Neutrophils are the most abundant immune cells found in actively inflamed joints of patients with rheumatoid arthritis (RA), and most animal models for RA depend on neutrophils for the induction of joint inflammation. Exogenous IL-4 and IL-13 protect mice from antibody-mediated joint inflammation, although the mechanism is not understood. Neutrophils display a very strong basal expression of STAT6, which is responsible for signaling following exposure to IL-4 and IL-13. Still, the role of IL-4 and IL-13 in neutrophil biology has not been well studied. This can be explained by the low neutrophil surface expression of the IL-4 receptor α-chain (IL-4Rα), essential for IL-4- and IL-13-induced STAT6 signaling. Here we identify that colony stimulating factor 3 (CSF3), released during acute inflammation, mediates potent STAT3-dependent neutrophil IL-4Rα up-regulation during sterile inflammatory conditions. We further demonstrate that IL-4 limits neutrophil migration to inflamed joints, and that CSF3 combined with IL-4 or IL-13 results in a prominent neutrophil up-regulation of the inhibitory Fcγ receptor (FcγR2b). Taking these data together, we demonstrate that the IL-4 and CSF3 pathways are linked and play important roles in regulating proinflammatory neutrophil behavior.

Rheumatoid arthritis (RA) is a severe chronic autoimmune disease affecting 0.5 to 1% of the global population, thus ∼50 million people worldwide. RA is characterized by progressive inflammation of small joints in the hands and feet, but also often spreads to larger joints (1). Systemic effects are furthermore commonly seen, including symptoms affecting the lung, blood vessels, and heart (2, 3). A major proportion of RA patients are characterized by having disease-specific autoantibodies, and the presence of such autoantibodies is linked to an expected worsening of disease progression (4). Of note, these autoantibodies are associated with exposure to defined environmental factors, such as smoking, and genetic risk factors related to the immune system, such as specific HLA-DR alleles (5).

Studies with several animal models for RA have identified a central role for neutrophils in the induction and propagation of experimental autoimmune joint inflammation (6–10). The role of neutrophils in RA patients is harder to define, although neutrophils are typically making up the largest proportion of immune cells found in inflamed joints of patients with active disease (also seen in animal models for RA) (11–13). One hypothesis states that neutrophils are responsible for an inflammatory amplification loop. Here, an initial trigger results in neutrophil migration into the joint, local neutrophil activation, and release of proinflammatory effector molecules in the joint, in turn resulting in more inflammation and more migration of neutrophils into the joint, in a self-propagating inflammatory loop (10, 14). Of importance, activated neutrophils are known to release several modified proteins that are targets for autoantibodies found in RA patients, including citrullinated histones and vimentin (15, 16). In these patients, the release of such modified proteins locally in the joints is thought to result in the formation of antibody–antigen immune complexes (ICs), further propagating inflammation by interaction with activating Fcγ receptors (FcγRs) and by activating the complement system (17–19).

We and others have shown a protective role for IL-4 receptor (IL-4R) signaling in animal models for RA, induced by both IL-4 and IL-13 (20–26). However, it is not known how the IL-4R pathway is regulated during autoimmune inflammation. Combining gene-expression data and a targeted CRISPR screen, we here identify colony stimulating factor 3 (CSF3) as a potent positive regulator of neutrophil IL-4Rα expression. We further demonstrate that the neutrophil IL-4Rα up-regulation, seen during experimental joint inflammation (21), can be attributed to CSF3-induced STAT3 signaling. Importantly, we find that IL-4 and CSF3 synergize to up-regulate the inhibitory FcγR2b on neutrophils | IL-4 | inflammation | arthritis | FcγR2b

Significance

Joint inflammation is the defining characteristic of the autoimmune disease rheumatoid arthritis. Here we use experimental systems to show that the Th2 cytokine IL-4 suppresses the neutrophil potential of causing acute joint inflammation, including suppressing their migration to the inflamed joint, and by upregulating their expression of the inhibitory IgG Fc receptor. We also identify that the pathway is regulated by colony stimulating factor 3. Identifying strategies for early intervention in patients before they develop chronic, debilitating disease is a key focus of the current research in autoimmune diseases. Our study identifies mechanisms that could be exploited therapeutically in patient groups where IgG autoantibodies and neutrophils are thought to play an important role in the pathology.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE134047 for Affymetrix expression data). Plasmids LentiGuide-Puro-P2A-EGFP and LentiGuide-Puro-P2A-EGFP, mRFPSuF have been deposited to AddGene (accession nos. 137729 and 137730, respectively).

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neutrophils and that the interaction also affects neutrophil migration into inflamed joints. This is of importance since CSF3 is essential for joint inflammation in these models (27, 28). Taken together, our data identify that neutrophil IL-4Rα signaling suppresses several of the proinflammatory effects of CSF3 in the context of experimental joint inflammation, similar to what has been shown in models of infection (29). Neutrophil IL-4Rα thus seemingly behaves functionally as a paired inhibitory receptor to the CSF3 receptor, a context commonly seen related to potent activating receptors expressed on immune cells, allowing for a fine-tuned activation and down-modulation of inflammation (30).

Results

IL-4 Protects Mice from Antibody-Mediated Joint Inflammation, and Neutrophil IL-4Rα Expression Is Potently Up-Regulated by a Serum Protein Related to Acute Inflammation. A single injection of IL-4, formulated in a complex form, protects both C57BL/6 and BALB/c mice from joint inflammation in the K/BxN serum transfer model (Fig. 1 A and B) (20, 21). The acute joint inflammation triggered in this model is characterized by the infiltration of a large number of neutrophils into the joint, and the joint inflammation is related to FcγR-mediated activation (8, 10, 31). We have previously shown that these infiltrating neutrophils strongly up-regulate the IL-4Rα and that this is a general phenomenon for neutrophils triggered by a diverse range of acute inflammatory stimuli, including following the injection of LPS (21). Ex vivo stimulation of neutrophils with serum collected from mice injected with LPS ∼20 h earlier ("LPS serum") results in a strong up-regulation of the IL-4Rα chain at concentration as low as 0.02% (Fig. 1 C and D) (21). Neutrophils incubated with serum from K/BxN mice similarly up-regulate the IL-4Rα, although several magnitudes lower compared to the LPS serum (Fig. 1 E).

Due to the strong activity of the LPS serum, we decided to initially use the LPS serum to study the characteristics of the IL-4Rα-regulating components. We ran LPS serum over several different protein-purification columns in a liquid chromatography system and found that the active component reproducibly segregates into defined fractions, behaving as expected from a protein or a stable protein complex. The active component showed, for example, strong binding to hydrophobic interaction columns (Butyl HIC), but no binding to Hepab columns (SI Appendix, Fig. S1). Taken together, these results suggest that a hydrophobic serum protein, or stable protein complex, released during acute sterile inflammation affects neutrophil IL-4Rα expression and that this protein could play a role controlling excessive inflammation.

Identification of CSF3 as a Potent IL-4Rα Regulator in Neutrophils. For more clues to the nature of the IL-4Rα regulating protein, we screened through supernatants of a panel of cell lines for their ability to up-regulate the IL-4Rα on neutrophils. The cell lines were stimulated overnight with or without LPS, and the cell-free conditioned supernatant was then transferred to ex vivo cultures with bone marrow cells, which contain a high proportion of neutrophils (Fig. 2 A). Here, we usually used bone marrow cells from MyD88 knockout (KO) mice to eliminate the risk of residual LPS in the transferred conditioned supernatant interfering with the neutrophils through the Toll-like receptor 4 (TLR4)/MyD88 signaling pathway. Two cell lines were identified, from which the conditioned medium was able to potently up-regulate the IL-4Rα on neutrophils: 1) MH-S (32), a mouse alveolar macrophage cell line, and 2) HoxB8 Macroph (33), a mouse bone marrow-derived CSF2-dependent progenitor line that can be differentiated to macrophages (Fig. 2 B and D and SI Appendix, Fig. S2 A). This is consistent with our previous observation showing that cells in the

![Fig. 1. IL-4 protects mice from antibody-mediated joint inflammation, and an inflammation-related serum factor regulates neutrophil IL-4Rα expression. (A) Joint inflammation in C57BL/6 and BALB/c mice injected intravenously with K/BxN serum (200 μL C57BL/6, 100 μL BALB/c) and IL-4 or control. (B) Functional test assessing the ability to hang on to a mesh at day 4. (C) Outline of experimental setup testing how serum from K/BxN mice or LPS injected mice affects neutrophil IL-4Rα expression. (D and E) Flow cytometry analysis of neutrophil IL-4Rα expression; cells isolated from bone marrow incubated overnight with different concentrations of serum collected from (D) LPS injected mice, and (E) K/BxN mice with active disease, as well as serum from naive control C57BL/6 mice. Data presented as mean and SEM in A (n = 4 to 5) and D and E (n = 3), and as mean and individual mice in B (n = 4 to 5). *P < 0.05, **P < 0.01, and ***P < 0.005 by Mann–Whitney U test (day 7 in A, and day 4 in B). Data are representative of at least two independent experiments. Differences in the basal IL-4Rα levels in D and E reflect the use of different flow cytometry machines with different sensitivities and settings.](https://www.pnas.org/cgi/doi/10.1073/pnas.1914186117)
lung (and epididymal fat pads), isolated from mice injected with LPS, could readily activate the IL-4Rα regulating protein (21). We could further confirm that the IL-4Rα regulatory activity of the conditioned medium was mediated by a protein, as the activity was abrogated by treatment with proteinase K (Fig. 2C and SI Appendix, Fig. S2B). We concluded that following activation of certain types of macrophages, a neutrophil IL-4Rα regulating protein is secreted.

We then generated single-cell colonies of the MH-S cell line and identified a set of clones that secreted higher amounts of the IL-4Rα regulating proteins ("high-activity clones") and a set of clones that secreted lower amounts ("low-activity clones"). Comparing these two groups revealed that the high-activity clones (n = 3) produced at least four times more of the active IL-4Rα regulating proteins compared to the low clones (n = 3) based on the ability to up-regulate neutrophil IL-4Rα at different dilutions (SI Appendix, Fig. S2C). Next, we performed a global gene-expression analysis comparing high- and low-activity clones stimulated with or without LPS. We had seen that the IL-4Rα regulating proteins accumulates in the supernatant of LPS-stimulated cell lines over several days. We, therefore, collected samples at a later time point (16 h) in order to limit picking up the burst of early response genes. At the assayed time point, 49 genes annotated as "extracellular" were significantly up-regulated more than twofold compared to unstimulated cells (SI Appendix, Fig. S2D). Of these, only seven genes (Csf3, Ili2b, Hn, Tnfsf15, Otm2, Lcn2, and Mmp9) showed higher expression in the high-activity clones compared to the low-activity clones. Based on these assumptions, we performed a small custom CRISPR screen targeting the differentially expressed genes and identified that Csf3, also known as G-CSF, was a plausible candidate for the IL-4Rα regulating protein (Fig. 2E). We subsequently generated individual Csf3 KO clones (n = 4) of the MH-S cell line, using a guide RNA (gRNA) sequence that differed from the ones used in the screen, and validated that the cells lacked a functional Csf3 gene (SI Appendix, Fig. S3A-C).

Stimulating the four different Csf3 KO clones with LPS and testing their supernatant for the ability to up-regulate IL-4Rα on neutrophils confirmed that the IL-4Rα regulating protein secreted by MH-S cells was Csf3 (Fig. 2F). Using recombinant proteins, we could further confirm that Csf3 has a potent ability to induce up-regulation of IL-4Rα on neutrophils as has been recently reported (29, 34), while no activity of the STAT3-activating cytokines IL-6 or LIF was seen (Fig. 2G). Notably, potent neutrophil IL-4Rα up-regulation can be observed at Csf3 levels as low as 1 ng/mL, indicating that this regulation is very sensitive (SI Appendix, Fig. S3D). In contrast, macrophages do not alter their IL-4Rα expression when incubated with Csf3, while IL-6 has a strong IL-4Rα up-regulating effect on macrophages (SI Appendix, Fig. S3 E and F) (35), hence highlighting differential regulatory properties of the IL-4Rα in myeloid cells. Finally, expanding and culturing bone marrow precursor cells, as described by Wang et al. (33), and then differentiating them in the presence of Csf3 further showed that the ability of Csf3 to up-regulate IL-4Rα is mainly confined to the final stage of differentiation when the cells express strong surface...
levels of CXCR2 and the neutrophil-specific protein Ly6G (SI Appendix, Fig. S4A). We concluded that the cytokine CSF3 is released upon stimulation of certain macrophage cell lines and that CSF3 can potently up-regulate the IL-4Rα on neutrophils.

**CSF3 Is an Inflammation-Related Neutrophil IL-4Rα-Regulating Factor in Vivo and Has a Conserved Activity on Human Neutrophils.** To study how CSF3 levels are regulated in vivo, we measured CSF3 protein levels in sera from LPS-injected mice. We found that the cytokine level was significantly up-regulated 24 h after LPS injection (SI Appendix, Fig. S5A) and that its mRNA transcripts were kept elevated ∼10-fold in lung tissue at that same time point (SI Appendix, Fig. S5B). Nevertheless, this up-regulation was not lung-specific, as we could also observe CSF3 mRNA up-regulation in the spleen and liver (SI Appendix, Fig. S5B). To directly examine whether CSF3 in the LPS serum was responsible for the observed neutrophil IL-4Rα up-regulation, we incubated serum from LPS-injected mice with a neutralizing anti-CSF3 antibody before coculture with neutrophils. We found that the sera treated with neutralizing anti-CSF3 antibody lost its ability to up-regulate neutrophil IL-4Rα (Fig. 3A). In line with this, neutrophils from CSF3 receptor (CSF3R) KO mice did not up-regulate the IL-4Rα upon incubation with LPS serum (Fig. 3A). Furthermore, we found that the in vivo up-regulation of neutrophil IL-4Rα following injection of LPS could be abrogated by coinjecting a neutralizing anti-CSF3 antibody (Fig. 3B). Since CSF3 plays a central role in inducing inflammation in the K/BxN serum transfer model (27, 28), the administration of a neutralizing anti-CSF3 antibody in this model in order to test whether CSF3 is responsible for the observed IL-4Rα up-regulation on neutrophils is not a conclusive experiment. In this scenario, with low CSF3 levels, limited joint inflammation can be observed, and consequently, no activation of neutrophils can occur. Instead, we tested how the anti-CSF3 antibody affected neutrophil IL-4Rα up-regulation during ex vivo culture with serum from K/BxN mice and found that the up-regulation was entirely abolished by the anti-CSF3 antibody (Fig. 3C).

Performing another targeted CRISPR screen in the HoxB8 Macpro macrophage cells, targeting 22 genes linked to TLR signaling, we identified that clones lacking Myd88, Ituk-4, Akt3, Rela, and Nfkβ1/p50 did secrete low levels of CSF3 following stimulation with LPS (SI Appendix, Fig. S6 A–C). Although we did not further validate this, this observation indicates that the canonical NF-kB pathway (35) is likely responsible for the CSF3 secretion in the used experimental setup. Consistent with this, neutrophils in MyD88 KO mice did not up-regulate the IL-4Rα following LPS injection but still retain the ability to up-regulate the receptor upon CSF3 exposure (SI Appendix, Fig. S6D).

Similar to mice, human neutrophils show a low basal expression of the IL-4Rα, while B cells show high expression (Fig. 3D). Human blood cells incubated with human CSF3, but not IL-6, resulted in clear neutrophil IL-4Rα up-regulation, although to a seemingly lower extent than observed in mice (Fig. 3D). Similarly to in mice, human CSF3 and IL-6 also do not affect IL-4Rα on human B cells (Fig. 3E).

STAT3 is the canonical signaling pathway downstream of CSF3R (37), and IL-6–induced STAT3 signaling has been linked to IL-4Rα up-regulation in macrophages (35). To test whether the CSF3-mediated up-regulation of neutrophil IL-4Rα can be attributed to STAT3 signaling, we used the STAT3 inhibitor Static (38) and found that Static inhibits the CSF3-mediated up-regulation of IL-4Rα on neutrophils in a dose-dependent manner (Fig. 3F). We also found that neutrophil survival is significantly decreased by Static (Fig. 3F). This emphasizes the
sensitivity of neutrophils, traditionally seen as very short-lived, and the central role for CSF3 and STAT3 in neutrophil biology (37, 39). Taken together, our data identify that CSF3 is the factor directly affecting the expression of IL-4Rα on neutrophils during acute inflammation and that this regulatory activity is conserved between mice and humans, as it been recently described (34). In this context, it is also notable that the ImmGen expression database (40) identifies that both mouse and human neutrophils have the highest expression of the IL-4R signaling adaptor Stat6/STAT6 seen in any immune cell (SI Appendix, Fig. S7). This suggests that the neutrophils are hard-wired to respond to signals from the IL-4R and, thus, that CSF3-mediated IL-4Rα up-regulation can have profound effects on the cell behavior.

**CSF3 and IL-4 Synergize to Up-Regulate the Inhibitory FcγR2b on Neutrophils.** Inflammation triggered by autoantibodies depends on several processes, which are initiated by the formation of antibody-antigen ICs (41). In the K/BxN serum transfer model, IgG IC-mediated cross-linking of activating FcγRs on neutrophils is essential for the development of joint inflammation (8, 10, 42), which in turn can be suppressed by the inhibitory FcγR2b (43, 44). Mouse neutrophils have a clear basal expression of FcγR2b (CD64) (45, 46), which in turn can be suppressed by the inhibitory FcγR2b (CD16-2) (SI Appendix, Fig. S8 A and B), and no evident expression of the activating FcγR1 (CD64) (SI Appendix, Fig. S8 C) or the inhibitory FcγR2b (CD32B) (Fig. 4 A–C). Taking into consideration that IL-4 is known to affect the expression of FcγR2b in certain myeloid cells (45, 46) and that CSF3 induces the up-regulation of IL-4Rα on neutrophils, we hypothesized that CSF3 and IL-4 could act synergistically to induce the expression of FcγR2b on neutrophils. Neutrophils isolated from naïve mice stimulated ex vivo with IL-4 overnight did not show any apparent up-regulation of FcγR2b. Intriguingly, neutrophils isolated from mice with inflammation, induced by LPS injection ~20 h earlier, showed a strong up-regulation of FcγR2b following IL-4 stimulation (Fig. 4A). Based on our findings where CSF3 was identified as responsible for the neutrophil IL-4Rα up-regulation, we repeated these experiments in the presence of a neutralizing anti-CSF3 antibody. Under these conditions, anti-CSF3 could substantially block the IL-4-induced FcγR2b up-regulation in LPS injected mice, showing that the CSF3-mediated IL-4Rα up-regulation is central to the enhanced FcγR2b expression induced by IL-4 following LPS injection (Fig. 4B). A similar result was seen when first injecting mice with CSF3 and the next day injecting IL-4. In these conditions, a major synergistic activity on FcγR2b expression can be observed by CSF3 and IL-4, while only a minor effect was detected on other FcγRs (SI Appendix, Fig. S8 A–C).

Importantly, exposure of neutrophils to CSF3 and IL-4 results in surface levels of FcγR2b that are higher than those observed on B cells, which are considered to be among the highest FcγR2b-expressing cells (Fig. 4C). Moreover, B cells display an opposite regulation of FcγR2b expression and instead down-regulate the receptor after IL-4 exposure (Fig. 4C and SI Appendix, Fig. S8D). The up-regulation of the FcγR2b protein induced by IL-4 could also be observed by qRT-PCR analysis in CSF3-stimulated neutrophils (SI Appendix, Fig. S4B), showing that the regulation is at least partly mediated at a transcriptional level.

Another known target locus induced by IL-4 is MHC-II (47). Expression of MHC-II by neutrophils and subsequent antigen presentation to CD4+ T cells has been described in various contexts, including in the inflamed joints of RA patients (48, 49). Hence, we assessed whether the CSF3/IL-4 combination could
also affect neutrophil MHC-II expression. Expectedly, IL-4 induced the up-regulation of MHC-II on B cells; however, no change in expression of the protein was observed on neutrophils (Fig. 4D). This observation shows that the up-regulation of MHC-II on neutrophils is insufficiently triggered by IL-4, even in a context where the neutrophils have up-regulated IL-4Rα by CSF3 exposure, possibly due to an inappropriate epigenetic state of the MHC-II locus. Overall, our data establish that CSF3 and IL-4 have a synergistic effect on the expression of the inhibitory FcyR2b on neutrophils, which is important for suppressing IC-mediated inflammation. Moreover, it shows that neutrophils can express levels of FcyR2b that surpass those seen on FcyR2b high-expressing B cells, although neutrophils have traditionally been described as not expressing FcyR2b.

IL-4 Drives Neutrophil FcyR2b Expression Directly Downstream of the IL-4Rα and Dictates Neutrophil Migration to the Inflamed Joint. To further explore the mechanisms involved in the FcyR2b regulation, we generated mixed bone marrow chimeric mice, where irradiated CD45.1 WT mice were grafted with CD45.1+ WT and CD45.2+ IL-4Rα KO bone marrow. The resulting mice thus contain immune cells with a WT expression of IL-4Rα as well as immune cells with absent IL-4Rα expression. Importantly, each of these cells can be easily distinguished by flow cytometry using CD45.1 and CD45.2 antibodies (SI Appendix, Fig. S9A). Injecting the chimeric mice with K/BxN serum and IL-4 or control showed that the binding of IL-4 to the IL-4Rα is directly responsible for the neutrophil FcyR2b up-regulation, as only IL-4Rα WT cells but not IL-4Rα KO cells up-regulated FcyR2b (Fig. 5A). Similar results were obtained in ex vivo cocultures of CD45.1 WT and CD45.2 IL-4Rα KO bone marrow cells, originated from mice previously injected with CSF3 or control the day before and then exposed to IL-4 or IL-13 in culture (Fig. 5B and SI Appendix, Fig. S9B). The bone marrow chimeric mice also allowed us to study how IL-4 affects neutrophil migration into different compartments by interacting with the IL-4Rα. Injection of K/BxN serum and IL-4 showed that neutrophil egress from the bone marrow and migration into the joint was significantly suppressed by the IL-4 injection. Migration was calculated as the frequency of WT neutrophils decreasing in competition with the IL-4Rα KO neutrophils in the K/BxN + IL-4 compared to the K/BxN + control group (Fig. 5C). Similarly, IL-4 could partly inhibit neutrophil migration toward CXCL2 and CSa chemotactants in an in vitro transwell assay (SI Appendix, Fig. S4C). These data extend recent findings showing that IL-4 signaling in neutrophils inhibits their migration during inflammation and infection (29, 34), and at the same time illustrates this as a relevant mechanism also in the context of joint inflammation. We concluded that both IL-4 and IL-13 potently up-regulate the inhibitory FcyR2b in CSF3 primed neutrophils directly downstream of signaling through the IL-4Rα, and that IL-4 suppresses neutrophil egress from the bone marrow and migration into the joint in the context of K/BxN mediated joint inflammation.

CSF3 is known to be elevated in contexts of acute inflammation, including infection, sepsis, trauma, as well as in RA patients (50) and animal models for RA (28). To test whether

![Image: Fig. 5. IL-4 affects neutrophil FcyR2b expression directly downstream of the IL-4Rα and affects neutrophil migration to the inflamed joint. (A) Neutrophil FcyR2b expression in mixed bone marrow chimeric mice, CD45.1 WT, and CD45.2 IL-4Rα KO cells, injected with 200 μL K/BxN serum and IL-4 or control day 0, IL-4 or control day 2, and finally analyzed by flow cytometry day 3. Lines connect WT and IL-4Rα KO neutrophils in the same mouse. (B) Neutrophil FcyR2b expression from mixed ex vivo cultures of WT (CD45.1) and IL-4Rα KO (CD45.2) bone marrow from mice injected intravenously with 2 μg CSF3 or control the day before and incubated overnight with IL-4 or IL-13 (20 ng/mL). Lines connect WT and IL-4Rα KO neutrophils in the same sample. (C) The proportion of WT (CD45.1) and IL-4Rα KO (CD45.2) neutrophils in different compartments day 3 in mixed bone marrow chimeric mice injected day 0 with K/BxN and IL-4 or saline, and IL-4 or saline day 2. Data shows percent CD45.1 (WT) neutrophils based on flow cytometry analysis of CD45.1+ and CD45.2− neutrophils. (D) Joint inflammation in C57BL/6 mice injected with a suboptimal dose of K/BxN serum (100 μL) with CSF3 and IL-4. (E) A model describing inhibitory effects of IL-4 on neutrophils during acute inflammation. Data presented as individual mice in A (n = 2 to 5), and B (n = 4 to 5), as well as mean and SEM in C (n = 6 to 7), and D (n = 5). *P < 0.05, **P < 0.01, ***P < 0.005, n.s. = not significant by one-way ANOVA with Tukey’s post hoc test (A–D) and by Mann–Whitney U test in C and D (at day 6). Data are representative of at least two independent experiments.
IL-4 can suppress joint inflammation in a context with increased CSF3 level, WT mice were injected with a suboptimal dose of K/BxN sera together with CSF3 and further treated with IL-4. Despite the suboptimal dose of K/BxN serum used, robust joint inflammation was observed in combination with CSF3, supporting the known role for CSF3 in this model (27). Nevertheless, the administration of IL-4 still suppressed the induced joint inflammation (Fig. 5D). Hence, we conclude that IL-4 can affect several proinflammatory features of CSF3 primed neutrophils with relevance for antibody-mediated joint inflammation, including in inflammatory conditions with high CSF3 levels (Fig. 5E).

**Discussion**

IL-4 and its close relative IL-13 are central to T helper cells type 2 (TH2) responses, commonly associated with parasitic infections and allergies (51, 52). Here we identify the IL-4/IL-4R signaling pathway as having several important effects related to proinflammatory neutrophil biology in the context of sterile inflammation. IL-4 has been widely studied in relation to its effect on lymphoid cells, centrally involved in T helper cell differentiation, B cell class switch (52, 53), as well as effects on non-immune stromal cells (54). An emerging field related to IL-4R signaling in neutrophils is broadening the implications under conditions with increased IL-4 and IL-13 levels, such as allergic reactions and parasitic infections, but also related to therapeutic interventions affecting this pathway (55, 56). For example, an interesting connection between the increased susceptibility to skin infections in patients with the TH2 centric disease atopic dermatitis and the suppression of neutrophil migration by IL-4R signaling has been proposed (29, 34). Importantly, blockade of the IL-4R by the clinically approved antagonist antibody Dupilumab decreases the risk of skin infections in atopic dermatitis patients, something that could be speculated to relate to better neutrophil activation in the absence of IL-4R–induced signaling, and as a consequence better control of the infection (57). In contrast, in a context where neutrophil activation is contributing to pathology, therapeutic activation of the IL-4R signaling pathway could be beneficial. Our data from mixed bone marrow chimeras extend the published observations of IL-4 suppressing neutrophil migration also in the context of joint inflammation (59).

The role of neutrophils in RA is debated. Neutrophils are typically making up the most abundant cell population in the synovial fluid from inflamed joints of patients with active disease, as well as in mouse models of acute joint inflammation. Furthermore, neutrophil deficiency in animal models for experimental joint inflammation results in significant suppression of the inflammation in most used models (6–10), suggesting that neutrophil localization to the joint in human RA patients could play an important role in the disease. Neutrophils are rapidly recruited to sites of inflammation and are activated in a rather nonspecific fashion compared to many other immune cells. During an infection, this behavior is often beneficial, helping to contain the invading infectious agent rapidly. In the context of sterile inflammation, such as during an autoreactive attack on the joint, neutrophil recruitment and activation is likely unfavorable, amplifying the unwanted inflammation. Limiting the migration of neutrophils into inflamed joints could thus be an interesting therapeutic approach in RA patients.

The IL-4R signaling pathway is fairly simple when compared to many other cytokine signaling pathways. It involves a type 1 IL-4 receptor made up of the IL-4Rα (CD124) and the common cytokine γ-chain CD132 that interacts with IL-4, and a type 2 IL-4 receptor made up of the IL-4Rα and IL-13Rα1 that interacts with both IL-4 and IL-13. Both type 1 and type 2 IL-4Rs activate STAT6 (58). Furthermore, a relative to the IL-13Rα1 called IL-13Rα2 (CD213A2) also exists, which only directly interacts with IL-13 (59). Importantly, the IL-4Rα protein is essential for all signaling pathways induced by IL-4. Neutrophils do not express high levels of the IL-4Rα at steady state compared to, for example, B cells. However, we and others have shown that IL-4Rα is highly up-regulated on several myeloid cell populations during different inflammatory conditions (21, 29, 35). Up-regulation of cytokine receptor α-chains on immune cells is well described where, for example, the up-regulation of IL-2Rα (CD25) on T cells is central to T cell activation and proliferation (60). In this example, the up-regulation of the receptor (IL-2Rα) results in an increased sensitivity to lower levels of the cytokine (IL-2), something that is important as many cytokines are found at low concentrations and are often rapidly consumed. The expression of the IL-4Rα is known to be regulated by several factors in B and T cells, including IL-2 and IL-4, and induced STAT5 and STAT6 signaling, respectively (61, 62). IL-2 and IL-4 do, however, not regulate the IL-4Rα on neutrophils (21).

Here we identify that activation of an alveolar macrophage cell line, as well as a CSF2/GM-CSF–dependent bone marrow–derived macrophage cell line, induces the releases a protein that in turn up-regulates the IL-4Rα on neutrophils. Combining gene expression analysis and a targeted CRISPR screen, we identified this IL-4Rα–inducing factor as CSF3/G-CSF. Furthermore, we were able to show that also in vivo, CSF3 is the protein responsible for the up-regulation of IL-4Rα on neutrophils seen during sterile inflammation and that this is related to CSF3–induced STAT3 signaling. Previously, we identified that conditioned supernatant from lung and epididymal (vesicular) fat pad organ cultures from mice injected with LPS were able to produce a factor that up-regulated the IL-4Rα on neutrophils (21). Interestingly, the Genotype-Tissue Expression (GTEx) project database (63) identifies that the lung has the highest basal CSF3 expression of 53 tested human organs, while visceral fat is among the top 5 organs in that same list. Following LPS injection into mice, we saw robust CSF3 levels in serum and broad Csf3 mRNA up-regulation in all tested organs (liver, lung, and spleen).

The majority of RA patients display a spectrum of specific autoantibodies that characterize the patient as having seropositive RA. Notably, this subset of patients has an expected worsening disease progression (4, 5). An explanation for the accelerated disease progression of these patients has been speculated to relate to the fact that autoantibodies being involved in forming antibody–antigen ICs locally in the joints, which in turn further amplify the inflammation by interacting with activating FcyRs on different immune cells, such as neutrophils (8, 10). Animal studies have shown that activating FcyRs play a central role in antibody-mediated joint inflammation (10, 18, 19, 43) and that the inhibitory FcyR2b (CD32b) suppresses this interaction (20, 44). Significantly, here we identify that CSF3-activated neutrophils respond to IL-4 with strong up-regulation of the inhibitory FcyR2b while activating FcyRs are not majorly affected. FcyRs are expressed on most immune cells, and myeloid cells often express both activating and inhibitory FcyRs (41). The consequence of an interaction between an FcyR-expressing cell and an IC depends on the relative interaction of the IC with activating and inhibitory receptors on the specific cell. This is affected by multiple parameters, such as the subclass and glycoform of the IgG antibodies, but also by the expression levels of activating and inhibitory FcyRs on the cells (64, 65). We have previously shown that both IL-4 and IL-13 protect from antibody-mediated joint inflammation in WT mice, while this protection is not seen in mice lacking IL-4Rα, STAT6, or FcyR2b (20). Moreover, we have also previously shown that IL-4 does not protect mice where the IL-4Rα is deleted in LysM-Cre–expressing cells, such as macrophages and neutrophils (21). Therefore, the conclusion has been that IL-4R–induced STAT6 signaling results in the up-regulation of FcyR2b, which in turn is responsible for limiting the antibody-mediated joint inflammation. Due to the low basal expression of FcyR2b on neutrophils (66), we...
have hypothesized that the IL-4-mediated protection could mainly be related to the up-regulation of FcγR2b on macrophages. However, the data presented here clearly show that also neutrophils can be induced to express very high levels of FcγR2b when primed by CSF3, a phenomenon commonly occurring during acute inflammation of various sorts.

Activated neutrophils are known to release targets for autoantibodies found in seropositive RA patients, such as citrullinated histones and vimentin, in a process referred to as netosis (15, 16). Limiting neutrophil recruitment, activation, and netosis in inflamed joints, could, therefore, be a therapeutic approach to suppress joint inflammation associated with local IC formation. Interestingly, it was recently shown that IL-4 could suppress neutrophil netosis (34), showing that IL-4–induced signaling in neutrophils results in several behavioral changes that could be expected to be beneficial in RA. Together, our data identify a regulatory loop where CSF3, known to activate neutrophils and to enhance neutrophil-mediated inflammation [including anecdotally resulting in flares of disease after injection into patients with RA (67)], also prime neutrophils for IL-4R signaling by up-regulating the IL-4Rα. In the presence of IL-4, CSF3-primed neutrophils up-regulate the inhibitory FcγR2b, and their egress from the bone marrow and migration into the inflammatory site is suppressed. Both these phenotypes are of significant relevance to antibody-mediated inflammation both involving neutrophils. The suppressive activity of IL-4Rα signaling in neutrophils is thus directly linked to the activating signal induced by CSF3. Such paired activating/inhibitory pathways are commonly found in the immune system, exemplified by CD28/CTLA4 and activating/inhibitory FcγRs, important for fine-tuning the activation and down modulation of immune responses. Considering these types of regulatory mechanisms could have important therapeutic implications, as shown by the many novel immune modulatory drugs targeting such pathways. We thus propose that the converging IL-4/CSF3 pathways could be explored therapeutically to both enhance and suppress immune responses involving neutrophils.

Materials and Methods

Animals. Eight- to 12-wk-old, sex- and age-matched mice were used for experiments. All animal experiments have been approved by the local animal ethical board at the Karolinska Institute. Mice were housed under specific-pathogen-free conditions in a 12-h/12-h light-dark cycle and fed standard chow diet ad libitum. WT (C57BL/6, BALB/c) or modified mice on C57BL/6 background from the Jackson Laboratory were used. MyD88 KO (CD192). In the presence of IL-4, CSF3-primed neutrophils up-regulate the inhibitory FcγR2b, and their egress from the bone marrow and migration into the inflammatory site is suppressed. Both these phenotypes are of significant relevance to antibody-mediated inflammation both involving neutrophils. The suppressive activity of IL-4Rα signaling in neutrophils is thus directly linked to the activating signal induced by CSF3. Such paired activating/inhibitory pathways are commonly found in the immune system, exemplified by CD28/CTLA4 and activating/inhibitory FcγRs, important for fine-tuning the activation and down modulation of immune responses. Considering these types of regulatory mechanisms could have important therapeutic implications, as shown by the many novel immune modulatory drugs targeting such pathways. We thus propose that the converging IL-4/CSF3 pathways could be explored therapeutically to both enhance and suppress immune responses involving neutrophils.

Injection. K/BxN serum transfer experiments were performed by intravenous injection of 100 or 200 μL serum as indicated in the figure legends. Clinical score of joint swelling and functional test was performed as described previously (21). IL-4 complexes with prolonged half-life were generated by incubating mouse IL-4 (Peprotech) with the anti-IL-4L clone 1B11 (BioXcell) as described previously (20). IL-4 complexes (5 μg mouse IL-4 + 25 μg 1B11) and/or mouse CSF3 (2 μg, Peprotech) in sterile PBS were intravenously injected 30 min before K/BxN serum or 2 d after K/BxN, as indicated in the figure legends. TLR4-specific LPS [Salmonella Minnesota R595 (Re); Enzo Life Sciences] diluted in sterile PBS was injected intraperitoneally (2.3.5, or 10 μg) as indicated in the figure legends. Anti-CSF3 (984CSF, functional grade, ebioScience) or isotype control (eBR2a, functional grade, ebioScience) was diluted in sterile PBS and injected intravenously, at doses indicated in the figure legends; 30 min before LPS injection. Mouse CSF3 (2 μg, Peprotech) diluted in sterile PBS was injected intravenously. When IL-4 complexes (5 μg mouse IL-4 + 25 μg 1B11) were injected in the same experiment as LPS or CSF3, the IL-4 was intravenously injected 20 to 24 h after the LPS/CSF3 injection. The effect of the injections was measured by flow cytometry 20 to 24 h after the IL-4 injection.

Thioglycollate-elicited macrophages were generated as described previously (21) with the modification that 0.5 ml 4% thioglycollate solution was injected intraperitoneally.

Cell Lines and Ex Vivo Stimulations. A549 (human lung epithelial carcinoma), Jurkat (human T cell leukemia), and MH-S (mouse alveolar macrophage cell line) were cultured in RPMI-1640 (Sigma-Aldrich) with 10% heat-inactivated bovine serum, 1% penicillin-streptomycin-glutamine, and 0.1% β-mercaptoethanol (Gibco; referred to below as crPMI). The ER-HoxB8 Macrophage cell line (mouse bone marrow macrophage precursor, a kind gift from Mark P. Kamins, University of California, San Diego) was cultured in crPMI with 1 μM β-estradiol (Sigma-Aldrich) and 10% conditioned medium from a CSF2/10GM-CSF expressing B16 cell line, as described previously (33). Cells were seeded at 250,000 cells per well in 6-well plates in 1.5 to 3 mL medium, and 50,000 cells per well in 96-well plates in 0.2 mL medium and split every 2 to 3 d. To differentiate the HoxB8 Macrophage cells to mature macrophages, the cultures were washed three times to remove steroid-β-estradiol, and cells cultured in crPMI with CSF2/CSF3 without β-estradiol for >5 d before being stimulated with LPS. Cells line at 50 to 80% confluency cultured in six-well plates were stimulated with 0.2 μg/mL LPS [Salmonella Minnesota R595 (Re); Enzo Life Sciences] overnight. Conditioned supernatant was collected and separated by centrifugation (500 g × 5, 5 min). Single-cell colonies of the Macpro and MH-3 cell lines were generated by limiting dilution. High- and low-producing MH-5 clones were identified by assaying the conditioned media for the ability to up-regulate the IL-4Rα on neutrophils after LPS stimulation. For the generation of C9a-expressing Macpro cells, the cells were spin-infected with Lenti-Cas9 Blast viral particles, described below, and selected after ~72 h with 10 μg/mL blasticidin for 10 d. All cell lines were tested negative for mycoplasma contamination.

Bone marrow cells from WT or MyD88 KO mice were collected by flushing femur/tibia with a syringe and a 25-G needle with sterile PBS. Blood cells were collected from PVP tubes (Biochrom) without whole blood contamination through tail vein bleeding. Blood cells were used fresh, from the heart or by cardiac puncture. Red blood cells (RBCs) were lysed using 1× RBC lysis buffer (ebioScience), and cells stimulated overnight in 96-well plates (1:100 if cells from two femur were used per well in a 96-well plate). In Fig. 5B, bone marrow cells were mixed from WT (CD45.1) and IL-4Rα KO (CD45.2), injected or not with CSF3 as indicated in the figure legend. Stimulations include dilutions of serum from K/BxN mice (from mice with active disease), and mice injected with LPS (“LPS serum,” collected 20 to 24 h after intraperitoneal injection of 10 μg LPS into WT C57BL/6 mice), 20% conditioned medium from cells stimulated overnight ±0.2 μg/mL LPS, and cytokines (mouse: IL-4, IL-6, CSF3, LIF at 20 ng/mL; and human: CSF3 [10 and 25 ng/mL], and IL-6 [25 ng/mL], all from Peprotech). To degrade proteins in conditioned medium, the sample was incubated with protease K-agarose (2 kU/mL) at 37 °C for 45 min, and CSF3 was measured by ELISA. Blood cells were supplemented with 0.5 mL 4% thioglycolate solution was injected intraperitoneally 20 to 24 h after the LPS/CSF3 injection. The effect of the injections was measured by flow cytometry 20 to 24 h after the IL-4 injection.

Flow Cytometry, Sorting, and ELISA. Single-cell preparation of mouse organs was generated as described previously (21), and RBCs were lysed using 1× RBC Lysis buffer (ebioScience). Cells isolated from joints of mice with K/BxN-induced inflammation were collected from mice perfused with cold PBS to
remove blood from tissues. Cells were stained with FC-blocker (clone 2.4G2, importantly not included if cells were stained for FcRy), and labeled with the anti-CD11b/CD18 (M1/70), Ly-6G (1AB), CD4 (RM4-5), IL-4Rα, and the triple-positive staining (CD11b/CD18/IL-4Rα) of the macrophages. As clones expanded, part of the cells was frozen for future use.

For introducing gRNAs into pX459 V2.0, paired sense and antisense gRNA oligonucleotides were combined with either pCgpV, psPAX2 (Addgene #12260), and pMD2.G (Addgene #12259) (both kind gifts from Didier Trono, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) at a 2:2:1 ratio (the first number indicates the ratio of the transfer plasmid), and transfected into the HEK293T cells using the TransIT-LT1 Transfection Reagent (Mirus) according to the manufacturer’s protocol. Approximately 12 h later, the medium was removed and replaced by 5 mL of fresh DMEM with 10% serum and 1% glutamine. After another ~36 h, the supernatant containing the virus was collected, briefly centrifuged to remove cellular debris, and used for spin-infection of bone marrow cells.

For CRISPR screen experiments, the multiplicity of infection (MOI) of the gRNA library was adjusted to 1000:1. Viral particles were tested by infection with serial dilutions of virus particles, to find a dilution resulting in a low MOI suitable for a screen. Fifty thousand cells per well in 12-well plates were spin-infected (1,200 x g, 90 min) in the presence of 8 μg/mL polybrene (Sigma-Aldrich). The Mcp pro cell line is difficult to infect, and an infection efficiency of ~25% was used for the screens, as measured by the percentage of GFP + cells after 96 h.

gRNA Design and Cloning. gRNAs with matching overhangs for the respective plasmids were extracted from the Green Listed software (http://greenlisted.cmm.ki.se), described in Panda et al. (69), using the Brie reference library (70). gRNA oligonucleotides for pSpCas9(2A)-Puromycin (PX459) V2.0 (Addgene plasmid #62988; kindly provided by Feng Zhang, Broad Institute of MIT and Harvard, Cambridge, MA) experiments were ordered as sense and antisense 80-mers (SI Appendix, Table S5) and the genotype correlated to the studied phenotype. Colonies having more than 50% of the expected genotype of the different single-cell clones was identified by Sanger sequencing, and the genotype correlated to the studied phenotype. Colonies having more than one gRNA were excluded from the analysis. Details of the PCR conditions and primers were listed in SI Appendix, Table S5.

Materials and Data Availability. The Affymetrix expression data of MH-S clones stimulated with LPS have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus and are accessible through GEO series accession number GSE134047. The plasmids lentiGuide-Puro-P2A-EGFP and lentIGuide-Puro-P2A-EGFP_mRFPstuf have been deposited to the nonprofit plasmid repository AddGene.

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