Cytokines are key regulators of tumor immune surveillance by controlling immune cell activity. Here, we investigated whether interleukin 4 (IL4) has antileukemic activity via immune-mediated mechanisms in an in vivo murine model of acute myeloid leukemia driven by the MLL–AF9 fusion gene. Although IL4 strongly inhibited leukemia development in immunocompetent mice, the effect was diminished in immune-deficient recipient mice, demonstrating that the antileukemic effect of IL4 in vivo is dependent on the host immune system. Using flow cytometric analysis and immunohistochemistry, we revealed that the antileukemic effect of IL4 coincided with an expansion of F4/80+ macrophages in the bone marrow and spleen. To elucidate whether this macrophage expansion was responsible for the antileukemic effect, we depleted macrophages in vivo with clodronate liposomes. Macrophage depletion eliminated the antileukemic effect of IL4, showing that macrophages mediated the IL4-induced killing of leukemia cells. In addition, IL4 enhanced murine macrophage-mediated phagocytosis of leukemia cells in vitro. Global transcriptomic analysis of macrophages revealed an enrichment of signatures associated with alternatively activated macrophages and increased phagocytosis upon IL4 stimulation. Notably, IL4 concurrently induced Stat6-dependent upregulation of CD47 on leukemia cells, which suppressed macrophage activity. Consistent with this finding, combining CD47 blockade with IL4 stimulation enhanced macrophage-mediated phagocytosis of leukemia cells. Thus, IL4 has two counteracting roles in regulating phagocytosis in mice; enhancing macrophage-mediated killing of leukemia cells, but also inducing CD47 expression that protects target cells from excessive phagocytosis. Taken together, our data suggest that combined strategies that activate macrophages and block CD47 have therapeutic potential in acute myeloid leukemia.
antileukemic activity by inducing Stat6-dependent apoptosis of AML cells. Elevated IL4 levels in mice eradicate AML cells in both the spleen and bone marrow, resulting in increased survival. Under physiological conditions, IL4 is a pleiotropic cytokine that regulates several immunological processes, such as B-cell class switching, T helper cell maturation, alternative activation of macrophages, and activation of NK cells. IL4 can bind to the IL4 receptor (IL4R) type I receptor complex, a heterodimer of the IL4R alpha (IL4RA) and IL2 receptor subunit gamma (IL2RG) chains, or to the IL4R type II receptor complex, a dimer of IL4RA and IL13RA1. Whether immune cells also mediate the antileukemic activity of IL4 has not been previously explored.

In this study, we show that IL4 regulates phagocytosis by enhancing macrophage-mediated killing of AML cells and increasing CD47 expression on leukemia cells, which inhibits macrophages. Combined blockade of CD47 and IL4 stimulation enhanced macrophage-mediated killing of AML cells. Hence, our data suggest that combined strategies that activate macrophages and block CD47 have therapeutic potential in AML.

Methods

The murine leukemia model

All animal experiments were conducted according to the protocol approved by the Animal Care and Use Committee of the Lund/Malmö Ethical Committee. MLL–AF9 leukemias were generated in a dsRed C57BL/6 transgenic background (6051; Jackson Laboratory, Bar Harbour, NY, USA), as previously described. The MLL–AF9 leukemia was serially propagated in sublethally irradiated (600 cGy) C57BL/6 recipient mice and leukemia stem cells were enriched as previously described. All experiments involving murine leukemia cells were performed using tertiary or quaternary transplanted leukemia cells. As immunodeficient murine recipients, sublethally irradiated (250 cGy) NOD/SCID and NOD.Cg-Pkrdc<sup>-/-Il2rg<sup>tm1Wjl</sup>/SzJ</sup> (NSG) mice were used (in-house breeding). All mice in the survival experiments were sacrificed based on at least one of the following criteria: immobility, hunched back, hind leg paralysis, or dehydration.

Phagocytosis assay

For mouse phagocytosis assays, c-Kit<sup>+</sup> dsRed<sup>+</sup> murine MLL–AF9 leukemia cells were added to macrophage cultures in a 2:1 ratio. After 18 h, the cells were stained with a BV421-F4/80 antibody (BioLegend, San Diego, CA, USA), and the percentage of F4/80+dsRed<sup>+</sup> cells was determined by FACS analysis.

For the CD47 blocking experiments, we incubated c-Kit<sup>+</sup> dsRed<sup>+</sup> murine MLL–AF9 leukemia cells for 30 min with an anti-CD47 antibody or rat IgG2a isotype control (30 μg/mL; BioXCell, Lebanon, NH, USA), before co-culture with macrophages for 1 h at 37°C. The percentage of F4/80<sup>+</sup>dsRed<sup>+</sup> cells was determined by flow cytometry as described above.

For human phagocytosis assays, we labeled human leukemia cell lines with the PKH67 green fluorescent cell dye according to the manufacturer’s instructions (Sigma-Aldrich, Darmstadt, Germany) and stained macrophages with the PKH26 red fluorescent cell dye (Sigma-Aldrich). AML cells were mixed with human macrophages in a 2:1 ratio and incubated for either 2 h (Mono Mac 6 cells) or 18 h (MA9:16 cells). The percentage of PKH26<sup>+</sup> PKH67<sup>+</sup> macrophages was determined by FACS.

RNA sequencing analysis

Global gene expression profiling was performed on sorted F4/80<sup>+</sup> spleen cells from mice transplanted with IL4-overexpressing leukemia cells and non-transplanted irradiated controls. Cells were collected 12 days after irradiation. In addition, RNA sequencing was performed on macrophages produced in vitro by stimulating murine monocytes for 7 days with murine (m)CSF1 (25 ng/mL) and mIL4 (20 ng/mL) or only mCSF1.

Raw data and normalized gene expression data are available in the Gene Expression Omnibus database under accession number GSE155048.

Results

The antileukemic activity of interleukin 4 in vivo is predominantly mediated via immune cells

To determine whether immune cells contribute to the previously described antileukemic effects of IL4 in vivo, we used a murine AML model driven by the MLL–AF9 (KMT2A-MLLT3) fusion gene. The leukemia cells were generated in a dsRed<sup>+</sup> transgenic background, allowing for convenient tracking of leukemia cells upon serial transplantations. Serial passaging of leukemia cells in mice did not alter IL4RA expression on AML blasts (Online Supplementary Figure S1A). Consistent with previous results, we confirmed that elevated IL4 levels mediated by retroviral expression in c-Kit<sup>+</sup> AML cells transplanted into mice (IL4 group) resulted in strong in vivo antileukemic activity. The IL4 group showed prolonged survival compared to controls and had almost no leukemia cells in the bone marrow or spleen at the time of sacrifice (Figure 1A, B, Online Supplementary Figure S1B).

To address whether the antileukemic activity of IL4 in vivo was immune-mediated, we used two strains of immunodeficient recipient mice: NOD/SCID mice, which lack T and B cells and have decreased activity of both NK cells and macrophages, and NSG mice, which additionally lack NK cells. In NOD/SCID animals, the antileukemic effect of IL4 was reduced, and we observed increased levels of leukemia cells in the bone marrow and spleens compared to the levels in immunocompetent mice (Figure 1C, Online Supplementary Figure S1C). These findings suggest that immune cells at least partially mediate the antileukemic effect of IL4. To further characterize the antileukemic effect of IL4, we used the NSG mouse strain, which lacks a functional IL4 receptor type I complex because of deficiency in the Il2rg gene. Of note, in NSG mice, the antileukemic effect of IL4 was abolished, and survival was even shorter than in controls, with high levels of leukemia cells in both the bone marrow and spleens at the time of sacrifice (Figure 1D, Online Supplementary Figure S1D). These findings suggest that the antileukemic effect of IL4 in vivo depends on immune cells expressing the IL4 receptor type I complex.
Interleukin 4 expands macrophages in vivo

To identify the type of immune cell that mediates the IL4-induced antileukemic effects, we analyzed the hematopoietic compartment in mice receiving IL4-secreting AML cells. At day 19 after transplantation, we detected no IL4-induced alterations in blood cell lineages by flow cytometry (Figure 2A). Moreover, at this time-point, we detected no circulating leukemia cells in the blood of mice in the IL4 group (Figure 2B). In contrast, at day 27 after transplantation, the white blood cell, red blood cell, and platelet counts in the IL4 group were reduced compared to those in controls that had not been injected with leukemia cells (Figure 2C, Online Supplementary Figure S2A, B). Of note, at the time of sacrifice, when the mice had succumbed to disease (Figure 1B), there was significant expansion of F4/80+ macrophages in the bone marrow (on average, 2.4% vs. 1%; P < 0.001) and spleens (on average, 7.9% vs. 1.3%; P < 0.0001) of IL4 mice (Figure 2D, Online Supplementary Figure S2C, D). We confirmed this IL4-induced increase in the proportion of macrophages by immunohistochemistry (Figure 2E, Online Supplementary Figure S2E). We also confirmed IL4RA expression on the F4/80+ cells from both groups of mice, supporting that IL4 receptor signaling may directly stimulate macrophages in this model (Online Supplementary Figure S2F).

Interleukin 4 stimulation increases murine macrophage-mediated phagocytosis of leukemia cells

To assess whether the IL4-induced expansion of macrophages in vivo was responsible for the antileukemic activity of IL4, we depleted macrophages by intraperitoneal injections of clodronate liposomes, followed by injection of IL4-secreting AML cells (Figure 3A). Efficient depletion of macrophages was observed in the spleen but not in the bone marrow (Figure 3B). Consistent with the macrophage depletion, we found a proportional increase of leukemia cells in the spleen of these mice (on average, 33% vs. 6%; P < 0.05), but not in the bone marrow (Figure 3C). In contrast, depletion of macrophages had no effect on the level of leukemia cells in the MIG control group (Online Supplementary Figure S3A, B). These findings suggest that macrophages mediate the IL4-induced killing of leukemia cells.

Because macrophages kill cells by phagocytosis, we next assessed whether IL4 stimulation results in increased macrophage-mediated phagocytosis of leukemia cells in culture. Murine monocytes isolated from bone marrow were differentiated into macrophages for 7 days by supplementation of the culture medium with CSF1 (Figure 3D). The addition of IL4 to the medium resulted in increased phagocytosis of leukemia cells, as evident by macrophage acquisition of dsRed fluorescence (Figure 3E, F). Consistent with a more activated state, the IL4-stimulated...
Figure 2. Interleukin 4 stimulation increases the frequency of macrophages in vivo. C57BL/6 mice were transplanted with 30,000 sorted green fluorescent protein (GFP)+ MLL-AF9 acute myeloid leukemia (AML) cells 2 days after transduction with retroviral vectors co-expressing GFP and a murine interleukin 4 cDNA (MIG–IL4) or a control vector (MIG). (A) Percentages of blood cell populations within dsRed− cells 19 days after transplantation (n=3). (B) Percentage of leukemia (dsRed+) cells in the peripheral blood on day 19 after transplantation (n=3). (C) White blood cell counts at days 12 and 27 for MIG–IL4 and non-transplanted irradiated control mice (IL4 group, n=4; controls, n=3). (D) Percentage of F4/80+ cells within dsRed− cells in bone marrow and spleens of mice at the time of sacrifice (controls, n=4; IL4 group, n=5). (E) Representative immunohistochemistry staining of F4/80+ cells in bone marrow (40×; scale bar, 20 μm) and spleens (10x; scale bar, 100 μm). BM: bone marrow; N.D.: not detected; PB: peripheral blood; WBC: white blood cell; IHC: immunohistochemistry. **P<0.01; ***P<0.001; ****P<0.0001.
lated macrophages had an increased volume and were less irregular than unstimulated cells, as evaluated using phase holograph imaging (Online Supplementary Figure S4).

In contrast to its effect on murine macrophages, human IL4 is well known to differentiate human monocytes into anti-inflammatory macrophages.22 To assess how human IL4 affects phagocytosis of leukemia cells, human macrophages were stimulated with IL4 before mixing with AML cell lines. In line with a differential role of IL4 in mice and humans, IL4 suppressed human macrophage-mediated phagocytosis of the AML cells (Figure 3G-I).

**Interleukin 4 induces polarization of macrophages**

To investigate how IL4 affects the global gene expression of macrophages, we performed RNA sequencing of murine macrophages generated in vitro with or without IL4 stimulation. In addition, we performed RNA sequencing on sorted dsRed+ F4/80+ macrophages from mice transplanted with IL4-expressing leukemia cells and macrophages from leukemic control mice. In agreement with a described role for IL4 in promoting macrophage polarization, IL4 induced the expression of several genes associated with alternative activation of macrophages, including Arg1, Chil3, and Retnla (Figure 4A, Online Supplementary Figure S5A, B).22,23 which were among the most differentially upregulated genes (Online Supplementary Tables S1 and S2). Of note, IL4 also induced strong upregulation in vivo of the chemokine Ccl24, a biomarker for macrophages that originate from monocytes rather than tissue-resident macrophages (Figure 4A). Moreover, the IL4-induced macrophages showed downregulation of genes such as Ccl24, which is associated with tumor-associated macrophages (Figure 4B), indicating that IL4 differentiates macrophages into a phenotype that is distinct from tumor-associated macrophages.

We next performed gene set enrichment analysis to identify gene expression signatures enriched in the IL4-induced macrophages in vivo. In accordance with increased phagocytosis of macrophages stimulated with IL4 in vitro, we found an enrichment of phagocytosis signatures in macrophages harvested from mice in the IL4 group (Figure 4C). Moreover, IL4 stimulation resulted in enrichment of genes...
associated with major histocompatibility complex (MHC) proteins (Figure 4C). To determine the influence of the in vivo microenvironment, we compared the gene expression profiles of IL4-stimulated macrophages generated in vitro and those generated in vivo (Online Supplementary Table S3). Macrophages generated in vivo exhibited a preferential upregulation of several markers associated with inflammation and immune activation (Online Supplementary Figure S5C, D). Altogether, the gene expression data suggest that IL4 stimulation leads to an expansion of monocyte-derived macrophages with increased phagocytic activity.

Interleukin 4 upregulates CD47 in a Stat6-dependent manner

We next searched for IL4-induced mechanisms in leukemia cells that might affect their interactions with macrophages. Interestingly, the macrophage-inhibitory protein CD47 was upregulated on leukemia cells in the IL4 group compared to controls at the time of sacrifice (Figure 5A). Consistent with this finding, IL4 induced the expression of CD47 in leukemia cells in a dose-dependent manner, showing that IL4 activates signaling that induces CD47 expression (Figure 5B). Moreover, according to RNA sequencing data that we had previously generated, CD47 was upregulated in both c-Kit+ AML cells and normal c-Kit+ bone marrow cells stimulated with IL4 (Figure 5C).

We next explored the mechanistic basis of the IL4-induced upregulation of CD47. Because STAT6 is a critical downstream mediator of IL4R signaling, we used CRISPR/Cas9 genetic engineering to knock out Stat6 in Cas9-expressing MLL–AF9 AML cells using Stat6 gRNA that we had previously characterized. Stat6 disruption hindered the IL4-induced upregulation of CD47 (Figure 5D), demonstrating that IL4 upregulates CD47 in a STAT6-dependent manner. Thus, in addition to activating murine macrophages, we identified a previously unknown role of IL4 in protecting cells from phagocytosis via CD47 upregulation.

Combined interleukin 4 treatment and CD47 blockade results in enhanced macrophage-mediated phagocytosis of acute myeloid leukemia cells

Because CD47 protects cells from phagocytosis, we next evaluated whether the IL4-induced upregulation of CD47 on AML cells counteracts enhanced phagocytosis by IL4-stimulated macrophages. Consistent with this hypothesis, AML cells pre-treated for 24 h with IL4 and washed before co-culture with macrophages were partial-
ly resistant to phagocytosis (Figure 5E). To overcome the inhibitory signal provided by increased CD47 expression, we used an α-CD47 blocking antibody. Combined blocking of CD47 on AML cells and IL4 stimulation of macrophages resulted in enhanced phagocytosis of AML cells (Figure 5F). These findings show that IL4 has a dual role in murine phagocytosis by directly activating macrophages and enhancing their phagocytic activity, while also inducing CD47 expression that counteracts phagocytosis in target cells.

Discussion

Distinct types of macrophages control tumor development. Whereas tumor-associated macrophages promote tumor development by suppressing the immune system, other types of macrophages achieve tumor immune surveillance through phagocytosis of malignant cells. We found that IL4 has antileukemic effects in mice, primarily mediated by alternatively activated macrophages that normally play a key role in tissue repair and immune

Figure 5. Combined interleukin 4 stimulation and CD47 blockade result in enhanced macrophage-mediated phagocytosis of acute myeloid leukemia cells. (A) Representative histograms showing CD47 expression on acute myeloid leukemia (AML) cells in bone marrow (BM) and spleens of mice transplanted with dsRed+ leukemia cells transduced with the MIG-interleukin 4 (MIG-IL4) or control (MIG) vectors. (B) CD47 expression on AML cells following IL4 stimulation for 24 h. (C) Cd47 expression shown as FPKM values of normalized reads from RNA sequencing data of c-Kit+ dsRed+ leukemia cells and c-Kit+ normal BM cells stimulated with IL4 for 18 h. Data are presented as box and whiskers diagrams; the line indicates median, box limits are first and third quartiles, and bars indicate maximum and minimum values. (D) CD47 expression measured by flow cytometry after 24 h of stimulation with murine (m)IL4 (100 ng/mL) in cells transduced with lentiviral vectors expressing Stat6 or control sgRNA. (E) Phagocytosis assay with macrophages derived from murine BM monocytes stimulated with mCSF1 (25 ng/mL) and mL4 (20 ng/mL) for 7 days. The AML cells were treated with mL4 (100 ng/mL) or no IL4 (control) for 24 h prior to co-culture (n=3). Phagocytosis is presented as the percentage of dsRed+ cells within F4/80+ cells. (F) Phagocytosis assay with mouse BM monocyte-derived macrophages stimulated for 7 days with mCSF1 (25 ng/mL) and mL4 (20 ng/mL) or mCSF1 only (n=3). AML cells were cultured for 1 h with a blocking anti-CD47 antibody or corresponding isotype control and then mixed with the macrophages. FPKM, fragments per kilobase million; gMFI, geometric mean fluorescence intensity; NBM, normal bone marrow. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.
The observed expansion of alternatively activated macrophages is consistent with findings showing that IL4, via the IL4 receptor type I complex, promotes the outgrowth of macrophages beyond homeostatic levels in the setting of nematode infections. In contrast, the IL4-induced macrophages with antileukemic activity showed higher expression of Ccl24, Mrc1, and Pdkd1g2, suggesting that they are of monocytic origin, from either the bone marrow or peripheral blood. Among hematopoietic cells, only macrophages showed increased numbers following enforced expression of IL4 in vivo. IL4 also boosted the phagocytic activity of murine monocyte-derived macrophages in vitro, suggesting that IL4 acts directly on the monocytes/macrophages that mediate the antileukemic effect. Moreover, consistent with their increased phagocytic activity, the IL4-induced macrophages were functionally and molecularly distinct from tumor-associated macrophages, which are classically associated with an alternatively activated phenotype. Furthermore, the IL4-induced macrophages were functionally distinct from AML-associated macrophages, which polarize into a leukemia-supportive state. IL4-mediated phagocytosis of AML cells was further enhanced macrophage-mediated phagocytosis of AML cells. IL4 stimulation induced CD47 upregulation in a STAT6-dependent manner, and combined IL4 stimulation with CD47 blockade further enhanced macrophage-mediated phagocytosis of AML cells. These findings deepen our understanding of how IL4 regulates murine macrophages and suggest that strategies to combine macrophage activation with CD47 inhibition should be explored further as a therapeutic approach in cancer.

Disclosures
No conflicts of interest to disclose.

Contributions
PPM, RR, CH and CJ performed research, PPM and MJ analyzed data and wrote the manuscript, and all other authors contributed with valuable comments.

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Data-sharing statement
Raw data and normalized gene expression data are available in the Gene Expression Omnibus database under accession number GSE155048.

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