Interleukin 4 promotes phagocytosis of murine leukemia cells counteracted by CD47 upregulation

Pablo Peña-Martínez, Ramprasad Ramakrishnan, Carl Högberg, Caroline Jansson, David Gisselsson Nord and Marcus Järås

Division of Clinical Genetics, Department of Laboratory Medicine, Lund University, Lund, Sweden

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Correspondence: *MARCUS JÄRÅS* - marcus.jaras@med.lu.se

Supplementary information for:

IL4 promotes phagocytosis of murine leukemia cells counteracted by CD47 upregulation

Pablo Peña-Martínez[1], Ramprasad Ramakrishnan[1], Carl Högberg[1], Caroline Jansson[1], David Gisselsson Nord[1], and Marcus Järås[1]

[1]Division of Clinical Genetics, Department of Laboratory Medicine, Lund University, Lund, Sweden

SUPPLEMENTARY METHODS

Murine leukemia model

To enrich for leukemia stem cells, femurs from leukemic mice were crushed, red blood cells were lysed using NH_4Cl solution (STEMCELL Technologies, Vancouver, Canada), and c-Kit$^+$ cells were enriched by CD117 MicroBeads in MACS® Cell Separation Columns according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described.[1] Except for propagation of leukemia cells, all experiments involving murine AML cells were initiated using c-Kit$^+$ BM cells. dsRed$^+$ c-Kit$^+$ *MLL–AF9* leukemia cell cultures were grown in serum-free expansion medium (SFEM; Stemspan, STEMCELL Technologies) supplemented with 1% penicillin/streptomycin, 20 ng/mL murine (m)IL3, 25 ng/mL stem cell factor (mSCF), and 20 ng/mL human (h)IL6 (PeproTech, Rocky Hill, NJ, USA).

Viral vector generation and production

The murine stem cell virus gammaretroviral vector coexpressing a m*IL4* cDNA and *GFP* (green fluorescent protein) connected with an internal ribosome entry site (MIG–IL4), and *Stat6* and control single-guided RNA (sgRNA) vectors were previously generated.[2] The *Cas9*-expressing leukemia cells were generated as previously described.[2] Viral vectors were produced using standard protocols in 293T cells. Gammaretroviral vectors were pseudotyped with an ecotropic envelope and lentiviral vectors with a vesicular stomatitis virus G envelope. For transduction experiments, SFEM was supplemented with mIL3 (40 ng/mL), mSCF (50 ng/mL), and hIL6 (40 ng/mL) and mixed with the viral vectors. Transduction was performed by spinoculation at 600 ×*g* for 1 hour at 32°C, and 24 hours after transduction, the medium was replaced with fresh SFEM supplemented with cytokines.

Flow cytometric analysis and cell sorting

Flow cytometric analyses were performed using a LSRFortessa™ flow cytometer (BD Biosciences, San Jose, CA, USA), and cell sorting was performed using a FACSAria™ II cell sorter (BD Biosciences). To analyze cell populations in mice transplanted with leukemia cells transduced with retroviral vectors, we stained blood and BM cells using APC–CD3, PE/Cy7–CD4, BV510–CD8, APC/Cy7–CD19, and BV421–NK1.1 antibodies for lymphoid lineage stains, and APC–Ly6g, PE/Cy7–CD11c, BV421–CD115, and APC/Cy7–F4/80 antibodies for myeloid lineage stains (all from BioLegend, San Diego, CA, USA). Before flow cytometric cell sorting, we stained F4/80$^+$ cells with a BV421–F4/80 antibody (BioLegend). Staining of CD47 was achieved using an AF647–CD47 antibody (BioLegend). Staining of IL4RA was performed using a BV421–CD124 antibody (BD Biosciences).

Macrophage differentiation

To isolate both human and mouse monocytes, we used MACS® Cell Separation Columns with monocyte isolation kits according to the manufacturer's instructions (Miltenyi Biotec). Monocytes from mouse BM were enriched by negative selection, whereas we used CD14$^+$ selection to isolate human monocytes from the peripheral blood of healthy donors. Isolated monocytes were differentiated into macrophages in Roswell Park Memorial Institute (Gibco, Thermo Scientific, Waltham, MA, USA) medium supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, and 25 ng/mL murine or human colony-stimulating factor 1 (CSF1) for 7 days, and 20 ng/mL of murine or human IL4 for 7 days (cytokines from PeproTech). Half of the culture medium was replaced every 2-3 days.

Immunohistochemistry

Organs harvested from mice were fixed in 4% paraformaldehyde for 48 hours and stored in 70% ethanol. Formalin-fixed, paraffin-embedded tissue sections (4 µm) were dried on positively charged slides for 15 minutes at 60°C. The slides were then deparaffinized in xylene and hydrated in graded ethanol solutions. Endogenous peroxidase was blocked for 20 minutes with 1% H_2O_2 (Sigma-Aldrich) diluted in PBS pH 7.4 (Applichem, Darmstadt, Germany). Heat-induced epitope retrieval was performed by using target retrieval solution, pH 9.0 (Agilent DAKO, Santa Clara, CA, USA), and 0.2% Triton X-100 (Sigma-Aldrich) in a decloaking chamber (Biocare Medical, Pacheco, CA, USA) at 95°C for 20 minutes. Sections were incubated for 60 minutes with a rabbit anti-mouse F4/80 primary antibody (Thermo Fisher Scientific) in a 1:200 dilution in PBS containing 5% normal goat serum (Jackson Immuno Research, Ely, UK). Staining was obtained by using a horseradish peroxidase–conjugated anti-rabbit polyclonal antibody (AH Diagnostics, Tilst, Denmark) for 30 minutes, followed by incubation with the liquid DAB+ Substrate Chromogen System (Agilent DAKO) for 5 minutes, and counterstaining with Mayer's Hematoxylin (Histolab, Askim, Sweden) for 30 seconds. All incubations were performed at room temperature, and sections were washed three times with PBS after each incubation. Slides were mounted with Faramount Mounting Medium, Aqueous (Agilent DAKO). Images were acquired in an Olympus BX43 (Olympus, Waltham, MA, USA) with the Cellsens software (Olympus).

Phase holographic imaging

For morphologic analysis, a total of 5 000 macrophages were seeded per well in a 24-well plate and placed in a Holomonitor® M4 (Phase Holographic Imaging AB, Lund, Sweden). The microscope was located in an incubator at 37°C and 5% CO_2. Cells were allowed to attach for one hour, and then images were acquired and analyzed with the software Hstudio™ (Phase

Holographic Imaging AB). Individual cells were measured for volume and irregularity, a parameter based on the roundness of the cell.

RNA sequencing analysis

We used QIAshredder and RNeasy Microkit (QIAgen, Hilden, Germany) to extract RNA and validated RNA quality using a 2100 Bioanalyzer (Agilent Technologies, Inc).

To prepare RNA libraries from mouse cells, we used the TruSeq RNA sample prep kit v2 (Illumina, San Diego, Ca, USA) and performed sequencing in a NextSeq 500 Desktop Sequencer (Illumina) with the NextSeq 500/550 Mid Output v2 kit, 150 cycles (Illumina). The sequenced reads were aligned to the mm10 reference mouse genome using TopHat 2.0.13. For statistical analysis, differential gene expression, and visualization of the RNA sequencing data, we used Qlucore Omics Explorer 3.0 (Qlucore, Lund, Sweden). Gene set enrichment analysis (GSEA)[3] was performed with pre-ranked gene lists, based on the GSEA guidelines for RNA sequencing data, followed by pairwise t-tests for comparisons between groups.

Figure S1

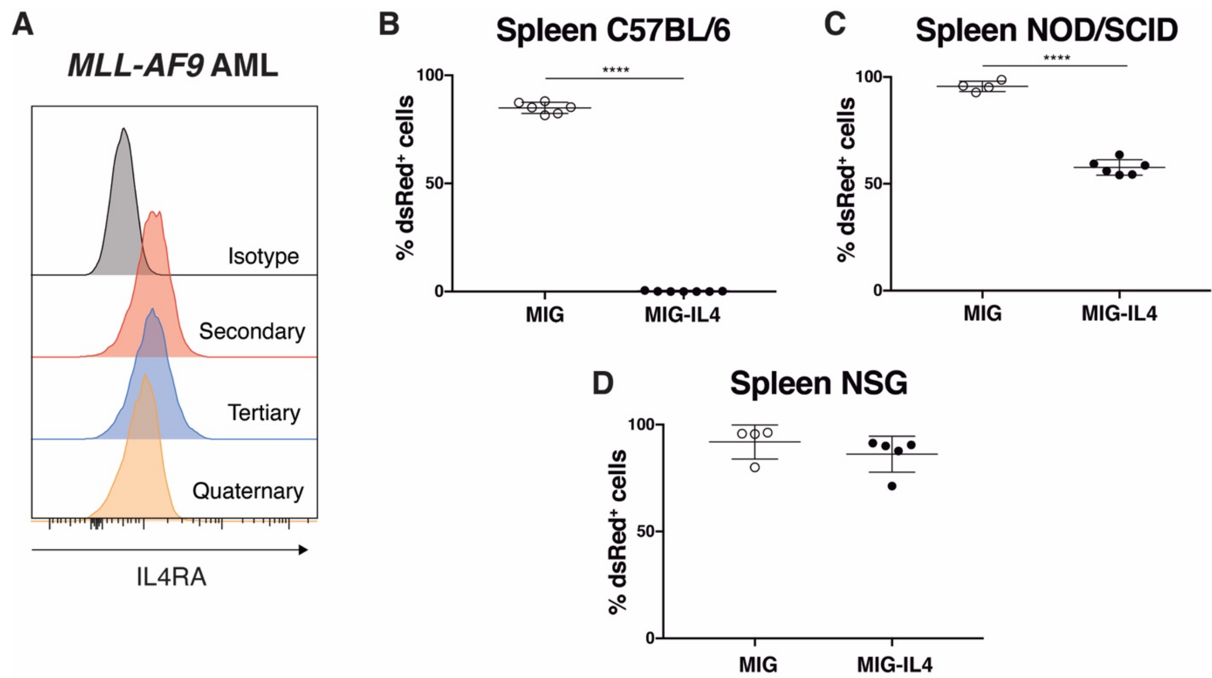

Figure S1. IL4 has antileukemic activity in a microenvironment-dependent manner. (A) Expression of IL4RA on dsRed$^+$ c-Kit$^+$ *MLL–AF9* AML from serial propagations. **(B–D)** Percentage of leukemia (dsRed$^+$) cells in the spleens of mice at the time of sacrifice. ****$P < 0.0001$.

Figure S2

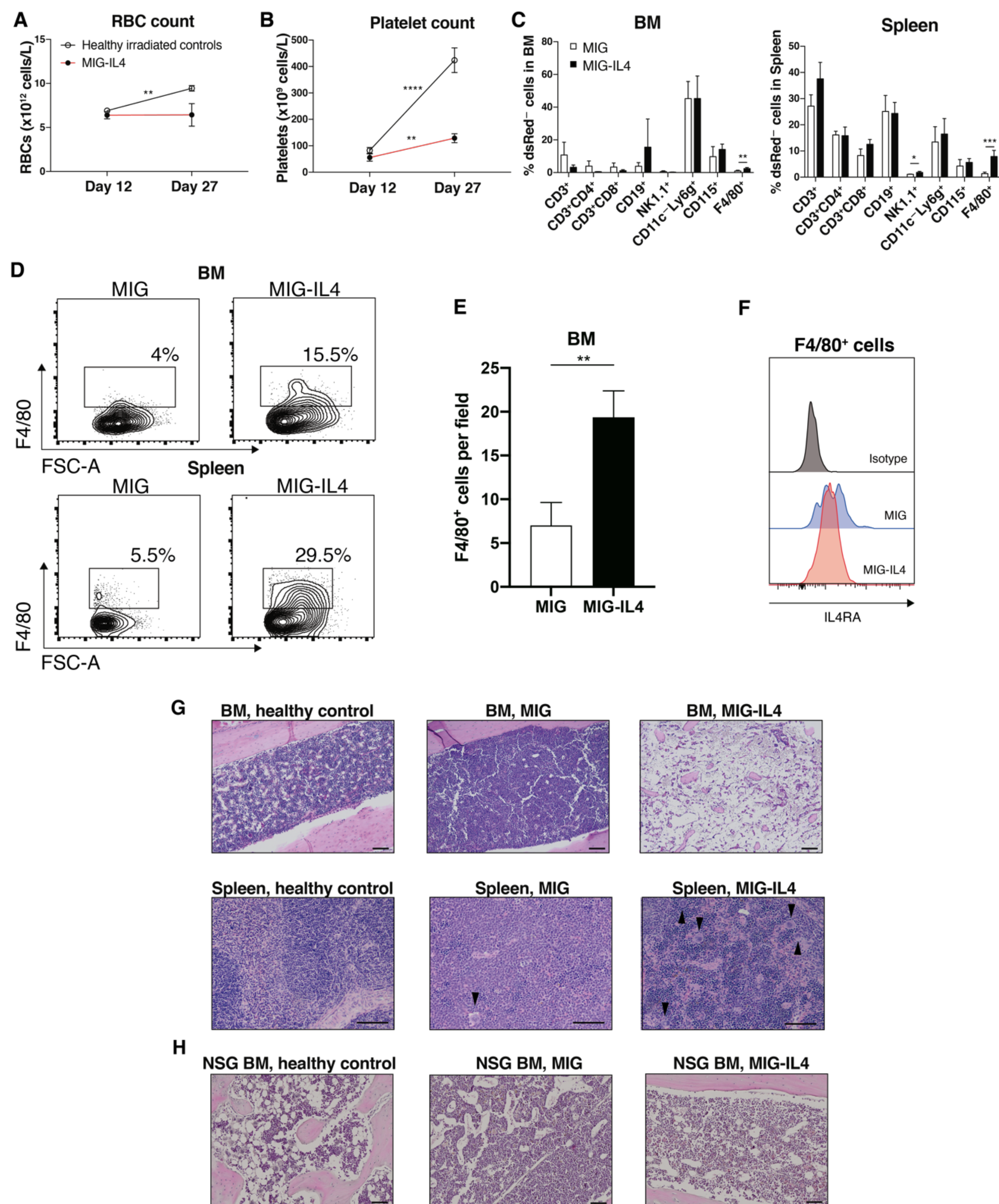

Figure S2. IL4 stimulation *in vivo* induces expansion of macrophages. (A–B) RBC and platelet count at days 12 and 27 for MIG–IL4 and non-transplanted irradiated mice as controls (controls, n = 3; MIG–IL4, n = 4). **(C)** Percentage of BM and spleen cell populations within the non-dsRed fraction at the time of sacrifice. **(D)** Representative contour plots of F4/80$^+$ cells in BM and spleen. **(E)** Count of F4/80$^+$ cells on IHC slides of BM per 20x magnification field (n = 3). **(F)** Expression of IL4RA on F4/80$^+$ cells harvested from mice at the time of sacrifice. (**G**) Representative hematoxylin and eosin (H&E) staining of BM and spleens (10 ×; scale bar, 100 μm). Arrowheads indicate megakaryocytes. (**H**) Representative H&E staining of BM from

NSG mice (10 ×; scale bar, 100 μm). BM, bone marrow; RBC, red blood cell. *$P < 0.05$, **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

Figure S3

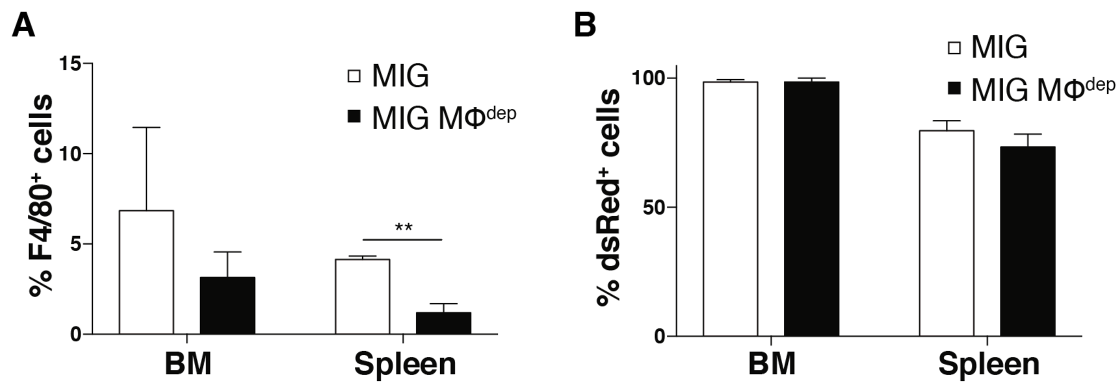

Figure S3. Depletion of macrophages *in vivo* did not affect leukemia levels in mice receiving non–IL4-expressing leukemia cells. C57BL/6 mice were transplanted with 30 000 sorted GFP$^+$ *MLL–AF9* AML cells transduced with the MIG control vector. One day prior to transplantation, mice received intraperitoneal (i.p.) injections of clodronate liposomes (MΦ^{dep} group) or PBS as control. Every tenth day, new i.p. injections were performed. **(A)** F4/80$^+$ cells at the time of sacrifice in BM and spleens of mice receiving clodronate liposomes. **(B)** Percentage of leukemia cells in BM and spleen of mice receiving clodronate liposomes. MΦ, macrophage. $^{**}P < 0.01$.

Figure S4

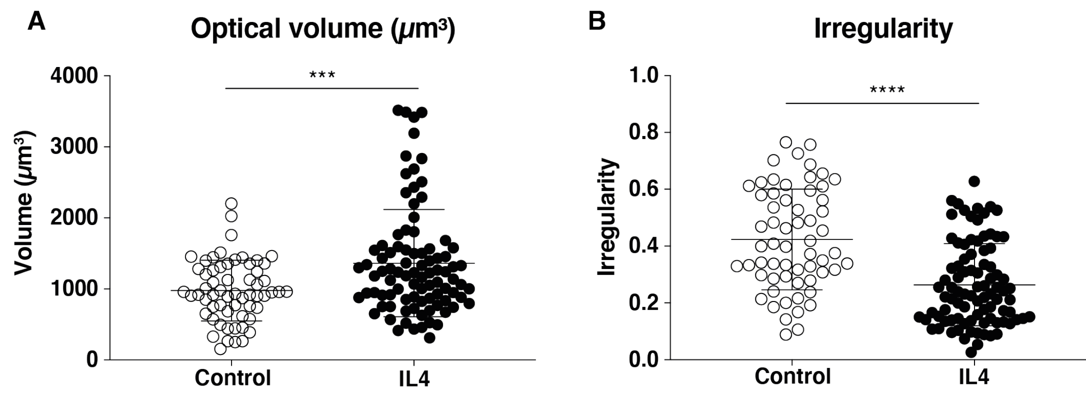

Figure S4. IL4 changes the morphology of macrophages. Monocytes were isolated from mouse BM and differentiated into macrophages in culture with mCSF1 (25 ng/mL) and mIL4 (20 ng/mL) or mCSF1 only (control) for 7 days and then analyzed using holograph imaging. **(A)** Cell volume and **(B)** irregularity of control (n = 60) and IL4-stimulated (n = 90) cells. ***$P < 0.001$; ****$P < 0.0001$.

Figure S5

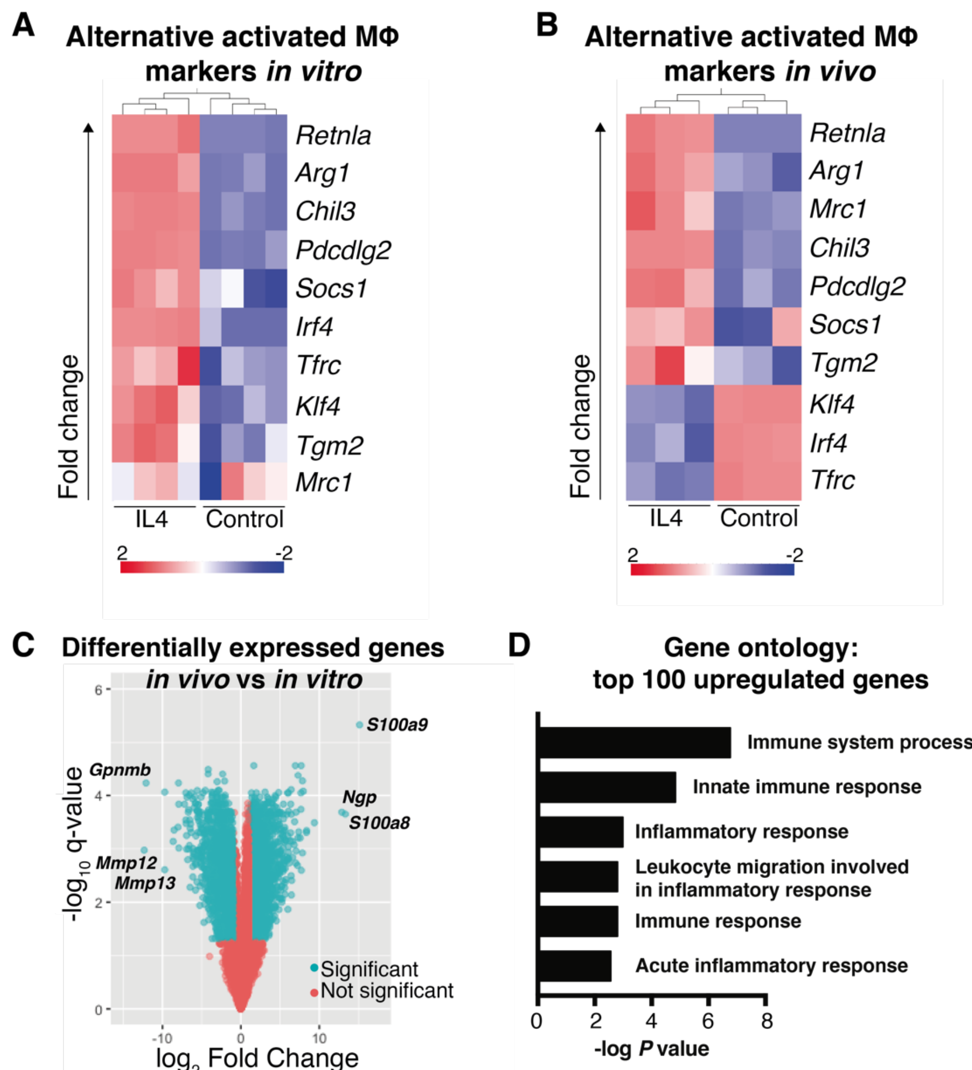

Figure S5. Gene expression of macrophages stimulated with IL4 *in vitro* versus *in vivo*. (A-B) Heatmaps showing expression of alternative activated macrophage–associated markers in IL4-stimulated macrophages and control macrophages *in vitro* and *in vivo*. **(C)** Volcano plot displaying differential gene expression between macrophages stimulated with IL4 *in vivo* versus *in vitro*. **(D)** Gene ontology enrichment associations based on the 100 most upregulated genes in IL4-stimulated macrophages *in vivo* versus *in vitro*.

SUPPLEMENTARY TABLES

Table S1. Differentially expressed genes in IL4 macrophages versus control macrophages generated *in vitro* (provided as Excel files).

Table S2. Differentially expressed genes in IL4 macrophages versus control macrophages generated *in vivo* (provided as Excel files).

Table S3. Differentially expressed genes in IL4 macrophages generated *in vivo* versus *in vitro* (provided as Excel files).

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