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Macrophage function in tissue repair and remodeling requires IL-4 or IL-13 with apoptotic cells

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Abstract

Tissue repair is a subset of a broad repertoire of interleukin-4 (IL-4)- and IL-13-dependent host responses during helminth infection. Here we show that IL-4 or IL-13 alone was not sufficient, but IL-4 or IL-13 together with apoptotic cells induced the tissue repair program in macrophages. Genetic ablation of sensors of apoptotic cells impaired the proliferation of tissue-resident macrophages and the induction of anti-inflammatory and tissue repair genes in the lungs after helminth infection or in the gut after induction of colitis. By contrast, the recognition of apoptotic cells was dispensable for cytokine-dependent induction of pattern recognition receptor, cell adhesion, or chemotaxis genes in macrophages. Detection of apoptotic cells can therefore spatially

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S15

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compartmentalize or prevent premature or ectopic activity of pleiotropic, soluble cytokines such as IL-4 or IL-13.

For a successful immune response, macrophages act in a sequential and coordinated cascade to remove a pathogen, resolve inflammation, and repair damaged tissue. When damage occurs, tissue-resident macrophages release inflammatory cytokines and chemokines and recruit effector cells such as neutrophils. Subsequently, macrophages at the damaged site up-regulate a gene expression program including *Retnla*, *Chil3*, and *Arg1*, consistent with their anti-inflammatory and tissue repair functions (1,2). The cytokines interleukin-4 (IL-4) and IL-13 induce host defense responses against helminth infections and the anti-inflammatory and tissue repair phenotype in macrophages (3, 4). How these pleiotropic and soluble cytokines coordinate time-resolved functions of macrophages and restrict the anti-inflammatory and tissue repair program to the damaged site is not understood. Clearance of apoptotic cells has also been linked to resolution of inflammation (5). However, apoptotic cells are not only generated during inflammation but also under physiological conditions (5). Therefore, we posited that coordination of IL-4 or IL-13 with apoptotic cell sensing induces the anti-inflammatory and tissue repair program.

Thus, bone marrow-derived macrophages (BMDMs) were either untreated or stimulated with apoptotic neutrophils (aN), IL-4, or IL-4 and aN (aN+IL-4). RNA sequencing (RNA-seq) analysis identified increased expression of 61 genes—including *Retnla*, *Chil3*, *Ear2*, and *Fnl* (fold change of >1.25)—on concomitant detection of aN+IL-4 compared with IL-4 alone and aN alone (Fig. 1A). This gene set was enriched for the gene ontology annotations “response to wounding” and “wound healing.” By contrast, IL-4-only treatment induced multiple genes involved in translation, regulation of cell proliferation, chemotaxis, pattern recognition, and cell adhesion (Fig. 1, A and B). The expression of classical anti-inflammatory and tissue repair genes, such as *Retnla* and *Chil3* (6), as well as genes with potential anti-inflammatory and tissue repair function, such as *Fnl* and *Ear2* (7,8), was higher when BMDMs were stimulated with aN+IL-4 compared with IL-4 alone and aN alone (fig. S1A). RETNLA and CHIL3 amounts were also enhanced when BMDMs were stimulated with aN+IL-4 compared with IL-4 alone and aN alone (fig S1B). aN+IL-4 induction was specific, as the expression of genes, including *Clec7a* (pattern recognition receptor), *Ccl2* and *Ccl7* (chemokines), and *Cdh1* (cell adhesion molecule), was the same whether IL-4 alone or aN+IL-4 were used for stimulation (fig. S1C). Necrotic neutrophils did not synergize with IL-4 to potentiate *Retnla* or *Chil3* expression (fig. S1D).

Because the IL-4 receptor α (IL-4R α) subunit is shared by IL-4R and IL-13R, we evaluated whether a subset of IL-13-induced gene expression in macrophages was also potentiated by aN. IL-13-induced *Chil3* and *Ear2* expression was increased in the presence of aN+IL-13 compared with IL-13 alone and aN alone (fig. S2), although the increase in *Retnla* and *Arg1* expression was not statistically significant. Anti-inflammatory and tissue repair gene expression in macrophages, albeit potentiated by concomitant aN+IL-13 sensing, was less sensitive to IL-13 in comparison with IL-4 (fig. S3). Together these results indicate that although sensing IL-4 or IL-13 leads to the expression of a broad set of genes in

macrophages, the sensing of IL-4 or IL-13 together with the recognition of apoptotic cells enhances the expression of anti-inflammatory and tissue repair genes.

Macrophages recognize apoptotic cells by sensing phosphatidylserine (PtdSer) (5, 9,10). Hence, we treated BMDMs with IL-4 in the presence or absence of annexin V to block PtdSer exposed on the surface of apoptotic cells. We found that IL-4-mediated induction of anti-inflammatory and tissue repair genes was reduced by pretreatment of macrophages with annexin V (Fig. 1, C and D, and fig. S4A). By contrast, annexin V failed to significantly affect *Clec7a* expression (Fig. 1C). Thus, a subset of gene expression ascribed to IL-4 requires IL-4 together with sensing of apoptotic cells mediated by PtdSer. Our experiments also showed that there was a detectable background population of annexin V⁺, annexin V⁺PI⁺, and TUNEL⁺ cells in BMDM cultures (fig. S4, B and C). Perhaps this unrecognized presence of apoptotic cells in cell cultures can explain why stimulation with IL-4 or IL-13 appears sufficient to drive the macrophage anti-inflammatory and tissue repair response.

The PtdSer-dependent receptor tyrosine kinases (RTKs) AXL and MERTK, found in both BMDMs (fig. S5A) (11) and tissue-resident macrophages (11,12), are receptors that function in the phagocytosis of apoptotic cells (13). We stimulated BMDMs from wild-type (WT) and *Axl*^{-/-}*Mertk*^{-/-} mice with IL-4 for 24 and 48 hours. BMDMs from both genotypes expressed similar levels of IL-4Rα in basal conditions, and this receptor subunit was down-regulated upon IL-4 stimulation, indicating that IL-4 sensing was preserved in the absence of AXL and MERTK (fig. S5B) (14). Nevertheless, *Axl*^{-/-}*Mertk*^{-/-} BMDMs expressed lower amounts of *Retnla*, *Chil3*, and *Arg1* mRNA when stimulated with IL-4, compared with WT BMDMs (fig. S6). By contrast, IL-4-mediated induction of *Clec7a*, *Ccl2*, and *Ccl7* was not diminished in the absence of AXL and MERTK (fig. S6). Incubation with annexin V further reduced *Retnla* mRNA and protein in *Axl*^{-/-}*Mertk*^{-/-} BMDMs, indicating that other PtdSer-dependent phagocytic receptors also participate in this process (Fig. 2, A and B). As expected, *Clec7a* expression was unaffected by annexin V (Fig. 2A). AXL and MERTK do not directly bind PtdSer. GAS6 and PROS1 bridge PtdSer to AXL and MERTK (13). We used recombinant AXL and MERTK extracellular domain-Fc fusion proteins to sequester GAS6 and PROS1 while incubating WT BMDMs with IL-4. This significantly reduced IL-4 induction of *Retnla* and *Arg1* when compared with results for IgG₁-Fc control-treated cells (Fig. 2C). IL-4-induced expression of *Retnla* and *Arg1* was also significantly reduced in BMDMs deficient for GAS6 (*Gas6*^{-/-}) (Fig. 2D), similar to *Axl*^{-/-}*Mertk*^{-/-} BMDMs.

Infection with the helminth *Nippostrongylus brasiliensis* causes substantial lung tissue damage in mice and induces a rapid IL-4 and IL-13 response, critical for resolution of inflammation and repair of the affected tissue (15–17). Lung-resident macrophages express AXL and MERTK (fig. S7A). When we examined the bronchoalveolar lavage (BAL) of *N. brasiliensis*-infected mice, we found significant hemorrhage 2 days postinfection (dpi) in both WT and *Axl*^{-/-}*Mertk*^{-/-} mice (Fig. 3A). Although red blood cell counts in the BAL were significantly reduced by day 4 and returned to uninfected baseline levels by day 7 postinfection in WT mice, *Axl*^{-/-}*Mertk*^{-/-} mice continued to bleed even at day 7 postinfection, indicating defective wound repair (Fig. 3A). Histological analyses also revealed enhanced peribronchial and perivascular inflammatory responses in *Axl*^{-/-}*Mertk*^{-/-} lungs by comparison with WT lungs, following *N. brasiliensis* infection (Fig. 3B).

N. brasiliensis infection also resulted in increased accumulation of apoptotic neutrophils (fig. S7, B to D) and reduced amounts of *Arg1* (Fig. 3C) in the lungs of *Axl*^{-/-} *Mertk*^{-/-} mice compared with those of WT mice. Similar to previous reports (18), we detected a significant increase in the percentage of Ki67⁺ proliferating alveolar macrophages in infected WT mice by comparison with their noninfected counterparts (Fig. 3D). Genetic ablation of *Axl* and *Mertk* significantly reduced this IL-4-dependent proliferation of alveolar macrophages (Fig. 3D). RNA-seq of sorted CD45⁺CD11c⁺SiglecF⁺Ly6G⁻CD11b^{lo} tissue-resident macrophages at 7 dpi revealed substantial reduction in the expression of anti-inflammatory and tissue repair genes in *Axl*^{-/-} *Mertk*^{-/-} mice compared with WT animals (Fig. 3E). Quantitative polymerase chain reaction (qPCR) analysis confirmed this result (fig. S8). RETNLA induction was significantly lower in *Axl*^{-/-} *Mertk*^{-/-} alveolar macrophages by comparison with WT macrophages after *N. brasiliensis* infection (fig. S9).

To examine the macrophage-specific function of *Axl* and *Mertk*, we crossed *Axl*^{f/f} *Mertk*^{f/f} mice with mice harboring Cre recombinase expressed in mononuclear phagocytes (*Csf1r*-Cre⁺). We confirmed that *Axl* and *Mertk* mRNA were undetectable in macrophages isolated from the peritoneum and lungs or differentiated from bone marrow progenitors from *Csf1r*-Cre⁺ *Axl*^{f/f} *Mertk*^{f/f} mice (fig. S10, A and B). Lower levels of anti-inflammatory and tissue repair genes were detected in lung-resident macrophages isolated from *N. brasiliensis*-infected *Csf1r*-Cre⁺ *Axl*^{f/f} *Mertk*^{f/f} mice by comparison with infected control mice (fig. S11A). By contrast, the expression of IL-4-induced genes that were independent of apoptotic cell sensing was not impaired in the absence of *Axl* and *Mertk* (fig. S11B).

IL-4 induces T helper 2 cell (T_H2) responses (19). The production of IL-4, IL-13, and IL-5 by T cells isolated from mediastinal lymph nodes was comparable between *N. brasiliensis*-infected WT and *Axl*^{-/-} *Mertk*^{-/-} mice (Fig. 3F). Thus, although the sensing of apoptotic cells is essential for IL-4- and IL-13-dependent induction of an anti-inflammatory and tissue repair program in macrophages, it is not required for adaptive type 2 immune responses.

Apoptotic cells, IL-4, and IL-13 are not restricted to the lungs during *N. brasiliensis* infection in mice (fig. S12) (20). Adipose tissue macrophages can also up-regulate anti-inflammatory and tissue remodeling genes (20). We observed a significant reduction in the expression of RETNLA, ARG1, and the mannose receptor CD206 in adipose macrophages from *N. brasiliensis*-infected *Csf1r*-Cre⁺ *Axl*^{f/f} *Mertk*^{f/f} mice, unlike in macrophages from infected *Csf1r*-Cre⁻ *Axl*^{f/f} *Mertk*^{f/f} mice (Fig. 4A). Protein amounts were unchanged regardless of the presence or absence of AXL and MERTK in macrophages from uninfected mice (fig. S13).

During the resolution phase of dextran sodium sulfate (DSS)-induced colitis, CD11b⁺F4/80^{hi} macrophages in the lamina propria acquire an anti-inflammatory and tissue repair signature (21). Expression of AXL and MERTK in these macrophages is required for colitis to resolve (21). Colitis is typically accompanied by the presence of aN in the intestinal lamina propria (21). We injected IL4, complexed to anti-IL4 antibody (IL-4c) (18), intraperitoneally at days 3 and 5 after the induction of colitis with DSS. A significant increase in the expression of CD206 on lamina propria macrophages was observed compared with DSS-treated WT mice

injected with phosphate-buffered saline (PBS). By contrast, *Axl*^{-/-}*Mertk*^{-/-} macrophages Med to up-regulate CD206 (Fig. 4B).

Next, we treated mice with an intraperitoneal injection of thioglycollate to induce inflammation. Of note, apoptotic neutrophils were detected in the peritoneum of both WT and *Axl*^{-/-}*Mertk*^{-/-} mice (fig. S14, A and B). Two days after thioglycollate injection, we administered IL-4c, which induced RETNLA and CD206 in peritoneal CD11b⁺F4/80⁺ macrophages in WT mice. By contrast, *Axl*^{-/-}*Mertk*^{-/-} macrophages induced less RETNLA and CD206 (Fig. 4C). Following thioglycollate injection, the administration of aN+IL-4c by comparison with IL-4 alone potentiated the expression of RETNLA and CD206 in WT mice (fig. S15), similar to our in vitro observations with BMDMs. In conclusion, our results indicate that AXL- and/or MERTK-dependent sensing of apoptotic cells, combined with IL-4 signaling, launches the anti-inflammatory and tissue repair response in macrophages in a wide range of contexts.

The pleiotropic effects of cytokines such as IL4 and IL-13 on macrophages are well known (3). Context-specific codetectors will therefore be important to localize such responses to sites of infection and wounding. However, the molecular signaling mechanism that integrates IL-4, IL-13, and apoptotic cell sensing remains unknown. IL-4 and IL-13 also coordinate discrete responses in cells beyond macrophages, such as T_H2 polarization in T cells and immunoglobulin class switch in B cells (19, 22). It is tempting to speculate that integration of cell type-specific components with cytokine receptor signals imparts specificity for cell-selective functions. In principle, signaling through coincidence circuits can structure cytokine responses into sequential and coordinated outputs during a multicellular, systemic response, such as host defense and resolution of inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Codetection of IL-4 and apoptotic cells is required for the induction of an anti-inflammatory and tissue repair program in BMDMs

(A) Scatterplot of relative expression of genes induced in bone marrow-derived macrophages (BMDMs) upon exposure to apoptotic neutrophils (aN) or IL-4 or both, as determined by RNA-seq. Genes induced by aN, IL-4, or aN+IL-4 are shown in gray. Genes potentiated (>1.25-fold) by aN+IL-4 compared with both aN alone and IL-4 alone are shown in red. Representative genes induced by IL-4 but not potentiated by aN are shown in blue, $n = 2$ biological replicates. (B) Gene ontology assignments of genes potentiated in BMDMs exposed to aN+IL-4 or genes whose expression was induced in BMDMs treated with IL-4. Dashed lines represent $P = 0.05$. (C) mRNA expression normalized to *Gapdh* in BMDMs untreated or treated with indicated doses of annexin V (Ann-V) before stimulation with IL-4, as determined by qPCR. (D) Representative flow cytograms and independent data depicting frequency of RETNLA⁺ BMDMs untreated (Unt.) or treated with annexin V (1 μ g/ml) before stimulation with IL-4 (Ann-V+IL-4). Each data point is from an independent sample. Mean $\pm$ SEM (error bars). * $P < 0.05$; ** $P < 0.01$: Mann-Whitney U rank sum test.

Fig. 2. IL-4 induction of anti-inflammatory and tissue repair genes in vitro depends on the activation of AXL and MERTK RTKs in BMDMs

(A) Expression of *Retnla* and *Clec7a* mRNA determined by qPCR and (B) representative flow cytograms and independent data of the frequency of RETNLA⁺ BMDMs from WT and *Axl^{-/-} Mertk^{-/-}* mice untreated (Unt.) or treated with annexin V at indicated doses (A) or 1 µg/ml (B) before stimulation with IL-4. (C) Expression of *Retnla* and *Arg1* mRNA determined by qPCR in WT BMDMs incubated with IL-4 in the presence of AXL and MERTK extracellular domain-Fc chimeric proteins or IgGrFc control (IgG, immunoglobulin G). (D) Expression of *Retnla* and *Arg1* mRNA determined by qPCR in BMDMs from WT and *Gas6^{-/-}* mice upon stimulation with IL-4. *Gapdh* expression was used for normalization. Each data point is from an independent sample. Mean ± SEM (error bars). **P* < 0.05; Mann-Whitney *U* rank sum test.

Fig. 3. IL-4 and/or IL-13 induction of anti-inflammatory and tissue repair responses in macrophages upon infection with *N. brasiliensis* is dependent on AXL and MERTK RTKs
 WT and *Axl*^{-/-} *Mertk*^{-/-} mice were infected with *N. brasiliensis*. (A) Representative images and independent data showing red blood cell (RBC) counts at the indicated number of days postinfection (dpi) in the bronchoalveolar lavage. (B) Representative images and histopathological score of lung sections stained with hematoxylin and eosin. Scale bars, 5 μ m. (C) *Arg1* expression in the lungs, as detected by qPCR. (D) Flow cytometric analysis showing the frequency of Ki67⁺ lung macrophages (M ϕ) (CD45⁺CD11c^{hi}SiglecF⁺CD11b^{lo}) from WT and *Axl*^{-/-} *Mertk*^{-/-} mice, untreated (Unt.) or 9 dpi. *N.b.*, *N. brasiliensis*. (E) Heat map representing the most differentially expressed genes [$\log_2$ of expression levels, fragments per kilobase of transcript per million mapped reads (FPKM)] in lung macrophages from WT and *Axl*^{-/-} *Mertk*^{-/-} mice at 7 dpi, as determined by RNA-seq. (F) IL-4, IL-13, and IL-5 production by T cells from WT and *Axl*^{-/-} *Mertk*^{-/-} mice at 7 dpi. unstm., unstimulated. Each data point is from an independent sample. For (A) and (B), one experiment, which is representative of three independent experiments, is reported. Mean $\pm$ SEM (error bars). n.s., nonsignificant; * P < 0.05; Mann-Whitney U rank sum test.

Fig. 4. IL-4 and AXL or MERTK RTK codetection functions across multiple tissues to induce anti-inflammatory and tissue repair-associated genes in macrophages

(A) Macrophages ($CD45^{+}CD11b^{+}F4/80^{+}$) isolated from the visceral adipose tissue of $Csf1r-Cre^{-/-} Axl^{fl/fl} Mertk^{fl/fl}$ and $Csf1r-Cre^{+/+} Axl^{fl/fl} Mertk^{fl/fl}$ animals at 7 dpi with *N. brasiliensis* were analyzed for expression of RETNLA, ARG1, and CD206 by flow cytometry. Histograms and independent data, representative of two independent experiments, are reported. MFI, mean fluorescence intensity. (B) Expression of CD206 on lamina propria macrophages ($CD45^{+}CD11b^{+}F4/80^{hl}$) isolated from the large intestine of WT and $Axl^{-/-} Mertk^{-/-}$ mice treated for 7 days with 1.5% DSS and injected on days 3 and 5 with IL-4c or PBS intraperitoneally. Histograms and independent data are reported. (C) Frequency of RETNLA⁺ and CD206⁺ $CD45^{+}CD11b^{+}F4/80^{+}$ peritoneal macrophages from WT and $Axl^{-/-} Mertk^{-/-}$ mice injected intraperitoneally with 4% thioglycollate on day 0, followed by intraperitoneal injection of IL-4c or PBS on day 2 and isolation on day 4. Histograms and independent data, representative of two independent experiments, are shown. Mean $\pm$ SEM (error bars). n.s., nonsignificant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Mann-Whitney *U* rank sum test.