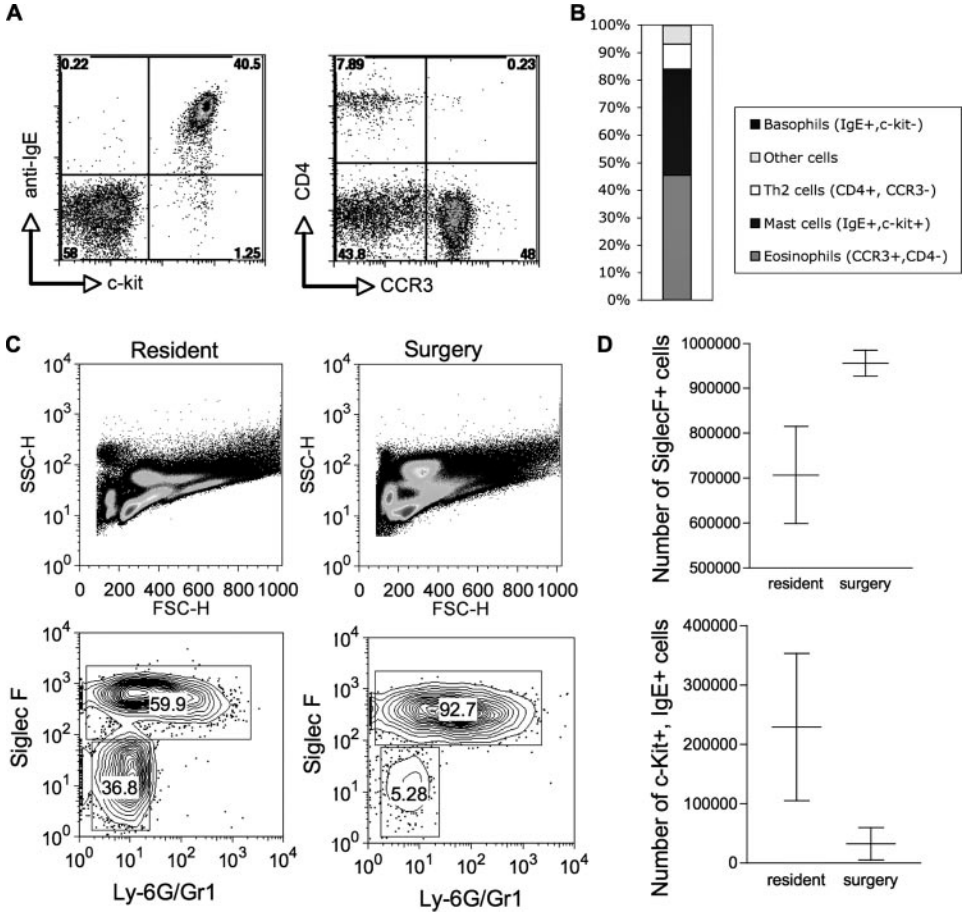


**FIGURE 5.** Identification of IL-4-expressing cells in the peritoneal cavity with the 4get mice. *A*, Representative plots showing c-*kit* and anti-IgE staining to identify basophils (anti-$IgE^+$, c-$kit^-$) and mast cells (anti-$IgE^+$, c-$kit^+$) within the $GFP^+$ population as well as showing CD4 and CCR3 staining to identify Th2 cells ($CD4^+$) and eosinophils ($CCR3^+$) that are $GFP^+$. *B*, Relative proportions of $GFP^+$ cells in PEC populations. *C*, Representative plots showing changes in cellular populations postsurgery. Plots show forward and side scatter, as well as Siglec F and Ly6G/Gr1 staining to identify mature eosinophils. *D*, Whisker plots showing the range and median total number of $GFP^+$ Siglec $F^+$ cells (eosinophils) and $GFP^+$, c-$Kit^+$, and $IgE^+$ (mast cells) in either resident peritoneal cells or 72 h after surgery.

IL-13 signaling was required (our unpublished observation). We hypothesized that in the absence of IL-4, C57BL/6 mice fail to develop Th2 cells that produce IL-13, but in the more Th2-prone BALB/c mouse, IL-13 may still be produced. To address this, we measured IL-13 production by splenocytes from naive and implanted WT and IL-4-deficient mice on both C57BL/6 and BALB/c backgrounds. Indeed, we found that IL-4-deficient mice on the BALB/c background make considerable amounts of IL-13 in response to *B. malayi* implant, whereas on the C57BL/6 background little IL-13 is produced in the absence of IL-4 (Fig. 3). These data suggested that in vivo IL-13 may have an equal or greater role to play than IL-4 in the induction of Ym1 and Fizz1. We thus chose to use the IL-4R$\alpha$-deficient mice to assess whether the inability of IL-4 to affect Ym1 expression during the early injury response could be explained by the presence of IL-13. These experiments were performed on the BALB/c background because this was the only strain available to us. We found that indeed in the absence of IL-4R signaling, there was a near complete ablation of Ym1 expression relative to BALB/c controls, whereas Fizz1 and arginase 1 were entirely absent (Fig. 2*C*). Thus, the IL-4R$\alpha^{-/-}$ mice demonstrate that innate expression of Ym1, Fizz1, and arginase 1 requires either IL-4 or IL-13. Different target cells for IL-4 vs IL-13 may explain the subtle differences in expression kinetics for these alternative activation markers.

Thus, the cells recruited to injury had the hallmarks of alternatively activated cells, because they expressed Ym1, Fizz1, and arginase 1, and blocked the proliferation of bystander cells. Furthermore, expression of the alternative activation markers was strictly dependent on the Th2 cytokines IL-4 and IL-13. We analyzed the cell types present in the peritoneal cavity in the first 5 days following surgery in these gene-deficient mice. In the control WT mice, neutrophils and macrophages represented the dominant cell types at day 1 and peaked by day 3 (Fig. 4). Macrophage numbers continued to rise and exceeded the neutrophil numbers by day 5. With the exception of a complete absence of lymphocytes, RAG$^{-/-}$ mice showed near identical recruitment patterns to the WT mice at these early time points, whereas in the IL-4$^{-/-}$ and IL-4R$\alpha^{-/-}$ mice, granulocyte numbers (both eosinophils and neutrophils) were reduced relative to WT (Fig. 4).

To determine the potential source of early IL-4, we utlilized the 4get mice that express GFP as part of a bicistronic IL-4 internal ribosomal entry site (IRES)-GFP mRNA (26). GFP$^+$ cells are rare in naive mice (26) and reflect cells expressing IL-4 transcript. Flow cytometric analysis of GFP$^+$ cells with markers for basophils, mast cells, eosinophils, and T cells (27, 36) allowed us to delineate which cells in the peritoneal cavity of naive mice have the capacity to produce IL-4. The composition of the GFP$^+$ cells was ~50% eosinophils and 40% mast cells (Fig. 5, *A* and *B*). Ten percent were Th2 cells, but these are not a possible source of early IL-4 in the RAG$^{-/-}$ experiments. In addition, we assessed the GFP$^+$ cells after surgery and found that the mast cell population was dramatically reduced, consistent with our previous cytospin analyses, and that now eosinophils represented >90% of the cells positive for IL-4 mRNA.

### *Sustained presence of AAMΦ during nematode infection requires the adaptive immune response*

Because our surgery experiments demonstrated the absence of a requirement for the adaptive immune system in the generation of the alternatively activated phenotype, we chose to examine whether the sustained activation of macrophages we see during nematode infection could also occur independently of adaptive immunity. To test this, we implanted *B. malayi* into the peritoneal cavities of RAG1$^{-/-}$ mice for 3 wk. Control RAG1$^{-/-}$ mice injected with thioglycolate recruited equal numbers of peritoneal cells (Fig. 6*A*), with similar

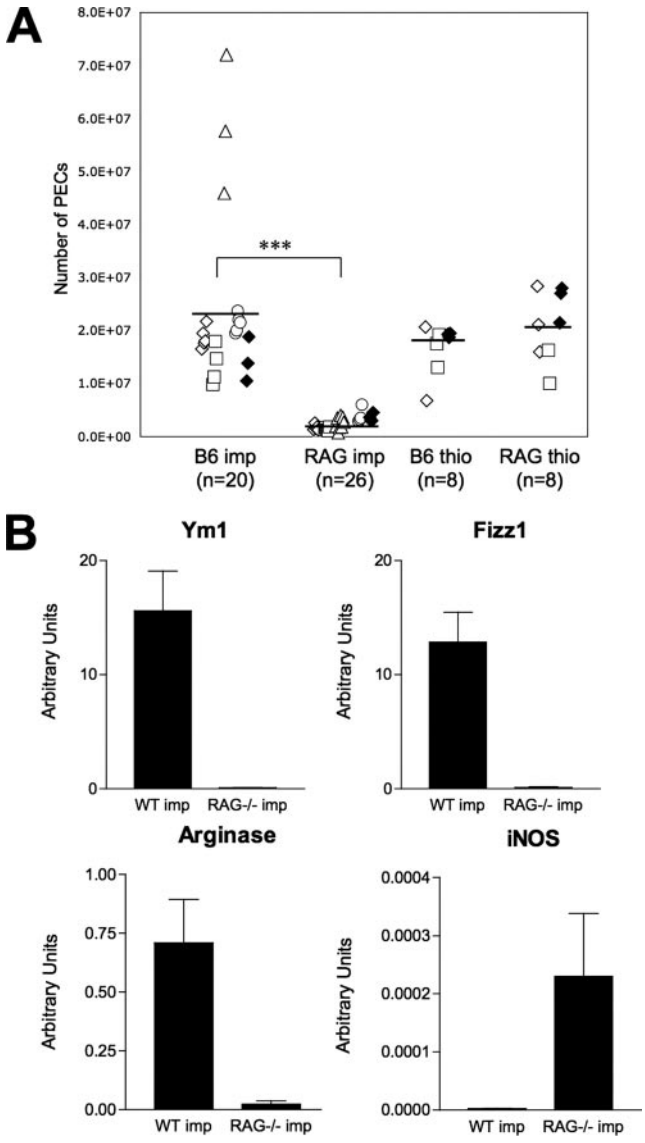


**FIGURE 6.** Alternative activation by *B. malayi* requires adaptive immunity to be sustained and is absent in RAG1-deficient mice. *A*, Parasite-implanted C57BL/6 or RAG1$^{-/-}$ mice were compared with mice either injected with 4% thioglycolate or left naive (data not shown). The PEC were counted on a Coulter counter. Data shown are compiled from five experiments with parasites and three experiments with thioglycolate, and are indicated by separate symbols being used for separate experiments. *B*, Expression of AAMΦ marker genes Ym1, Fizz1, and arginase 1 was determined by real-time RT-PCR analysis of total PEC. Data shown are a representative of five separate experiments with identical results. The error bars show the SD between individual mice ($n = 3$ for WT imp, and $n = 5$ for RAG1$^{-/-}$ imp for this experiment). ***, $p < 0.0005$.

percentages of F4/80$^+$ macrophages (data not shown) as WT mice, indicating that there is no inherent macrophage recruitment defect in RAG1$^{-/-}$ mice. In contrast, there was a dramatic difference in the number of cells recruited by the parasites in WT vs RAG1$^{-/-}$ mice (Fig. 6*A*). Although peritoneal cells in RAG1$^{-/-}$ naive mice increased from $5 \times 10^5$ cells per mouse (compared with $2.5 \times 10^6$ cells in WT mice), to a mean of $1.55 \times 10^6$ ($\pm 4.4 \times 10^5$) cells after infection. This was much less than the mean of $1.63 \times 10^7$ ($\pm 3.8 \times 10^6$) cells recruited in WT mice.

To determine whether the inflammatory cells recruited in RAG1$^{-/-}$ mice contain AAMΦ, we looked by real-time PCR for

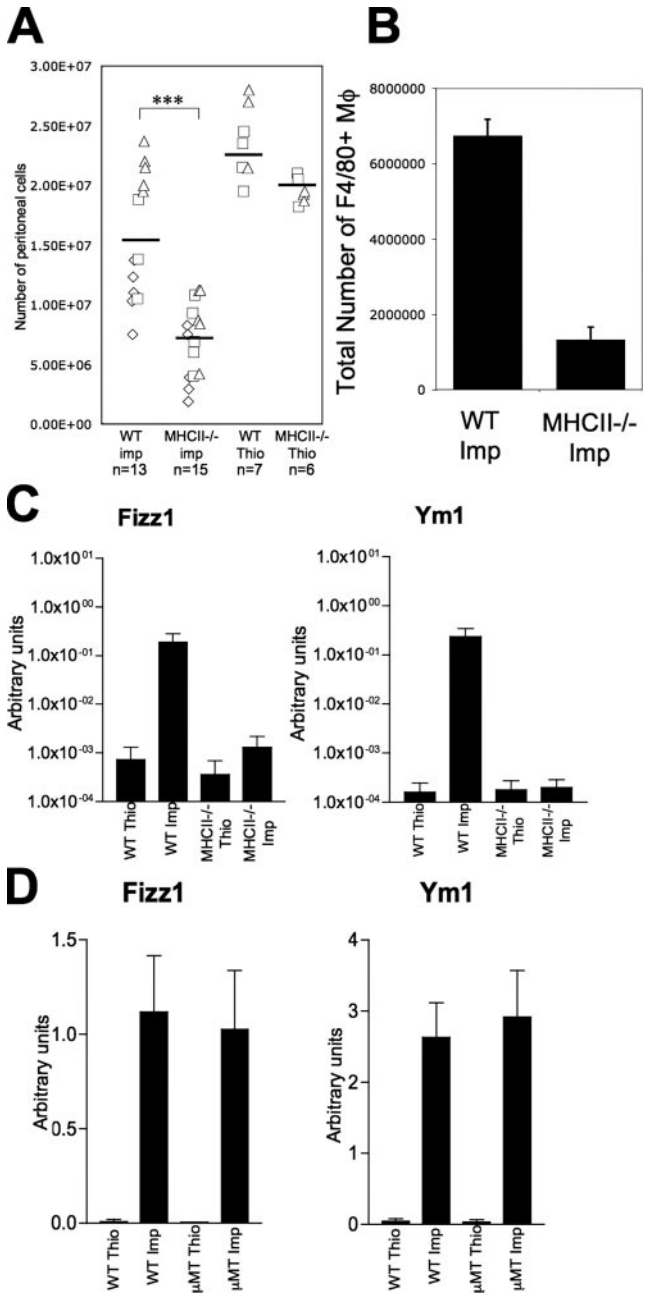


**FIGURE 7.** Recruitment of AAMΦ requires $CD4^+$ T cells, but not B cells. *A*, *B. malayi* parasites were implanted into MHC class II-deficient mice and C57BL/6 mice, as above, and were compared with thioglycolate injection. The numbers of PEC were counted using a Coulter counter. Data shown are compiled from three experiments with parasites and two experiments with thioglycolate, and are indicated by separate symbols being used for separate experiments. *B*, The total number of $F4/80^+$ macrophages was determined by FACS. Data shown are a representative of three separate experiments. *C*, MHC class II deficient mice do not recruit AAMΦ after *B. malayi* implantation. This graph is plotted on a log scale. Data shown are a representative of three separate experiments with identical results. Error bars represent SDs between individual mice ($n = 3$ for WT imp and $n = 5$ for $MHC^{-/-}$ imp for this particular experiment). *D*, B cell deficient μMT mice recruit AAMΦ after *B. malayi* implantation. Expression of the marker genes Ym1 and Fizz1 in adherent macrophage (>80% $F4/80^+$) populations was determined by real time PCR analysis as described above. Data shown are a representative of three separate experiments with identical results. Error bars represent SDs between individual mice ($n = 3$ for WT imp and $n = 5$ for μMT imp for this particular experiment). ***, $p < 0.0005$.

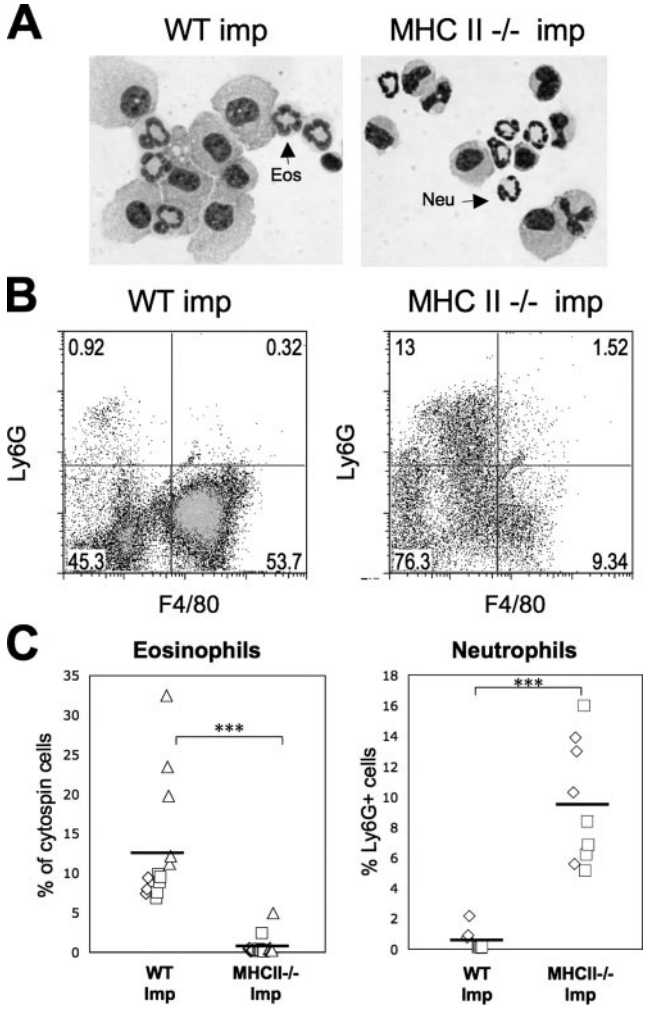


**FIGURE 8.** Increased neutrophilia and decreased eosinophilia in *B. malayi*-implanted MHC class II-deficient mice. *A*, Example of the cellular composition of PEC from parasite-implanted C57BL/6 (WT) mice and MHC $II^{-/-}$ mice. Eosinophils (Eos) and neutrophils (Neu) are indicated with arrows. *B*, Representative FACS analysis of PEC showing the proportion of $F4/80^+$ macrophages and Ly6G $(Gr1)^+$ neutrophils. *C*, The proportion of eosinophils from implanted mice was determined by counting >300 cells from randomly selected fields per Giemsa-stained cytospin. Data are compiled from three separate experiments indicated by separate symbols. The proportion of neutrophils was determined by FACS, as described above. Data are compiled from two separate experiments.

the expression of Ym1, Fizz1, and arginase 1. Although cell recruitment was very low in $RAG1^{-/-}$ mice, sufficient cells were present for mRNA analysis. Cells isolated from $RAG1^{-/-}$ mice did not display significant expression of Ym1, Fizz1, or arginase 1 (Fig. 6*B*) and were similar to thioglycolate controls (data not shown). Analysis of adherent cells enriched further for macrophages showed similar results (data not shown). These results suggest that either B or T cells are required for a sustained presence of AAMΦ. Of note, inducible NO synthase was elevated in the implanted $RAG^{-/-}$ mice, suggesting that the small numbers of cells recruited were in a classical activation state.

### *$CD4^+$ T cells, but not B cells, are required to maintain AAMΦ during an adaptive immune response*

We predicted that $CD4^+$ T cells were the most likely component of adaptive immunity that would be required for AAMΦ recruitment. Hence, we implanted parasites into mice lacking class II

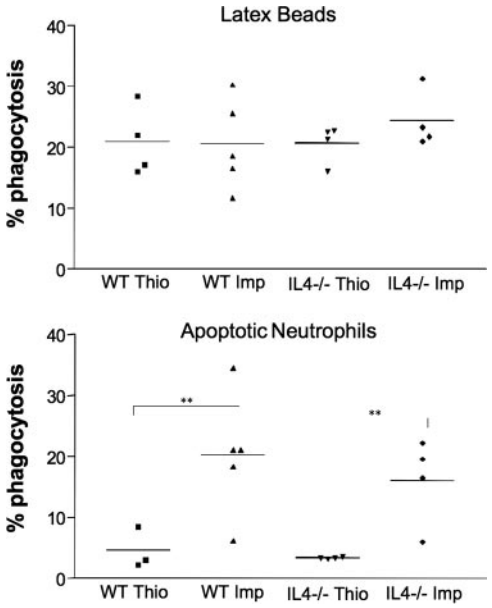


**FIGURE 9.** Thioglycolate-recruited macrophages (Thio) or parasite-recruited macrophages (Imp) were incubated with FITC-labeled latex beads or apoptotic neutrophils. The percentage of macrophages that had phagocytosed beads or neutrophils was determined by FACS. Data shown are representative of three separate experiments. **, $p < 0.01$.

MHC (MHC II$^{-/-}$), which are defective in selecting for CD4$^+$ T cells (37) and are a better model for T cell help than CD4-deficient mice, in which the CD8 population is heavily contaminated with MHC class II-restricted T cells (38). There was a striking reduction in the number of total cells recruited after implantation of MHC II$^{-/-}$ mice (Fig. 7*A*). There were also fewer F4/80$^+$ macrophages recruited in these mice (Fig. 7*B*), consistent with our previous study of nude mice (22). Thioglycolate-injected MHC II$^{-/-}$ mice recruited similar numbers of macrophages and PEC as WT mice (Fig. 7*A*). Expression of Ym1 and Fizz1 mRNA was reduced to control levels in parasite-implanted MHC II $^{-/-}$ mice (Fig. 7*C*). Thus, class II-restricted T cell help is apparently required for the sustained recruitment and differentiation of AAMΦ in vivo.

Because RAG1$^{-/-}$ mice lack both B and T cells, we chose to also assess whether B cells are an important component of the adaptive response required for AAMΦ differentiation and maintenance. We therefore implanted parasites into B cell-deficient $\mu$MT mice and looked for the expression of AAMΦ marker genes. In contrast to the analysis of RAG1$^{-/-}$ and MHC II $^{-/-}$ mice, identical numbers of peritoneal cells, including F4/80$^+$ macrophages, were recruited in $\mu$MT mice (data not shown), which expressed comparable levels of AAMΦ genes as WT mice after parasite implantation (Fig. 7*D*).

### *AAMΦ induced during an adaptive immune response may mediate clearance of early neutrophilia*

To determine whether there were any changes in the composition of cell types in MHC II$^{-/-}$ mice, we compared the inflammatory PEC by FACS and cytospin analysis. We observed an increase in neutrophils and a marked decrease in eosinophils (Fig. 8). The reduction of eosinophils was not surprising and is consistent with the known role of Th2 cytokines in eosinophil development and recruitment (39). Ramalingam et al. (30) have also shown that CD4$^+$ cells play a role in recruiting eosinophils in the closely related *Brugia pahangi* infection model. However, the increase in neutrophilia was unexpected. Parasite-implanted RAG$^{-/-}$ mice also had elevated neutrophils similar to MHC II$^{-/-}$ mice (data not shown). The sustained presence of neutrophils in mice lacking AAMΦ suggested that AAMΦ might also play a role in the clearance of neutrophils. Hence, we used an in vitro system to determine whether AAMΦ could phagocytose neutrophils. We found that AAMΦ from parasite-implanted WT mice were significantly better at phagocytosing neutrophils than thioglycolate-induced macrophages (Fig. 9). In contrast, there was no difference in their ability to phagocytose latex beads (Fig. 9). Macrophages from C57BL/6 IL-4$^{-/-}$ mice were as capable as macrophages from WT mice at phagocytosing neutrophils. This is consistent with the fact that we did not observe an increase in neutrophilia in C57BL/6 IL-4$^{-/-}$ mice, even though macrophages from these mice do not possess the markers of alternative macrophage activation (2, 3, 23, 31) These data suggest that macrophages in the nematode infection setting are influenced by factors other than IL-4 alone.

## Discussion

We have used a surgical implant model of filarial nematode parasite infection to illustrate the requirements for both innate and adaptive cells for alternative activation during acute vs chronic inflammatory responses. These studies reveal that there is an innate alternative activation pathway that does not require adaptive immunity and can be triggered solely by tissue injury. Although activation of innate cells by microbial products or proinflammatory cytokines has long been documented, activation by early sources of Th2 cytokines or Th2-inducing pathogen products remains ill defined. Metazoan parasites are well known to induce early and rapid IL-4 (40–43), and there is also evidence that their secreted products can lead to alternate macrophage activation (29, 44). However, we show in this study, the markers of alternative activation could be induced in the absence of parasite products and can occur in response to a tissue injury, without the presence of T cells. The IL-4R$\alpha^{-/-}$ mice demonstrated that innate expression of Ym1, Fizz1, and arginase 1 requires either IL-4 or IL-13, as previously reported for allergy, fibrosis, and infection (45–48).

The innate source of IL-4 or IL-13 triggering this injury response has yet to be identified, but is likely to be eosinophils or mast cells. Analysis of 4get mice revealed that eosinophils, mast cells, and Th2 cells were the only populations in the peritoneal cavity of naive mice expressing IL-4 mRNA. Very few basophils were present. T cells can be excluded because we observed the IL-4R$\alpha$-dependent injury response in RAG-deficient mice. Following surgery, there was a dramatic increase in the numbers of IL-4$^+$ eosinophils accompanied by a drop in mast cell numbers. Eosinophils have been previously identified as innate IL-4-producing cells in *Nippostrongylus brasiliensis* (49), *Schistosoma mansoni* (42), and *Heligomosoides polygyrus* infection (36). Although these data implicate eosinophils as the trigger for alternative activation, mast cells cannot be ruled out. Mast cells are considered sentinels of tissue injury (50) and are known to rapidly produce IL-4 or IL-13 (27, 51). The rapid decline in mast cells following surgery may reflect degranulation or activation-induced adherence to the peritoneal wall with release of cytokine stores.

Recently, Reece et al. (52), using the *N. brasiliensis* model, demonstrated innate expression of *ym1*, *fizz1*, and *arg1* in the lungs of infected SCID mice. Because *N. brasiliensis* migration leads to severe lung damage, the study supports a role for these proteins as responders to tissue injury. However, it is not possible to determine whether the response was the result of tissue damage or helminth Ags. Our study demonstrates that an innate type 2 response will occur solely in response to physical trauma. An intriguing twist to this story is the recent discovery that chitin, a molecule found in many pathogens and allergens, but not in mammals, leads

to rapid alternative activation through recruitment of IL-4-expressing cells (53). Because chitin is unlikely to be found in our sterile surgical procedure, it raises the possibility that endogenous activators of IL-4 include chitin-like sugars. Ym1 itself is a molecule that can bind chitin, but also has the ability to bind an array of host sugars (54). The finding that Ym1 is needed for Th2 cell activation (55) suggests that it may play a role in triggering activation by these sugars. This raises the question as to whether there are fundamental differences in alternative activation by endogenous ligands vs pathogen-derived molecules. We have found that the ability of some parasite-derived molecules to induce AAMΦ (29) is entirely dependent on IL-4 or IL-13 (our unpublished observation) as is the innate wounding response described in this work. Thus, the key factor seems to be the ability to induce IL-4 and/or IL-13. Whether parasite and host factors are acting through distinct (and thus potentially additive) pathways has yet to be determined. However, in our study, the sham surgery response was as great as that seen with surgical parasite implant, suggesting that the wounding response was dominant and the parasite at these early stages was not augmenting this response.

The main difference between sham surgery and parasite implant was the sustained Th2 cell-dependent response. With the knowledge that alternatively activated macrophages are part of the innate response, this raises an additional question about their role in Th2 cell induction. We have shown in vitro that macrophages from *Brugia*-implanted mice can prime naive T cells to differentiate into Th2 cells (56), and Ym1 has been shown to be an important DC factor involved in Th2 differentiation (55). These data along with the knowledge that inflammatory macrophages can traffic to the draining lymph nodes to prime T cell responses (57) would support a role for these macrophages in Th2 induction. However, we have also shown that AAMΦ, unlike T regulatory cells, do not become detectable in the draining lymph nodes of filarial-infected mice until the infection becomes systemic (58), arguing against a role in early induction of Th2 cells. Regardless of whether the AAMΦ play a role in Th2 induction, the data do suggest they are involved in orchestrating the sequence of events. We have previously found that there is an early influx of neutrophils during the acute phase of the response, but they are gradually replaced by eosinophils and are essentially gone after 2 wk (29), and this pattern was observed again in this study (data not shown). A recent study demonstrated that AAMΦ contribute significantly to eosinophil recruitment (59). Put together with our finding that AAMΦ may also mediate neutrophil clearance, a model is suggested whereby macrophages orchestrate the cell recruitment profile during type 2 inflammation, through an initial clearance of neutrophils, followed by the recruitment of eosinophils, with Th2 cells required for the second phase. Whether they are also responsible for Th2 induction and whether wound AAMΦ vs parasite AAMΦ differ in this regard still needs to be addressed.

Our previous work points to AAMΦ as the main source of Fizz1, Ym1, and arginase 1 in the peritoneal population (3, 23). However, neutrophils may be an important source of these proteins at early time points because they have been shown to contain Ym1 (60) and are the predominant cell in WT and $RAG^{-/-}$ mice in the first 24–48 h (this study) (29), but are significantly reduced in the IL-$4R^{-/-}$ mice that failed to express AAMΦ-associated markers. Evidence also suggests that neutrophils can be classified into classical or alternative categories (61). Additionally, eosinophils were significantly elevated at 1 and 3 days postsurgery and can express both Fizz1 (49) and Ym1 (our unpublished observations). Although arginase expression has been described in human granulocytes, in mouse studies to date, Th2-inducible arginase has only been described in macrophages (62). Differential expression of Ym1 (peaking at day 1) as compared with Fizz 1 and arginase 1 (peaking at day 3) could suggest that different cell types are responsible for the early production of these proteins.

Although our data provide strong evidence for alternative activation as a part of the innate response to injury, the expression of these markers was transient. Only in the presence of the helminth parasite were macrophage numbers as well as AAMΦ-associated markers sustained. This required the adaptive immune response because AAMΦ were absent in the $RAG^{-/-}$ mice at later time points, contrasting directly with the injury response. Consistent with this finding, expression of AAMΦ markers during *N. brasiliensis* infection declines after 1 wk in SCID, but not WT mice (52). Our results show that the component of the adaptive immune response required to sustain alternative macrophage activation are class II-restricted $CD4^+$ T cells with no requirement for B cells. Specifically, because the expression of these genes is dependent on type 2 cytokines, it is Th2 cells that are required. Interestingly, costimulation through CD28 is not necessary for this component of $CD4^+$ T cell function (63). This is consistent with the completely normal Th2 response observed in CD28-deficient mice infected with a different nematode parasite *H. polygyrus* (64). Additionally, we have shown that despite a role in the induction of type 2 Abs, ICOS costimulation is also not required for the AAMΦ phenotype (63).

The observation that T cell help is required for alternative macrophage activation is important because $CD4^+$ T cells are known to be essential for the formation of immune granulomas, which play a role in protecting both the host and the parasite (30, 65). In the mouse schistosomiasis model, $CD4^+$ T cells have been shown to play a role in granuloma formation through studies with MHC class II-deficient mice and through anti-CD4 depletion experiments (66, 67). More recently, macrophages expressing high levels of arginase 1 have been implicated in the deposition of collagen and formation of fibrotic granulomas (7). Our results suggest that the recruitment of AAMΦ could be a crucial mechanism through which $CD4^+$ T cells are involved in fibrotic granuloma formation. Our results would suggest that AAMΦ could also be key intermediaries between $CD4^+$ Th2 cells in asthma and downstream effects such as eosinophil recruitment (59) and tissue remodeling (through arginase 1/Ym1) (10).

It is important to stress that although IL-4/IL-13-mediated responses may be important in tissue repair, they do not appear to be essential. Although we have never directly quantified the rate of wound repair in our surgery model, the incision is effectively healed in the mice that lack IL-4 or IL-4R. Nonetheless, the importance of type 2 cytokines in damaging tissue remodeling and fibrosis is increasingly well documented (1, 68). It now becomes important to ask the following: what is the role of type 2 cytokines in normal repair, and why do helminth parasites induce an immune response that has as a primary function wound healing? The answer to the second question may lie with the propensity of many parasites to induce potentially lethal tissue damage. Hookworm parasites penetrate the gut wall to feed, whereas schistosome eggs use proteolytic enzymes to enter the gut. Both situations could potentially lead to sepsis, unless the repair of gut wall was rapid and effective, thus providing sufficient evolutionary pressure for the development of a worm-specific tissue repair process (69). Indeed, *S. mansoni* infection of mice that lack AAMΦ is fatal due to endotoxemia (11), potentially explained by the inability of these mice to repair a breach of the intestinal wall. Similarly, many helminthic parasites have a migratory stage through the lung that leads to substantial pulmonary hemorrhaging that is repaired remarkably quickly (70).

We hypothesize that IL-4 and/or IL-13 may mediate a more rapid form of tissue repair that is necessary to maintain tissue

integrity. This may be at the expense of higher quality repair. Consistent with this hypothesis, Eming et al. (71) demonstrated accelerated closure of skin punch biopsy wounds in IL-10-deficient mice, but at the cost of biomechanical strength. Importantly, Ym1-expressing macrophages were more abundant in the rapidly healing mice. The well-described anti-inflammatory properties of AAMΦ (1) are also consistent with a role for these cells in accelerating the rate of tissue repair, because effective healing cannot progress until classical inflammation has ended (32–35). To test these hypotheses, it will be necessary to develop or identify both helminth and nonhelminth models in which the rate of repair is a determining factor in outcome.

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## Disclosures

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