CELL INJURY, REPAIR, AGING, AND APOPTOSIS

Uptake of Neutrophil-Derived Ym1 Protein Distinguishes Wound Macrophages in the Absence of Interleukin-4 Signaling in Murine Wound Healing

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The determination of regenerative wound-healing macrophages as alternatively activated macrophages is currently questioned by the absence of IL-4 in wound tissue. Yet, murine wound tissue expressed high levels of Ym1 (chitinase 3-like 3), an established marker of the IL-4-induced alternatively activated macrophage phenotype. Ym1 was expressed in wound neutrophils but not in macrophages. Initially, Ym1-free wound-healing macrophages, invading from the wound margins, became gradually positive for the protein in the absence of IL-4 signaling and Stat6 activation, as they entered the neutrophil-populated wound regions. IL-4 failed to induce Ym1 protein in *ex vivo*-cultured wound tissue explants containing wound-healing macrophages. Recombinant Ym1 protein was selectively taken up by macrophages but not by keratinocytes and endothelial cells. Cultured macrophages lost the ability to take up the recombinant protein when four highly conserved residues and the 70-amino acid small α - β domain essential for Ym1 function were removed. The data suggest that the IL-4/Stat6-independent presence of Ym1 protein in wound-healing macrophages is of exogenous origin, with Ym1 taken up from wound neutrophils as the cellular source. The data suggest that *in situ* determination of wound-healing macrophages, often defined by Ym1, might not essentially describe an IL-4-dependent macrophage phenotype. Consequently, wound-healing macrophages should not be classified by the established categories of the well-accepted but simplified paradigm of M1/M2 macrophage activation. (*Am J Pathol* 2014, 184: 3249–3261; <http://dx.doi.org/10.1016/j.ajpath.2014.08.011>)

Starting in the 1970s and based on the groundbreaking work of Leibovich and colleagues¹ using a guinea pig model of incisional wound healing, the concept and functional implications of the wound-healing macrophage seemed to be intriguingly simple. Using glucocorticoids or an antimacrophage serum, the authors were able to show the pivotal role of the wound-healing macrophage for normal development of granulation tissue and removal of cellular debris from the site of injury.¹ However, it soon turned out that macrophages respond to different physiologic or pathologic conditions with remarkably expressed plasticity. Moreover, it is now evident that even circulating macrophage predecessors display diverse subpopulations of monocytes in the vasculature.² Two functional subpopulations have been described among murine blood monocytes, with differential properties to be recruited into inflamed or non-inflamed tissue.³ Early in tissue injury, inflammatory monocyte precursors specified as lymphocyte antigen (Ly)-6C⁽⁺⁾, C-C chemokine receptor 2⁽⁺⁾, and CX3C chemokine receptor 3⁽⁻⁾

rapidly infiltrate sites of inflammation.^{4,5} Maturation of these Ly-6C⁽⁺⁾ and C-C chemokine receptor 2⁽⁺⁾ monocytes into wound-healing macrophages adds expression of the mannose receptor and results in proinflammatory cytokine release (tumor necrosis factor α and IL-6) and phagocytosis.⁴ Later stages of wound tissue are then subsequently populated by wound-healing macrophages derived from Ly-6C⁽⁻⁾, C-C chemokine receptor 2⁽⁻⁾, and CX3C chemokine receptor 3⁽⁺⁾ infiltrating monocytes, which secrete factors to stimulate fibrosis (eg, transforming growth factor- β 1) and angiogenesis (eg, vascular endothelial growth factor).^{3,4,6}

The concept of classically activated proinflammatory macrophage and alternatively activated repair-type macrophage phenotypes that emerge during macrophage maturation

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under varying physiologic and pathologic conditions is now an integral part of the respective literature.⁷ However, the early description of wound-healing macrophages as an alternatively activated macrophage phenotype⁸ now seems to be an attempt to shoehorn repair-associated macrophages into the established pattern of classically or alternatively activated macrophage phenotypes. This concept is no longer supported because murine wound-healing macrophages obviously show traits of both classically and alternatively activated phenotypes in the absence of wound-derived IL-4 and IL-13, cytokines proposed to essentially induce the wound-healing phenotype in macrophages.^{2,4}

However, the presence of arginase-1 (Arg-1) and Ym1 (chitinase 3-like 3) proteins constitutes a reliable marker of alternatively activated macrophages in mice.^{7,9–11} The notion to regard wound-healing macrophages as a specialized repair phenotype of an alternative macrophage activation, capable of controlling inflammation and stimulating regeneration, might be derived from the observation that murine wound-healing macrophages also express Arg-1 and Ym1 proteins.^{4,12} Yet, this approach seems to simplify macrophage activation in wounds, which also contradicts the IL-4-/IL-13-dependent alternative phenotype in Th2 immune response to parasites,¹³ as wound tissue is devoid of IL-4, IL-13, and active Stat6.⁴

Use of the Ym1 protein as a marker for wound-healing macrophages might again add to this confusing situation. Besides its expression in alternatively activated macrophages,^{7,9–11} Ym1 is also present at much higher concentrations in peritoneal and bone marrow-derived neutrophils.¹⁴ Moreover, Ym1 contains a secretory signal peptide and is secreted in large amounts from the cells.¹⁵ Thus, the dynamic of Ym1 protein appearance obviously challenges its use as a marker to define a wound-healing macrophage phenotype. In accordance with this notion, a recent study on wound-healing macrophages used Ym1 to state that the early and inflammatory phase of repair promoted an alternative activation of macrophages at the wound site.¹²

However, herein we show that Ym1 is clearly associated with the appearance and localization of neutrophils in normal and impaired wound tissue. These data suggest that neutrophil-released Ym1 protein is specifically taken up by infiltrating wound-healing macrophages. Importantly, wound-healing macrophages amid the granulation tissue could not be induced to express Ym1 protein by IL-4 stimulation as a result of skin-specific suppression of this particular signaling pathway. Thus, the localization of Ym1 in wound-healing macrophages only mimics an alternative activation of the cells in the absence of an IL-4/Stat6 signaling network in wounds.

Materials and Methods

Reagents

Phenylmethylsulfonyl fluoride, dithiothreitol, aprotinin, sodium fluoride, sodium orthovanadate, and EDTA were from Sigma-Aldrich Chemie GmbH (Taufkirchen,

Germany). Leupeptin and ocaidaic acid were from BIO-TREND Chemikalien GmbH (Cologne, Germany). Deoxynucleoside triphosphate, random hexamers, and DNA restriction endonucleases were obtained from Roche Diagnostics GmbH (Mannheim, Germany). Taq- and Pfu polymerase were purchased from Promega GmbH (Mannheim, Germany). Purified recombinant mouse cytokines were from PeproTech Inc. (Tebu-bio, Offenbach, Germany).

Antibodies

Rat anti-Ly-6G and anti-Ly-6C [granulocyte antigen-1 (Gr-1)] (553123; BD Biosciences, Heidelberg, Germany), rat anti-mouse F4/80 (MCA497; AbD Serotec, Düsseldorf, Germany), rat anti-mouse neutrophils (Ly-6B2, clone7/4) (MCA771; AbD Serotec), rat anti-mouse CD16/CD32 (553142; BD Pharmingen, Heidelberg, Germany), phycoerythrin-conjugated rat anti-mouse Ly-6G (551461; BD Pharmingen), PerCP-Cy 5.5 rat anti-mouse Ly-6C (560525; BD Pharmingen), rabbit anti-Arg-1 (generated by the authors and raised against peptide H₂N-CEGNHKPETDYLKPPK-COOH), rabbit anti-Ym1 (01404; STEMCELL Technologies SARL, Cologne, Germany), rabbit anti-resistin-like molecule α (ab39626; Abcam, Quartett, Berlin, Germany), rabbit anti-cyclooxygenase-2 (Cayman 160126; via Biozol, Eching, Germany), rabbit anti-nitric oxide synthase-2 (sc-650; Santa Cruz Biotechnology, Heidelberg, Germany), rabbit monoclonal anti-Stat6 (YE361, Epitomics; via Biomol GmbH, Hamburg, Germany), rabbit anti-p-Stat6 (Y-641) (sc-11762-R; Santa Cruz Biotechnology), rabbit anti-c-Myc (C3956; Sigma-Aldrich Chemie GmbH), rabbit monoclonal anti-Flag (F2555; Sigma-Aldrich Chemie GmbH), mouse monoclonal anti-Flag (F3165; Sigma-Aldrich Chemie GmbH), mouse monoclonal anti- β -actin (A5441; Sigma-Aldrich Chemie GmbH), nonspecific rat IgG (Santa Cruz Biotechnology), fluorescein isothiocyanate-labeled goat anti-rabbit (11-4839; eBioscience, Frankfurt, Germany), fluorescein isothiocyanate-conjugated rabbit IgG isotype antibody (11-4614; eBioscience), and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (2275-Pc; Trevigen, Frankfurt, Germany).

Animals

Female C57Bl/6J (wild-type), leptin-deficient (C57Bl/6J-*obese/obese*), and Stat6^{-/-}C57Bl/6 (B6.129S2-Stat6^{tm1Gru/J}) mice were obtained from Charles River Laboratories (Sulzfeld, Germany). At age 10 weeks, the mice were caged individually, monitored for body weight, and wounded as described in the following subsection.

Wounding of Mice

Wounding of mice was performed as described previously.^{16,17} Briefly, mice were anesthetized. Subsequently, six full-thickness wounds (5 mm in diameter, 3 to 4 mm apart) were made on the backs of the mice by excising the skin

and the underlying panniculus carnosus. The wounds were allowed to form a scab. An area of 7 to 8 mm in diameter, which included the granulation tissue and the complete epithelial margins, was excised at the indicated time points for analysis. Back skin from nonwounded mice served as a control. Twelve wounds isolated from four animals were used for RNA analysis. For immunoblot analysis, eight wounds from four individual mice were used. All the animal experiments were performed according to the guidelines and approval of the local ethics animal review board (Regierungspräsidium Hessen, Darmstadt, Germany).

Treatment of Mice

To induce a systemic depletion of neutrophils (neutropenia), purified rat monoclonal anti-neutrophil antibody Ly-6G/Ly-6C (Gr-1)¹⁸ (1 mg/g body weight) was injected i.p. in 0.5 mL of phosphate-buffered saline (PBS) 24 hours before wounding into C57Bl/6J wild-type mice. A nonspecific rat IgG served as control. *ob/ob* mice were treated i.p. by PBS or leptin (2 µg/g body weight) during wound healing as described in detail previously.^{19,20}

Ex Vivo Analysis of Skin and Lung Tissue from Nonwounded and Wounded Mice

Skin and lung tissues were isolated under sterile conditions from nonwounded and wounded mice (3 days after injury). Pieces of tissue (3 × 3 × 3 mm) were subsequently cultured *ex vivo* in RPMI 1640 medium with or without IL-4 (10 ng/mL) supplementation for the indicated periods. The tissue samples were directly fixed in formalin (4.5% buffered in PBS) or zinc fixative (see later in the Immunohistochemical Analysis section) for immunohistochemical analysis or were snap frozen for RNA and protein preparation.

Thioglycollate-Elicited Peritoneal Macrophages

Mice were injected i.p. with a single injection of 0.5 mL of a sterile thioglycollate solution (8% w/v in H₂O) (Sigma-Aldrich Chemie GmbH). Five days after injection, mice were sacrificed and peritoneal cells were isolated by peritoneal lavage using RPMI 1640 medium. Erythrocytes were lysed in ACK buffer (150 mmol/L NH₄Cl, 10 mmol/L KHCO₃, and 0.1 mmol/L Na₂EDTA), and the remaining leukocytes were collected by centrifugation (250 × g). Cells (3 × 10⁶ per well) were seeded in 6-well plates using RPMI 1640 medium and were incubated for an additional 16 to 20 hours before stimulation.

Bone Marrow—Derived Macrophages

Isolated femora and tibiae from nonwounded and wounded mice (see *Wounding of Mice*) were flushed out using RPMI 1640 medium to obtain bone marrow cells for culturing. Flushed cells were collected in RPMI 1640 medium by

centrifugation (250 × g). After lysis of erythrocytes in 0.83% NH₄Cl, the remaining cells were analyzed by fluorescence-activated cell sorting (see *Flow Cytometry*) or cultured in Dulbecco's modified Eagle's medium/F12 medium (#11039; Gibco Life Technologies, Darmstadt, Germany) in the presence of macrophage colony-stimulating factor (1 ng/mL) at 3.5 × 10⁵ cells/cm² for 8 to 10 days to obtain adherent cell fractions for further studies.

Flow Cytometry

Isolated bone marrow cells from nonwounded and injured mice (see *Wounding of Mice*) were recovered in fluorescence-activated cell sorting staining buffer [2% fetal calf serum (v/v) and 0.1% Na₃N (w/v) in PBS]. Cells (2 × 10⁶) were blocked with rat anti-mouse CD16/CD32 and were stained for Ly-6G using phycoerythrin-conjugated rat anti-mouse Ly-6G as previously described.²⁰ Cells were fixed and permeabilized using the BD Cytfix/Cytoperm kit (554714; BD Biosciences). Rabbit anti-Ym1 and fluorescein isothiocyanate-labeled goat anti-rabbit were used to detect Ym1 protein. Intracellular staining was controlled using a fluorescein isothiocyanate-conjugated rabbit IgG isotype antibody (11-4614; eBioscience). Labeled cells were analyzed using a FACSCantoII flow cytometer (BD Biosciences).

Cell Culture

The murine RAW 264.7 macrophage cell line was cultured in RPMI 1640 medium. The murine brain endothelial cell (BND3) and the human keratinocyte cell line HaCaT²¹ were cultured in Dulbecco's modified Eagle's medium. Dulbecco's modified Eagle's medium, RPMI 1640 medium, penicillin/streptomycin, and 0.05% Trypsin-EDTA were purchased from Gibco Life Technologies. Fetal bovine serum was obtained from Biochrom (Berlin, Germany).

RNA Isolation and RNase Protection Analysis

RNA isolation and RNase protection assays were performed as described previously.^{17,22} If not indicated otherwise, every experimental time point depicts 12 wounds isolated from four individual mice for all RNase protection assays analyzing wound tissues samples. All the samples were quantified using phosphorimager PSL counts per 15 µg of total wound RNA. The murine cDNA probes were cloned using RT-PCR. The probes corresponded to nucleotides 1174 to 1441 [for Ym1 (chitinase 3-like 3); GenBank (<http://www.ncbi.nlm.nih.gov/genbank>; Accession number NM_009892.2)] or to nucleotides 163 to 317 [for murine *GAPDH*; GenBank (<http://www.ncbi.nlm.nih.gov/genbank>; Accession number NM_008084.2)] of the published sequences. For RNA protection assays, 1000 cpm of the hybridization probe was used as a size marker. tRNA hybridization showed the specificity of the probe. Hybridization against *GAPDH* was used as a loading control.

RT-PCR

RT-PCR was performed to assess the expression of *Ym1* in isolated peritoneal macrophages. One microgram of total RNA was transcribed using random hexameric primers and Moloney murine leukemia virus reverse transcriptase (Promega GmbH) according to the manufacturer's instructions. For *Ym1*, 5'-GATCTCAATATACACAGTGC-3' (forward) and 5'-GAGCTTAGCCAAAGCTGAC-3' (reverse) and for GAPDH, 5'-CTGGCATTGCTCTCAATGAC-3' (forward) and 5'-TCTTACTCCTTGGAGGCC-3' (reverse), primers were used to amplify the murine sequences. PCR was performed using a T3000 thermocycler (Biometra, Göttingen, Germany).

Real-Time Quantitative PCR

Real-time quantitative PCR (qPCR) was performed to assess the expression of genes of interest. TaqMan fast advanced master mix (cat. No. 4444557) and the predesigned qPCR assays were purchased from Applied Biosystems (Darmstadt, Germany): *Ym1* (chitinase 3-like 3) (Mm00657889-mH), resistin-like molecule α (Mm00445109-m1), Arg-1 (Mm00475988-m1), lipocalin-2 (Mm01324470-m1), EGF-like module—containing mucin-like hormone receptor-like 1 (Mm00802529-m1), or 4352339E for GAPDH. qPCR was performed using a 7500 fast real-time PCR system (Applied Biosystems, Heidelberg, Germany) as follows: 95°C (20 seconds), 40 cycles; 95°C (3 seconds) and 60°C (30 seconds). Analyses of qPCR runs were performed using Sequence Detector software.

Preparation of Protein Lysates and Western Blot Analysis

Skin, lung, and wound tissue biopsy samples or cultured cells were homogenized in lysis buffer [1% Triton X-100 (Roche Diagnostics GmbH), 20 mmol/L Tris/HCl (pH 8.0), 137 mmol/L NaCl, 10% glycerol, 1 mmol/L dithiothreitol, 5 mmol/L EDTA, 10 mmol/L sodium fluoride, 2 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 5 ng/mL aprotinin, 5 ng/mL leupeptin, and 50 nmol/L okadaic acid]. Extracts were cleared by centrifugation. Protein concentrations were determined using the BCA protein assay kit (Thermo Scientific, Cologne, Germany). Total protein (25 to 50 μ g) was analyzed by SDS gel electrophoresis. After transfer to a nitrocellulose membrane, specific proteins were detected using primary antibodies. A secondary antibody against goat, rabbit, or mouse IgG coupled with horseradish peroxidase (Bio-Rad Laboratories GmbH, Munich, Germany) and the enhanced chemiluminescence detection system (Amersham, Freiburg, Germany) were used to visualize the proteins.

Enzyme-Linked Immunosorbent Assay

Quantification of murine IL-4 was performed using the murine Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

Immunohistochemical Analysis

Skin biopsy samples were isolated from the back, fixed in formalin or in zinc fixative solution (0.05% calcium acetate.2H₂O, 0.5% zinc acetate.2H₂O, 0.5% zinc chloride in 0.1 mol/L Tris-Cl, pH 7.4), and embedded in paraffin. Four-micrometer sections were counterstained with hematoxylin and eosin. For immunostaining, sections were incubated overnight at 4°C with antibodies raised against mouse neutrophils (Ly-6B2), F4/80, or *Ym1*. Primary antibodies were detected using a biotinylated secondary antibody. The sections were subsequently stained with the avidin-biotin-peroxidase complex system (Santa Cruz Biotechnology) using 3,3'-diaminobenzidine-tetra-hydrochloride or Fast Red substrate-chromogen system (Dako, Hamburg, Germany) as a chromogenic substrate. Finally, sections were counterstained with hematoxylin and were mounted.

Immunofluorescence

RAW 264.7 macrophages were grown on glass slides and incubated with supernatants from pCMV-mmYm1F stably transfected human embryonic kidney (HEK) 293 cells for 24 hours. Supernatants from mock-transfected HEK 293 cells served as controls. RAW cells were fixed using 2% paraformaldehyde for 15 minutes and were subsequently blocked with 5% goat serum diluted in PBS/Triton (0.1% w/v). A rabbit monoclonal anti-Flag antibody (Sigma-Aldrich Chemie GmbH) was incubated for 1 hour at room temperature. The fluorescence-coupled secondary antibody (Alexa Fluor 488; Molecular Probes, Leiden, the Netherlands) was diluted (1:250) in 5% goat serum/PBS and incubated in the dark for 30 minutes. Nuclei were counterstained using DAPI (Sigma-Aldrich Chemie GmbH).

Expression and Purification of Recombinant *Ym1* Wild-Type and Mutated Proteins

A full-length mouse *Ym1* open reading frame was amplified from total day 1 wound RNA using RT-PCR. *Ym1* forward primer 5'-CGATGTCGACCCACCATGGCCAAGCTCATTCTTG-3' and reverse primer 5'-GCATGGATCCATAAGGGCCCTTGCAACTTG-3' were used. Amplicons were digested with SalI and BamHI and were cloned into XhoI/BamHI sites of pCMV-FLAG-N3.²³ The resulting pCMV-mmYm1F vector was used to express a recombinant Flag-tagged *Ym1* wild-type protein. The pCMV-mmYm1F vector was used as a template to generate a *Ym1*-Myc-tagged construct. 5'-GCATGGTACCGCTAGCCCACCATGGCCAAGCTCATTCTTG-3' and 5'-GAATGGTGACCATAAGGGCCCTTGCAACTTG-3' were used as primers. Amplicons were cloned via KpnI/BstEII sites into pcDNA3.1-myc-HisC (Invitrogen, Karlsruhe, Germany) to finally generate a *Ym1*-Myc-six histidine (6xHis)-tagged expression plasmid (pcYm1-Myc-6xHisC).

Mutation of specific amino acid residues in the *Ym1* sequence residues was performed using the pCMV-mmYm1F

Figure 1 Ym1 expression in wounds. **A:** Wound tissue (13 days) of PBS- or leptin-treated diabetic *ob/ob* mice was analyzed for Ym1 mRNA by RNase protection assay or immunoblot for Ym1 protein expression in mice as indicated. Values were obtained from 12 wounds (nine mRNA and three protein) isolated from three animals. **B:** Analysis of Ym1 mRNA expression by RNase protection assay from skin and wound tissue at different time points in wild-type or diabetic *ob/ob* mice as indicated. Values were obtained from three independent experimental series representing 36 total wounds isolated from nine animals. **C:** Expression of Ym1 protein in skin and wound tissue at different time points in wild-type or diabetic *ob/ob* mice as indicated. β -Actin was used as a loading control. Real-time quantitative PCR quantification of Ym1 (**D**), lipocalin (**E**), and EGF-like module-containing mucin-like hormone receptor-like (Emr) 1 (**F**) mRNA in skin (ctrl), or wound margin and wound granulation tissue in wild-type mice. The time points after injury are indicated. Values were obtained from 12 wounds isolated from four animals. **G:** Double staining of 1-day wound tissue for Ym1 mRNA (*in situ* hybridization, red signals) and neutrophil-specific Ly-6G/Gr-1 (immunohistochemical analysis, brown signals). The Ym1⁽⁺⁾ strand probe served as a negative control. **H:** Immunoblot showed Ym1 protein in the presence (IgG) or absence (Gr-1) of neutrophils in wound tissue at the indicated time points on injury. Ponceau S (PonS) staining was used to control equal loading. Data are given as means $\pm$ SD. ** $P < 0.01$ (Student's unpaired *t*-test) compared with PBS-treated mice (**A**). $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ (analysis of variance, Dunnett method) compared with nonwounded control skin (ctrl) (**B**). $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$ (Student's unpaired *t*-test) compared with wound margin (**D** and **E**). Scale bar = 50 μ m (**G**).

vector as a template (QuikChange site-directed mutagenesis kit; Stratagene, Heidelberg, Germany) and the following primers (underlined letters determine mutated or changed nucleotides): 5'-TAACTTTGATGGCCTCGCCCTGGCCTGGGGGTAC-CCTGGGTCTCG-3' and 5'-CGAGACCCAGGGTACCC-CCAGGCCAGGGCGAGGCCATCAAAGTTA-3' (for mutation of N136 to A, D138 to A, and Q140 to G) or 5'-CAGGTCATGACATATGCTCTCCATGATCCTAAG-3' and 5'-CTTAGGATCATGGAGAGCATATGTCATGACC-TG-3' (for mutation of D213 to A). The resulting plasmid is referred to as pCMV-Ym1-N^{*}D^{*}Q^{*}D^{*}-213F. Deletion of the small α + β domain²⁴ was performed via PCR-directed DNA depletion using 5'-CTTGAAGCTCCTGACATTGTCATA-TGCTGGAAATCCCACAATGAG-3' and 5'-CTCATTGT-GGGATTTCAGCATATGACAATGTCAGGAGCTTCA-AG-3' together with the Ym1 forward and reverse primers (see *RT-PCR*) on the pCMV-Ym1-N^{*}D^{*}Q^{*}D^{*}-213F plasmid. The mutated open reading frame was cloned into XhoI/BamHI

sites of pCMV-FLAG-N3²³ to generate pCMV-Ym1- Δ S-N^{*}D^{*}Q^{*}D^{*}-213F. The NheI/BamHI-excised Ym1- Δ S-N^{*}D^{*}Q^{*}D^{*}-213F open reading frame was then cloned to a 6xHis linker (5'-GATCCCACCATCACCATCACCATTGAT-3') and into the NheI/XbaI sites of the pcDNA3.1 vector (Invitrogen) to finally generate pc-Ym1- Δ S-N^{*}D^{*}Q^{*}D^{*}-213-6xHis.

Expression and Purification of Recombinant Ym1 Protein

The HEK 293 cell line was stably transfected with the plasmids pcYm1-Myc-6xHisC, pc-Ym1- Δ S-N^{*}D^{*}Q^{*}D^{*}-213-6xHis, and pCMV-mmYm1F. The respective cell lines secreted Myc-6xHis-tagged Ym1, Δ S-N^{*}D^{*}Q^{*}D^{*}-213-6xHis-tagged Ym1, and Ym1-Flag-tagged protein into the cell culture supernatants. 6xHis-tagged Ym1 proteins were purified using Ni-NTA agarose (Qiagen, Hilden, Germany). Potential endotoxin contaminations in purified protein samples were removed using an

Figure 2 Localization of Ym1⁽⁺⁾ wound-healing macrophages in wounds. **A:** Sections from 1-day wounds isolated from wild-type mice were single stained using antibodies directed against macrophage-specific F4/80 or neutrophil-specific Ly-6B2 (clone 7/4) (red signals) or double stained by the additional use of an anti-Ym1 antibody (brown signals) as indicated. **B:** Five-day wounds were double stained using macrophage-specific F4/80 (red) and anti-Ym1 (brown) antibodies. The **white boxes** indicate the localizations of the respective magnifications. Quantification of Ym1⁽⁺⁾ wound-healing macrophages in the outer (outside), middle (midsection), and innermost (inside) parts of 5-day (**C**) and 7-day (**D**) wound tissue from wild-type mice. Ym1⁽⁺⁾ cells are given as percentage of total F4/80⁽⁺⁾ cells. The analyzed areas were 250 μm^2 in size. Bars indicate means $\pm$ SD. Values were obtained from three wounds isolated from three animals. ****** $P < 0.01$ (Student's unpaired *t*-test).

immobilized polymyxin B Detoxi-Gel filtration endotoxin-removing column (Thermo Scientific). Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Scientific). The purity of each protein fraction was determined by SDS-PAGE and silver staining.

Densitometric Analysis of Immunoblots

Immunoblots were analyzed for positive signals using the density analysis Quantity One software version 4.6.8 (Bio-Rad Laboratories).

Statistical Analysis

Data are shown as means $\pm$ SD. Data analysis was performed using the unpaired Student's *t*-test or analysis of variance (the Dunnett method) with raw data and GraphPad Prism software version 5.02 (GraphPad Software Inc., San Diego, CA).

Results

Ym1 in Normal and Nonhealing Skin Wounds in Mice

We first determined expression of the IL-4-inducible Ym1 protein as a marker to highlight the presence or absence of alternatively activated wound-healing macrophages^{7–11} in highly disturbed wounds of diabetic obese/obese (*ob/ob*) mice.^{19,25} On wounding, we established different healing

conditions in *ob/ob* mice by injection (i.p.) of PBS or the adipokine leptin concomitant to healing. As previously reported,^{19,25,26} we found highly disturbed wounds in PBS-treated diabetic animals. The impaired wound tissues showed marked expression of Ym1 mRNA and protein (Figure 1A) expression. In contrast, healing of wound tissue in *ob/ob* mice occurred during leptin supplementation.^{19,25} Healing wounds in *ob/ob* mice were characterized by low Ym1 mRNA expression and the absence of Ym1 protein (Figure 1A). These findings suggest that the most-disturbed wound tissues expressed the highest levels of the wound-healing macrophage marker Ym1^{7,8}; Ym1 was reduced or even completely absent in healing tissue.

To track this conflicting finding, we next determined Ym1 expression throughout a normal and disturbed wound-healing process in healthy or diabetic *ob/ob* mice. Again, normal repair in wild-type mice showed the rapid but transient appearance of Ym1 mRNA (Figure 1B) and protein (Figure 1C) during the early and acute phase of healing. Consistent with results from late wounds (Figure 1A), impaired wound tissue revealed a markedly prolonged and elevated presence of Ym1 mRNA (Figure 1B) and protein (Figure 1C).

Ym1 Co-localizes with Neutrophils in Undisturbed Acute Wounds

Next, we separated isolated wound tissue into wound margin (outer part of the wound) and granulation tissue (central part of

the wound) as described previously.²⁷ We did so to localize Ym1 and immune cells in the respective wound compartments (Figure 1, D–F). The induction of Ym1 mRNA expression on injury (Figure 1B) was strongly restricted to the inner granulation tissue compartment (Figure 1D) and markedly coincided with the constitutively expressed neutrophil marker lipocalin²⁸ (Figure 1E) within the first 24 to 36 hours of repair. Macrophage infiltration, as assessed by EGF-like module–containing mucin-like hormone receptor-like-1 mRNA, the transcript of the macrophage-specific marker F4/80,^{29,30} occurred later and did not preferentially co-localize to Ym1 mRNA expression in inner wounds (Figure 1F). The qPCR–based colocalization of Ym1 and neutrophils in wounds (Figure 1, D and E) was supported by combined *in situ* hybridization (for Ym1 mRNA) and immunohistochemical analysis [for the neutrophil surface marker Ly-6B2 (clone 7/4)]. We found distinct expression of Ym1 mRNA in infiltrating neutrophils in the granulation tissue (Figure 1G). The observed neutrophil-coupled Ym1 expression in early wounds could be strengthened experimentally as complement-driven depletion of wound neutrophils by targeting the surface marker Gr-1 using a systemic injection of the anti–Ly-6G/Ly-6C (Gr-1) monoclonal antibody¹⁸ nearly completely abolished the strong presence of Ym1 protein in early wounds (Figure 1H).

Staining of 1-day wound sections for F4/80 (macrophage marker), Ly-6B2 (neutrophil marker), and Ym1 antigens delineated the differential spreading of infiltrating neutrophils and macrophages into early wounds and a co-appearance of Ym1 protein to those wound regions particularly packed by neutrophils (Figure 2A). The colocalization of Ym1 protein and wound neutrophils notably intensified during later 5-day wound stages of repair. The Ym1-specific signals concentrated in the wound center (Figure 2B). In contrast, the whole entity of wound-located macrophages, migrating from below and from the edges into the wound site, was devoid of Ym1 protein (Figure 2B). Despite the finding of Ym1-free wound margin macrophages, we found nearly all wound center macrophages to hold strong Ym1-positive signals (Figure 2, B–D). This pattern might suggest a Ym1-based gradient that might interact with or even organize the macrophage influx during healing. In addition, data from granulocyte mobilization from the bone marrow of wounded mice again indicated neutrophils as an important cellular source of Ym1. Fluorescence-activated cell sorting analysis of Ly-6G⁽⁺⁾ bone marrow cells in mice showed significant mobilization of Ym1⁽⁺⁾ neutrophils (defined by Ly-6G) on wounding (Figure 3).

Wound Ym1 Induction in the Absence of IL-4–Mediated Stat6 Activation

The gradual appearance of Ym1 in infiltrating wound-healing macrophages might be explained either as induction at the transcriptional level or possibly in conjunction with a gradual uptake of neutrophil-derived Ym1 at the wound site. Herein, we confirm data from Daley et al,⁴ who

reported the lack of IL-4 and Stat6 activation in murine wounds, as we could not detect IL-4 protein (Figure 4A) or Stat6 (Figure 4B) activation in mouse wounds. Moreover, Ym1, a central marker of alternatively activated wound-healing macrophages,¹² was independent of a Stat6-mediated induction in wounds, as Stat6-deficient mice also showed marked induction of Ym1 mRNA (Figure 4C) and protein (Figure 4D) after wounding. In contrast, peritoneal macrophages from wild-type mice were responsive to IL-4 to express (Figure 4E) and secrete (Figure 4F) Ym1. The insignificant role of IL-4 in wounds is further strengthened by the robust expression of Arg-1, an additional marker of the well-established IL-4–/Stat6-mediated macrophage polarization,^{10,11} in Stat6 knockout mice (Figure 5). Also, the found in inflammatory zone 1/resistin-like molecule α marker protein of IL-4–mediated alternative macrophage polarization⁹ was not induced at the wound site throughout the healing period (data not shown).

Wound Tissue Macrophages Do Not Express Endogenous Ym1 Protein

We next assessed the general responsiveness of wound tissue to an exogenous IL-4 stimulus. IL-4 and activated Stat6 were absent in wound tissue (Figure 4), but that did not exclude a basic responsiveness of wound tissue to IL-4. Ym1 protein could serve only as a regular marker for the alternative maturation wound-healing macrophages, when wound-located macrophages will respond to an exogenous IL-4 signal. *Ex vivo*–cultured wound tissue expressed moderate levels of Ym1 mRNA on IL-4 addition (Figure 6A). Lung tissue–induced Ym1 expression from nonwounded mice was more sensitive to exogenously added IL-4. The Ym1 mRNA induction in lung tissue was even enhanced in wounded mice,

Figure 3 Mobilization of Ym1⁽⁺⁾ bone marrow granulocytes on skin wounding. Bone marrow cells from nonwounded (ctrl) and wounded mice were isolated. Isolated cells were analyzed for Ym1⁽⁺⁾ bone marrow granulocytes by fluorescence-activated cell sorting using anti–Ly-6G and anti-Ym1 antibodies. The time points after injury are indicated. Ym1/Ly-6G–positive cells are given as percentage of total bone marrow cells. Bars indicate means $\pm$ SD. $n = 3$ animals. * $P < 0.05$, ** $P < 0.01$ (Student's unpaired *t*-test) compared with ctrl.

Figure 4 Ym1 expression is independent of IL-4/Stat6 signaling in wounds. **A:** ELISA analysis for IL-4 in lysates of skin (ctrl) and wound lysates isolated from wild-type mice at indicated time points. The line (det.lim.) demonstrates the detection limit of the assay. **B:** Immunoblots showing activated Stat6 (p-Stat6, Y641) and total Stat6 in skin (ctrl skin) and wounds from wild-type mice at indicated time points. IL-4-stimulated primary macrophages served as a positive control. Ponceau S (PonS) staining was used to control equal loading. **C:** Real-time quantitative PCR quantification of Ym1 mRNA in skin (ctrl) and during skin repair in wild-type (C57Bl/6) or Stat6-deficient (Stat6^{-/-}) mice at indicated time points. Values were obtained from 12 wounds isolated from four animals. **D:** Immunoblots for total Stat6 and Ym1 protein in skin (ctrl skin) and wounds from Stat6^{-/-} and C57Bl/6 mice at time points as indicated. PonS staining was used to control equal loading. **E:** RT-PCR analysis of Ym1 mRNA expression in isolated primary peritoneal macrophages. Macrophages were left unstimulated for 24 hours (ctrl) or were treated with IL-4 (10 ng/mL) or LPS (100 ng/mL)/Th1 (20 ng/mL of interferon γ, 25 ng/mL of tumor necrosis factor α) stimuli for 24 hours. Macrophages before stimulation served as an additional control (0-ctrl). **F:** Primary macrophages were stimulated with IL-4 or with IL-4 and Th1 or were left untreated (ctrl) for 24 hours. Fifty micrograms of total cellular protein lysate (left panel) or 25 µL of the respective conditioned cell culture supernatant (right panel) was analyzed for the expression of Ym1. Bars indicate means ± SD (A and B). n = 3 animals (A). *P < 0.05 (analysis of variance, Dunnett method) compared with ctrl (C).

suggesting a systemic effect of skin wounding on internal organs (Figure 6A). In addition, we controlled the general sensitivity of macrophages to IL-4 from the respective animals. Isolated peritoneal and bone marrow macrophages showed elevated Ym1 mRNA levels on IL-4 stimulation (Figure 6B). Nevertheless, endogenous Ym1 protein expression, which seems critical to the alternatively activated wound-healing macrophage paradigm, did not subsequently follow its observed mRNA patterns in IL-4-stimulated wound and lung tissue. We could not find Ym1⁽⁺⁾ wound-healing macrophages in an IL-4-supplemented wound environment (Figure 6C). In contrast, lung tissue macrophages from the same individual animal expressed Ym1 protein on IL-4 stimulation (Figure 6D).

Biochemical analysis confirmed the absence of Ym1 protein in *ex vivo*-cultured skin tissue (Figure 7A) in the presence of transient Stat6 activation (Figure 7C). Importantly, the strong Ym1 protein signals in cultured wound tissue (Figure 7B) were completely independent of IL-4-stimulated Stat6 activation in the respective tissue (Figure 7D). The Stat6-independent Ym1 protein in skin wounds (Figure 7B) might,

therefore, be allocated to the neutrophil subset in the cultured wound tissue. In accordance with the IL-4-induced Ym1⁽⁺⁾ macrophages in lung tissue (Figure 6D), Ym1 protein increased over time in IL-4-treated lung tissue from normal and injured mice (Figure 7, A and B) in the presence of persistent Stat6 activation (Figure 7, C and D).

Macrophages Take Up Ym1 Protein

This study suggested the presence of Ym1 protein in wound-healing macrophages independent of its endogenous induction by IL-4.^{9,11} To assess potential Ym1 uptake into macrophages, we first expressed a recombinant Flag-tagged Ym1 protein in transfected HEK cells from which the recombinant protein was secreted (Figure 8A). RAW 264.7 macrophages, but not HaCaT keratinocytes and BND3 endothelial cells, took up the Flag-tagged Ym1 protein from HEK supernatants (Figure 8, B and C). We excluded that the Flag-tagged Ym1 protein induced the expression of endogenous Ym1 in macrophages because primary and RAW 264.7 macrophages did not express endogenous Ym1 mRNA

Figure 5 Arg-1 expression is independent of IL-4/Stat6 signaling in wounds. **A:** Real-time quantitative PCR quantification of Arg-1 mRNA expression in skin (ctrl) and during healing in wild-type (C57BL/6) or Stat6-deficient (*Stat6*^{-/-}) mice at indicated time points. Values were obtained from 12 wounds isolated from four animals. Immunoblots for Arg-1 protein in skin (ctrl skin) and wounds from wild-type (C57BL/6) (**B**) or Stat6-deficient (*Stat6*^{-/-}) (**C**) mice at indicated time points. Ponceau S (PonS) staining was used to control equal loading. Bars indicate the means $\pm$ SD (**A**). **P* < 0.05 (analysis of variance, Dunnett method) compared with nonwounded control skin (ctrl) (**A**). BM, bone marrow.

in the presence of exogenously applied Flag-tagged Ym1 protein (Figure 8D).

To assess a specific uptake of Ym1 into macrophages, we mutated the Ym1 protein by replacement of essential amino acid residues (D136 to A, D138 to A, Q140 to A, and D213 to A) and by depletion of the small α + β domain (Δ S), all pivotal to three-dimensional structure and protein function.²⁴ Finally, we added an in-frame 1.2-kDa Myc-tag for protein detection and 6xHis for protein purification. The resulting Ym1-N^{*}D^{*}Q^{*}D^{*} Δ S-H6—mutated protein is shown in Figure 8E. A Myc-tagged wild-type Ym1 protein served as control. Notably, the purified mutant Ym1 protein was not taken up by primary macrophages, whereas the purified wild-type Ym1 protein was effectively assimilated by the cells (Figure 8F). Moreover, the absorbed wild-type Ym1 protein interfered with an exogenously triggered low-grade inflammatory response of the cells: the Myc-tagged Ym1 protein was associated with a marked down-regulation of inducible nitric oxide synthase and

cyclooxygenase-2 expression in response to low amounts of lipopolysaccharide (LPS) (Figure 8G).

Discussion

Since the first studies on macrophage function in wound healing,¹ it is now beyond question that macrophages are pivotally involved in the control of tissue repair. Depletion of macrophages from wounds generated important knowledge on macrophage function in the control of wound neutrophil infiltration, inflammation, epithelialization, vascularization, and contraction.^{1,12,20,31,32} A recent review on wound macrophages as key regulators of repair² now placed emphasis on the request to reexamine the phenotype of wound-healing macrophages and their role in tissue repair. This review was based on a report that had assessed the phenotype of wound-healing macrophages.⁴ This report questioned the well-accepted pattern of macrophage maturation

Figure 6 IL-4 does not induce Ym1 protein expression in wound macrophages. Wild-type mice were wounded. Wound and lung tissues were isolated from the animals 3 days after injury. In addition, lung tissue was isolated from nonwounded wild-type mice. The isolated tissues were cultured *ex vivo* in the absence or presence of 10 ng/mL of IL-4 for 24 hours. **A:** Real-time quantitative PCR quantification of Ym1 mRNA expression in *ex vivo* wound tissue and lung tissue from nonwounded and wounded mice on IL-4 stimulation. **B:** Isolated peritoneal and bone marrow macrophages were cultured for the indicated periods in the absence or presence of 10 ng/mL of IL-4. Values were obtained from *ex vivo* tissue or cells isolated from four animals. Three-day wound (**C**) or lung (**D**) tissue isolated from the same individual mouse was cultured *ex vivo* in the absence (w/o IL-4) or presence (+IL-4) of 10 ng/mL of IL-4 for 24 hours. Sections were double stained using the macrophage-specific F4/80 (red signals) and an anti-Ym1 antibody (brown signals). **Arrowheads** indicate Ym1-free wound macrophages (**C**) or Ym1-expressing lung macrophages (**D**). Bars indicate means $\pm$ SD. ***P* < 0.01 (Student's unpaired *t*-test) compared with nonstimulated cells (-).

Figure 7 IL-4 activates Stat6 in *ex vivo*—cultured skin, wound, and lung tissue. Skin and lung tissue was isolated from nonwounded wild-type mice (**A** and **C**). Skin wounds and lung tissue were isolated from the same individual mice on day 3 after injury (**B** and **D**). All the tissues were cultured *ex vivo* in the absence or presence of 10 ng/mL of IL-4 for the indicated periods. Skin and lung tissues from nonwounded mice were analyzed by immunoblot for Ym1 (**A**) or activated Stat6 (*p*-Stat6) (**C**). Skin wounds and lung tissue from wounded mice were analyzed by immunoblot for Ym1 (**B**) or *p*-Stat6 (**D**). Ponceau S (PonS) staining, β-actin, and GAPDH were used to control loading.

toward an alternatively activated wound-healing macrophage phenotype. Most important, considerable events known to drive an alternative maturation of macrophages could not be substantiated in a murine wound model: essentially, the presence of IL-4 and the subsequent activation of Stat6 in the cells.⁴ Based on the widely accepted notion of Ym1 as a marker of alternative activation of macrophages,^{7–9,11} Lucas et al¹² proposed such an alternative activation of wound-healing macrophages in acute wounds. However, as IL-4 is missing in acute murine wounds,⁴ one has to be cautious to oversimplify the easy presence of alternative macrophage markers (such as Ym1) to define wound-healing macrophage phenotypes. This notion is further supported by a most recent finding showing that simultaneous adenosine A_{2a} receptor and LPS-mediated Toll-like receptor stimulation was also capable of driving an alternative activation of cultured peritoneal macrophages. Again, that adenosine/LPS-mediated switch toward an alternative macrophage polarization was independent of IL-4 signaling and characterized by the absence of Ym1 and resistin-like molecule α.³³

In addition, impaired wound tissue has not yet been analyzed with respect to standard markers in macrophage maturation.^{7,8} Therefore, it was interesting to observe elevated levels of Ym1 mRNA and protein particularly in

nonhealing wound tissue, as Ym1 is currently evaluated and used to define an IL-4–triggered wound-healing macrophage phenotype with beneficial properties in wound healing.¹² Herein, we confirmed the absence of IL-4 and activation of Stat6 from acute wounds in mice.⁴ Thus, our finding revealed wound conditions with elevated Ym1 and Arg-1 levels in the absence of the inducing cytokine. This notion was further strengthened in Stat6-deficient mice.³⁴ The mice revealed normal expression of Ym1 and Arg-1 in wound tissue. That finding again implicates that these established markers of alternative macrophage activation^{10,11} do not functionally couple to IL-4/Stat6 signaling in wound healing.

These data suggest infiltrating neutrophils as the main cellular source of Ym1 protein in wounds. Early studies on Ym1 have shown prominent expression of the protein in F4/80⁽⁻⁾ myeloid precursor cells of hematopoietic tissues, particularly in fetal and adult liver, spleen, and bone marrow, indicating that the protein was predominantly expressed in nonmonocytic cells.³⁵ In accordance with this report, we first observed a significant increase in Ym1⁽⁺⁾/Ly-6G⁽⁺⁾ cells in the bone marrow of wounded mice, representing neutrophil precursors.³⁶ This is consistent with observations from a genetic mouse model of chronic granulomatous disease,

Figure 8 Uptake of Ym1 is restricted to macrophages and is dependent on protein structure. **A:** Analysis of conditioned media from mock- and Ym1-Flag-transfected HEK cells. IL-4-treated (10 ng/mL) primary peritoneal macrophages served as a positive control for Ym1 detection. **B:** RAW 264.7 macrophages, endothelial cells (BMD3), and keratinocytes (HaCaT) were incubated with conditioned medium from Ym1-Flag-transfected HEK cells for 24 hours for the indicated periods. Cellular lysates were analyzed by immunoblot for the intracellular presence of Ym1. Twenty-five microliters of conditioned medium from Ym1-Flag-transfected HEK cells (Ym1-Flag) served as a positive control. Ponceau S (PonS) staining was used to control loading. **C:** RAW 264.7 macrophages were incubated with conditioned medium from Ym1-Flag- or mock-transfected HEK cells for 24 hours. Ym1 protein uptake was assessed by immunofluorescence. Nuclei were stained with DAPI. **D:** RT-PCR analysis of Ym1 mRNA in primary peritoneal and RAW 264.7 macrophages. Primary macrophages were left unstimulated (ctrl) or were treated with 10 ng/mL of IL-4 for 24 hours as a positive control. Primary and RAW 264.7 macrophages were incubated with fresh medium [Dulbecco's modified Eagle's medium (DMEM)] or conditioned medium from mock- or Ym1-Flag-transfected HEK cells for 24 hours as indicated. GAPDH mRNA served to control equal loading. **E:** Scheme of the expression vector for recombinant Ym1 protein. The Ym1 open reading frame (ORF) is shown in red, mutated amino acid residues are marked by asterisks, and the positions of the deleted small α+β domain (ΔS) and the Myc and 6xHis tags are indicated. **F:** Primary peritoneal macrophages were incubated with purified Myc-tagged mutated Ym1 (Ym1-N¹³⁶D¹⁴⁰Q²¹³D²⁶⁷ΔS-H6) or Myc-tagged wild-type Ym1 (Ym1-Myc-H6) protein for 24 hours. Cells were washed intensively. Cellular lysates were analyzed by immunoblot for the intracellular presence of recombinant Ym1 using an anti-Myc antibody. Three experiments (1-3) are shown. β-Actin was used to control loading. **G:** Primary macrophages were stimulated with increasing concentrations of LPS in the absence or presence of purified recombinant Ym1-Myc protein for 24 hours as indicated. Cellular lysates were assessed for inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (Cox-2) expression by immunoblot. β-Actin was used to control loading. IP, immunoprecipitation; peMφ, peritoneal macrophages.

where peritoneal and bone marrow-derived neutrophils expressed Ym1 at concentrations one order of magnitude higher compared with macrophages at the assessed sites of inflammation.¹⁴

This observation was also true for skin wounds (this study). A skin wound is a well-known site of acute inflammation,³⁷ which is often prolonged and aggravated in disturbed, non-healing conditions.^{25,28} The acute phase of cutaneous wound inflammation is paralleled by the presence of the neutrophil infiltrate. Neutrophils disappear as soon as the wound inflammation is resolved but markedly persist during impaired conditions.^{25,28,31} The observed Ym1 expression kinetics in normal and late diabetes-disturbed wounds perfectly matched the presence of neutrophils, and we could allocate the wound-derived Ym1 mRNA and protein to infiltrating wound neutrophils but not to macrophages (this study). Here, it is

tempting to argue that only those infiltrating F4/80⁽⁺⁾ wound-healing macrophages became positive for Ym1 protein in wound tissue that had been in close spatial vicinity to the observed Ym1⁽⁺⁾ wound neutrophils. This phenomenon thereby generated a visible gradient of Ym1⁽⁺⁾ wound macrophages, which peak in numbers in the center of the wound, essentially where the neutrophils crowded together.

Thus, two scenes might provide explanations for the observed Ym1⁽⁺⁾ wound-healing macrophage gradient in the absence of an IL-4/Stat6 activation in wounds. First, wound macrophages might acquire Ym1 by engulfment of apoptotic neutrophils at the wound site.³⁸⁻⁴⁰ The selective uptake of a secreted Ym1 protein might represent the second alternative to explain Ym1⁽⁺⁾ wound-healing macrophages in the absence of IL-4 at wounded sites. A 21-amino acid signal peptide for secretion^{15,24} and the

capability of neutrophils to secrete Ym1¹⁴ might argue for this notion. The selective uptake of the recombinant Ym1 protein into cultured macrophages, but not endothelial and epithelial cells, supports this hypothesis. The subsequent mutation of amino acid residues N¹³⁶, D¹³⁸, Q¹⁴⁰, and D²¹³ and deletion of the small α + β domain (Δ S), necessary for a correctly folded and functional Ym1 protein,²⁴ abrogated the uptake of recombinant Ym1 protein into cultured macrophages and speaks in favor of a specific uptake of Ym1 protein into macrophages. Recombinant Ym1 reduced a low-grade inflammatory response from macrophages to LPS. Thus, Ym1 protein from exogenous neutrophil sources might, therefore, contribute to anti-inflammatory changes in wound-healing macrophages in response to wound neutrophils.^{38–40}

The presented data suggest that the activation of wound-healing macrophages and expression of the Ym1 marker protein were not functionally connected to the IL-4–/Stat6-dependent signaling pathway, despite its accepted role in alternative activation of macrophages. Thus, the paradigm of an alternative activation of regenerative wound-healing macrophages might not provide a solid basis to categorize a common repair phenotype of the wound-healing macrophage.

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