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Mouse neutrophils express the decoy type 2 interleukin-1 receptor (IL-1R2) constitutively and in acute inflammatory conditions

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ABSTRACT

The proinflammatory activities of IL-1 are tightly controlled at different levels. IL-1R2 acts as a decoy receptor and has been shown to regulate the biological effects of IL-1 in vitro and in vivo. However, little is known about its natural expression in the mouse in physiologic and pathologic conditions. In this study, we examined IL-1R2 mRNA and protein expression in isolated cells and tissues in response to different stimulatory conditions. Data obtained using ex vivo CD11b$^+$Ly6G$^+$ peripheral blood cells and in vitro-differentiated CD11b$^+$Ly6G$^+$ BMG indicated that neutrophils are the major source of constitutively expressed IL-1R2 in the mouse. The expression of IL-1R2 on BMG and ex vivo Ly6G$^+$ peripheral blood cells was highly up-regulated by HC. IL-1R2 pull-down experiments showed that mouse rIL-1β binds to BMG IL-1R2, whereas binding of IL-1Ra could not be detected. Furthermore, LPS treatment induced shedding of IL-1R2 from the neutrophil membrane in vitro and in vivo, executed mainly by ADAM17. Finally, in in vivo models of inflammation, including thioglycolate-induced acute peritonitis and acute lung injury, infiltrating Ly6G$^+$ neutrophils, expressed IL-1R2. Our data show that in the mouse, neutrophils mainly express the decoy receptor IL-1R2 under naïve and inflammatory conditions. These data suggest that neutrophils may contribute to the resolution of acute inflammation.
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Introduction

The IL-1 system comprises two agonists, IL-1α and IL-1β (IL-1); a specific receptor antagonist (IL-1Ra); and two receptors, IL-1R1 and IL-1R2 [1]. In contrast to the 80-kDa IL-1R1, the 68-kDa IL-1R2 has a short 29-aa cytoplasmic terminus lacking the Toll/IL-1R domain and is therefore incapable of signaling [2]. IL-1R2 thus acts as a decoy receptor that regulates the proinflammatory activities of IL-1 by two different mechanisms [3]. First, it acts by competitively binding to IL-1 and thus, preventing its interaction with IL-1R1 and second, by sequestering IL-1R accessory protein and inhibiting the formation of a heterodimeric signaling complex with IL-1R1 [4, 5]. In addition, IL-1R2 binds to IL-1 with a higher affinity than to IL-1Ra, thus further enhancing the inhibitory activity of IL-1Ra [6].

Studies using human cells or artificial systems demonstrated that anti-inflammatory molecules, such as glucocorticoids or IL-4 and IL-13, augment the surface expression of IL-1R2 [7, 8]. In contrast, proinflammatory molecules, such as LPS [9, 10], fMLP [11], or TNF-α [12], induce in vitro proteolytic cleavage of membrane-bound IL-1R2 by different proteases, such as TNF-α-converting enzyme/ADAM17 [13, 14] or the aminopeptidase regulator of TNFR1 shedding [15]. This results in the shedding of the extracellular portion of IL-1R2, which has a molecular weight of ~45 kDa. Thus, shed IL-1R2 could buffer IL-1, leaking into the systemic circulation from the sites of inflammation, and dampen IL-1-mediated immune responses. Elevated levels of soluble IL-1R2 are also found in vivo in the circulation of sepsis patients [9] and in the synovial fluid of patients with rheumatoid arthritis [16]. In humans, a second soluble form of IL-1R2 is generated by alternative splicing [17].

In contrast to the ubiquitously expressed IL-1R1, expression of IL-1R2 is rather restricted. Natural expression of IL-1R2 has been described in activated human T and B cells, activated

Abbreviations: 7-AAD=7-aminoactinomycin, ADAM=a disintegrin and metalloprotease, ADAM17$^{ex/ex}$= a disintegrin and metalloprotease 17 hypomorphic mouse, BMDC=bone marrow-derived DC, BMG=bone marrow-derived granulocyte, BMMϕ=bone marrow-derived macrophage, BMN=bone marrow neutrophil, GI=GI254023X, GMFI=geometric mean fluorescence intensity, GW=GW280264X, HC=hydrocortisone, icIL-1Ra1=intracellular IL-1R antagonist isoform 1, icIl1rn1=intracellular IL-1R antagonist isoform 1 protein, IL-1Ra=IL-1R antagonist, PNGase F=peptide:N-glycosidase F, qRT-PCR=quantitative real time-PCR, sIL-1Ra=secreted IL-1R antagonist, sIl1rn=sIL-1R antagonist protein, Treg=regulatory T cell

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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human keratinocytes [2, 18], human monocytes [19] and basophils [20], human PMN cells [7, 19, 21, 22], activated human natural Tregs [23, 24], and human T and B cell lines [2, 25, 26]. Although some studies reported the expression of IL-1R2 in mouse cells, such as BMDCs [27], $CD4^+$ T cells from senescent mice [28] or monocytes, stimulated by *Listeria monocytogenes* infection [29], the expression and regulation of IL-1R2 in the mouse, remain poorly described. Transgenic mice, overexpressing IL-1R2 in keratinocytes, are protected from local acute cutaneous vascular leakage and chronic PMA-induced inflammation but not from IL-1-induced systemic inflammation [30]. Furthermore, the engraftment of human IL-1R2-transfected cells in the back of mice inhibits the development of collagen-induced arthritis [31]. As a result of its supposed anti-inflammatory function, IL-1R2 might be a new target for effective therapeutic intervention in IL-1-mediated inflammatory diseases. However, to design future animal experiments addressing the role of naturally expressed IL-1R2 in vivo, it is indispensable to investigate thoroughly the natural expression and regulation of the decoy receptor in mouse cells and tissue.

Herein, our results show that in the mouse, IL-1R2 is expressed mainly by neutrophils as a highly glycosylated protein. Its surface expression is augmented by the anti-inflammatory molecule HC, whereas LPS induces shedding from the surface in vitro and in vivo. In addition, we show that IL-1β binds to IL-1R2, whereas binding to IL-1Ra is not observed. By using inflammatory mouse models of peritonitis and acute lung injury, which involve an early recruitment of neutrophils to the site of inflammation, we present the expression of IL-1R2 on the surface of neutrophils in vivo. These results show for the first time the natural expression of IL-1R2 on neutrophils and its regulation in the mouse in vitro and in vivo.

MATERIALS AND METHODS

Mice

WT C57BL/6J mice were obtained from Janvier (Le Genest-St-Isle, France). $ADAM17^{ex/ex}$ mice, on the C57BL/6N background, were described previously [32]. WT littermates were used as controls. All mice were maintained under conventional conditions in the animal facility of the Geneva University School of Medicine, and water and food were provided ad libitum. Animal studies were approved by the Geneva cantonal authority for animal experimentation and were performed according to the appropriate codes of practice.

Biological reagents

Media used for mouse primary cell isolation and culture were obtained from Invitrogen Life Technologies (Basel, Switzerland). Mouse rGM-CSF was purchased from Immuno Tools (Friesoythe, Germany), mouse rG-CSF and mouse rIL-1β were purchased from Peprotech (London, UK), and mouse rIL-1Ra was purchased from R&D Systems (Abingdon, UK). Con A was purchased from Amersham Pharmacia Biotech (GE Healthcare, Little Chalfont, Buckinghamshire, UK), purified LPS (*Escherichia coli* serotype O55:B5) from Fluka (Buchs, Switzerland), HC for cell culture from Sigma-Aldrich (Buchs, Switzerland), and HC sodium succinate solution for in vivo injection (Solu-Cortef) from Pfizer (New York, NY, USA). The hydroxamate-based metalloprotease inhibitors GI (ADAM10-selective) and GW (ADAM10- and ADAM17-selective) [33] were synthesized by Iris Biotech (Marktredwitz, Germany).

T and B cell isolation and culture

Total $CD4^+$ and $CD8^+$ T cells and $CD19^+$ B cells were purified from spleen by negative or positive selection using immunomagnetic beads, according to the manufacturer's instructions ($CD4^+$ T Cell Isolation Kit II, $CD8a^+$ T Cell Isolation Kit II, CD19 MicroBeads; all purchased from Miltenyi Biotec, Bergisch Gladbach, Germany), and passed through a magnetic cell-sorting column (Miltenyi Biotec). The purity of total $CD4^+$, $CD8^+$ and $CD19^+$ cells was determined by extracellular FACS staining and was usually 75–85%. $CD4^+$ and $CD8^+$ T cells (5×10^5) were activated with plate-bound anti-mouse CD3 (1 μg/ml; BD PharMingen, Allschwil, Switzerland) and soluble anti-mouse CD28 (5 μg/ml; BD PharMingen) or Con A (5 μg/ml) in flat-bottom, 96-well plates for 24 h in RPMI, 10% FCS, 50 μM β-ME, penicillin/streptomycin. $CD19^+$ B cells (5×10^5) were activated with 1 μg/ml LPS and cultivated as described for T cells. Cells were collected, and RNA was isolated.

Generation, culture, and stimulation of BMG, BMDC, and BMMϕ

BMG, BMDC, and BMMϕ were obtained from 8- to 12-week-old mice. BMGs were cultured as described previously [34], except that HC was not added to the cell culture during generation. BMDC and BMMϕ were cultured as described previously [35]. Cells/well (1×10^6) were seeded into 24-well plates and cultured with or without 10 μM HC for 20 h unless specified otherwise. The percentage of $CD11b^+Ly6G^+$ cells (BMG), $CD11c^+CD11b^+$ cells (BMDC), or $CD11b^+$ cells (BMMϕ) was monitored by FACSCalibur and was usually ~65%, 75%, or 90%, respectively.

Morphologic analysis of BMG by cytospin

BMGs were washed with PBS, and 1×10^5 cells were centrifuged on microscope slides using Cytospin (Shandon Thermo Electron, Pittsburg, PA, USA). The cells were incubated with May-Grünwald (Sigma-Aldrich) for 2 min; the same volume of H_2O was added for a further 3 min. After rinsing the slides with H_2O, cells were stained with Giemsa (diluted 1:20 in H_2O; Fluka) for 20 min, washed, and embedded.

Phagocytosis assay

BMGs were seeded into a 24-well plate at 1×10^6 cells/well. The cells were loaded with the Latex Beads-Rabbit IgG-FITC Complex (Phagocytosis Assay Kit IgG FITC; Cayman Chemical, Ann Arbor, MI, USA) for 1 h, and the degree of phagocytosis was analyzed by flow cytometry.

Flow cytometry analysis

Fluorochrome-labeled mAb, specific for Gr1 (Clone RB6-8C5), Ly6G (Clone 1A8), CD11b (Clone M1/70.15), CD11c (Clone HL3), CD4 (Clone RM 4–5), CD8 (Clone 53-6.7), B220 (Clone RA3-6B2), IL-1R2 (Clone 4E2), and rat IgG2a as isotype control for IL-1R2, were obtained from Invitrogen Life Technologies, BD PharMingen, or eBioscience (Vienna, Austria). Cells were preincubated with a mAb specific for CD16/CD32 (Clone 2.4G2; BD PharMingen) to block FcγRs, diluted in washing buffer (PBS, 1% BSA, 1 mM EDTA). Fluorchrome-labeled mAb, diluted in washing buffer, were added. Labeled cells were fixed in PFA buffer (PBS, 0.2% BSA, 10 mM EDTA, 1% PFA), or 7-AAD (eBioscience), diluted in washing buffer, was added to living cells. Samples were run on a FACSCalibur, and data were analyzed using CellQuest software. Where peripheral blood cells were stained, blood was collected by cardiac puncture or by bleeding from the tail vein into PBS, 1% BSA, 1 mM EDTA to prevent clotting, and cell-surface staining was performed. Red blood cells were lysed by adding 155 mM NH_4Cl, 10 mM $KHCO_3$, 100 mM EDTA.

Western blot analyses

Total proteins were extracted from cells in lysis buffer [50 mM Tris/HCl, pH 8, 150 mM NaCl, 1% Triton X-100, 1× protease inhibitor cocktail (cOmplete, Mini, EDTA-free; Roche, Basel, Switzerland)] for 30 min on ice

and cleared by centrifugation. Samples were separated electrophoretically by a 4–12% gradient gel (NuPAGE; Invitrogen Life Technologies) and blotted onto PVDF membranes. Memberanes were blocked with 5% horse serum in TBS/0.05% Triton X-100 for 1 h at room temperature and then incubated with a goat anti-mouse IL-1R2 polyclonal antibody (R&D Systems) in TBS/0.05% Triton X-100 overnight at 4°C, unless otherwise specified. After washing with TBS/0.05% Triton X-100, the membranes were incubated with a donkey anti-goat IgG antibody conjugated to HRP (Santa Cruz Biotechnology, Heidelberg, Germany), followed by the development using a luminescent peroxidase substrate kit (Immun-Star WesternC kit; Bio-Rad, Cressier, Switzerland) and the detection of the signals by the imager LAS 4000 (Fujifilm Life Science). After stripping of the membrane with 50 μM Tris/HCl, 2% SDS, 100 mM β-ME for 30 min at 50°C and blocking with TBS/0.05% Triton X-100, 5% horse serum, the membrane was reprobed with a rabbit anti-human GAPDH polyclonal antibody (Santa Cruz Biotechnology).

Deglycosylation of IL-1R2

HC-treated BMGs were lysed in 100 mM Tris/HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA, 1% Nonidet P-40 for 30 min on ice and cleared by centrifugation. PNGase F (New England BioLabs, Ipswich, MA, USA) treatment was performed, according to the manufacturer's instructions.

Binding assay and coimmunoprecipitation

HC-activated BMGs (5×10^6) were incubated in 150 μl PBS with 10 μM HC and 400 ng mouse rIL-1β or mouse rIL-1Ra for 2 h at 4°C. The cross-linker solution, dithiobis[succinimidylpropionate] (Thermo Scientific, Rockford, IL, USA), diluted in DMSO, was added to the cells to a final concentration of 1 mM. After 30 min at room temperature, the cross-linking reaction was stopped by the addition of a final concentration of 20 mM Tris/HCl, pH 7.5. After 15 min at room temperature, the cells were harvested, washed three times with PBS, and lysed in nondenaturing lysis buffer (20 mM Tris/HCl, pH 8, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1×protease inhibitor cocktail) for 30 min on ice and cleared by centrifugation. Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) were loaded with goat anti-mouse IL-1R2 polyclonal antibody (R&D Systems) or goat IgG (Santa Cruz Biotechnology), used as an isotype control for 3 h at 4°C. This mixture was incubated with the lysate of BMG overnight at 4°C to immunoprecipitate IL-1R2. After washing, reducing sample buffer was added, and the beads were boiled for 5 min. The supernatant was loaded onto a SDS-PAGE, as described above. The membrane was blocked in TBS/0.05% Triton X-100, 5% horse serum, and probed with a goat anti-mouse IL-1β polyclonal antibody (detection antibody of IL-1β ELISA; R&D Systems) or a goat anti-mouse IL-1Ra polyclonal antibody (R&D Systems), followed by a HRP-conjugated donkey anti-goat IgG. After stripping of the membrane and blocking, as described above, the membrane was reprobed with a goat anti-mouse IL-1R2 polyclonal antibody (R&D Systems), followed by a HRP-conjugated donkey anti-goat IgG.

Shedding assay and immunoprecipitation

Shedding was induced in unstimulated or HC-treated BMGs by stimulation with 1 μg/ml LPS at 37°C for 1 h, unless specified otherwise. Where the metalloprotease inhibitors GI and GW were used, they were added at a concentration of 3 μM, 15 min before induction of shedding. Cells were collected and processed for RNA isolation or extracellular FACS staining, as described above, or lysed in 50 mM Tris/HCl, pH 8, 150 mM NaCl, 1% Triton X-100, 1× protease inhibitor cocktail for 30 min on ice and cleared by centrifugation.

For immunoprecipitation assays, horse serum-containing medium was replaced after 20 h of stimulation with HC by serum-free Opti-MEM (Invitrogen Life Technologies), supplemented with 5 ng/ml mouse rG-CSF and 10 μM HC, and shedding was induced with 1 μg/ml LPS at 37°C for 1 h. Cells were lysed in nondenaturing buffer. Immunoprecipitation with anti IL-1R2- or goat IgG-coated protein A/G PLUS-Agarose beads, preparation of the samples for SDS-PAGE, and the detection of IL-1R2 were performed as described above.

ELISA

IL-1α, IL-1β, and IL-1Ra protein levels in BMG cell lysates, lysed in 50 mM Tris/HCl, 150 mM NaCl, 1× protease inhibitor cocktail, were determined using ELISA DuoSet kits from R&D Systems.

RNA extraction and qRT-PCR

Total RNA was extracted from cell samples using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. Total RNA (500 ng) was treated with DNase RQ1 (Promega, Wallisellen, Switzerland) and then reverse-transcribed using SuperScript II RT (Invitrogen Life Technologies). *Il1r2*, *Il1r1*, *Il1a*, *Il1b*, *sIl1rn*, and *icIl1rn1* mRNA levels were examined by qRT-PCR using SYBR Green PCR Master Mix (Applied Biosystems, Zug, Switzerland). The primer sequences used were: *Il1r2*, forward primer 5′-GGTGCGGACAATGTTCATCTTG-3′, reverse primer 5′-GGGAACTGCTGGAGATCTCGGAGTG-3′; *Il1r1*, forward primer 5′-GAGTTACCCGAGGTCCAGTGG-3′, reverse primer 5′-GAGGGCTCAGGATAACAGG-3′; *Il1a*, forward primer 5′-GGGAAGATTCTGAAGAAGAG-3′, reverse primer 5′-GAGTAACAGGATATTTAGAGTCG-3′; *Il1b*, forward primer 5′-TGTGAAATGCCACCTTTTGA-3′, reverse primer 5′-GTGCTCATGTCCTCATCCTG-3′; *sIl1rn*, forward primer 5′-TTGCTGTGGCCTCGGGATGG-3′, reverse primer 5′-GACAGGCACAGCTTGCCCCC-3′; *icIl1rn1*, forward primer 5′-TCCACCCTGGGAAGGTCTGTG-3′, reverse primer 5′-GACAGGCACAGCTTGCCCCC-3′; *Gapdh*, forward primer 5′-AGGCCGAGAATGGGAAGCTTGT-3′, reverse primer 5′-TACTCAGCACCGGCCTCACCC-3′. PCR was performed with the SDS 2.2 system (Applied Biosystems). Gene expressions were calculated using the comparative method ($2^{-\Delta Ct}$) for relative quantification by normalization to *Gapdh* gene expression.

Immunohistochemistry

IL-1R2 expression was studied by immunohistochemistry on paraffin-embedded lung sections, as described previously [36], with slight modifications. Tissue sections were incubated with a goat anti-mouse IL-1R2 polyclonal antibody (R&D Systems) or rat anti-mouse Ly6G (Clone 1A8; BD PharMingen) or their appropriate isotype controls (goat IgG or rat IgG; Santa Cruz Biotechnology) for 2 h. The sections were rinsed and incubated with HRP-labeled donkey anti goat-IgG or goat anti-rat IgG (Santa Cruz Biotechnology) for 1 h, respectively.

Injection of HC or LPS into the peritoneum

Blood was taken from the tail vein before HC or LPS treatment. Twenty-four hours after the first bleeding, mice were injected i.p. with 125 mg HC sodium succinate/kg body weight or with 2 mg LPS/kg body weight and grouped into two groups, which were bled from the tail vein alternately at indicated time-points.

Thioglycolate-induced peritonitis

Mice were injected i.p. with 1 ml sterile 3% thioglycolate broth (Sigma-Aldrich). Four hours later, the mice were sacrificed, and the peritoneal cavity was flushed with 8 ml ice-cold PBS, 1.25 mM EDTA, to harvest cells.

Recruitment of neutrophils into the lung

LPS (200 μg), dissolved in 50 μl sterile 0.9% NaCl, was instilled intratracheally using a cannula into anesthetized mice. Sham mice were instilled intratracheally into anesthetized mice with 50 μl LPS-free, sterile 0.9% NaCl. Six hours after instillation, mice were intracardially perfused, and BAL was harvested by cannulating the trachea and infusing the lungs with cold 150 mM NaCl, 1× protease inhibitors. The lungs were dissected and fixed in 10% formalin and embedded in paraffin. Serial sections (3 μm) were stained with H&E, or immunohistochemistry was performed, as described above.

Statistical analysis

Significant variations were calculated using the unpaired or paired two-tailed Student's *t*-test or one-way ANOVA with Tukey's post-test for pairwise comparison when the ANOVA was statistically significant, as indicated in the figure legends. $P < 0.05$ was considered significant. Results are expressed as the mean ± SEM.

Online Supplemental material

The online Supplemental material contains Supplemental Figs. 1–4, together with their figure legends.

RESULTS

Mature neutrophils and their precursors are the main source of IL-1R2 in the mouse in vitro

To identify mouse cell types expressing IL-1R2, we first examined the expression of mouse IL-1R2 mRNA by qRT-PCR in different organs of naïve mice or 4 h after i.p. treatment with LPS. We observed that IL-1R2 mRNA was up-regulated significantly in lungs and the brain after LPS treatment (data not shown), as described already [37–39]. As i.p. administration of LPS leads to infiltration of neutrophils into the lung [40], we investigated the expression of IL-1R2 mRNA by qRT-PCR in in vitro-generated BMG in comparison with other immune cells. BMG expressed the highest levels of IL-1R2 mRNA (**Fig. 1A**, upper), whereas IL-1R1 mRNA levels were similar in all tested cells (Fig. 1A, lower). IL-1R2 expression has also been described in human M2 macrophages [41] and human natural Tregs [23, 24]. However, we could not detect IL-1R2 transcript or protein expression in mouse M2-polarized BMMϕ or in mouse natural Tregs (data not shown).

As the above data indicate that IL-1R2 mRNA is expressed mainly by BMG, we focused in our following experiments on this cell type. The protocol used for the in vitro generation of BMG leads to generation of a population of phagocytically active cells (Fig. 1B). To further characterize the BMG, cells were stained with antibodies against CD11b and the neutrophil-specific marker Ly6G and analyzed by flow cytometry. Most of the cells were $CD11b^+$, with ~65% also positive for Ly6G (Fig. 1C, gate G2). These findings are in line with the cytospin analysis, which showed that the majority of in vitro-generated BMG was mature neutrophils with typical lobed or ring-shaped nuclei (Fig. 1D, arrows), corresponding to $CD11b^+Ly6G^+$ cells. The other cells were neutrophilic precursors, metamyelocytes, corresponding to $CD11b^+Ly6G^{neg}$ cells (Fig. 1D, arrowhead). To investigate if the expression of high IL-1R2 transcript levels in BMG reflects the expression of IL-1R2 protein on the surface of BMG, cells were stained with an antibody against IL-1R2 and analyzed by flow cytometry. The whole BMG population stained positive for surface IL-1R2 protein (Fig. 1E, left; G1+G2). $CD11b^+Ly6G^{neg}$ cells (G1) express less IL-1R2 on

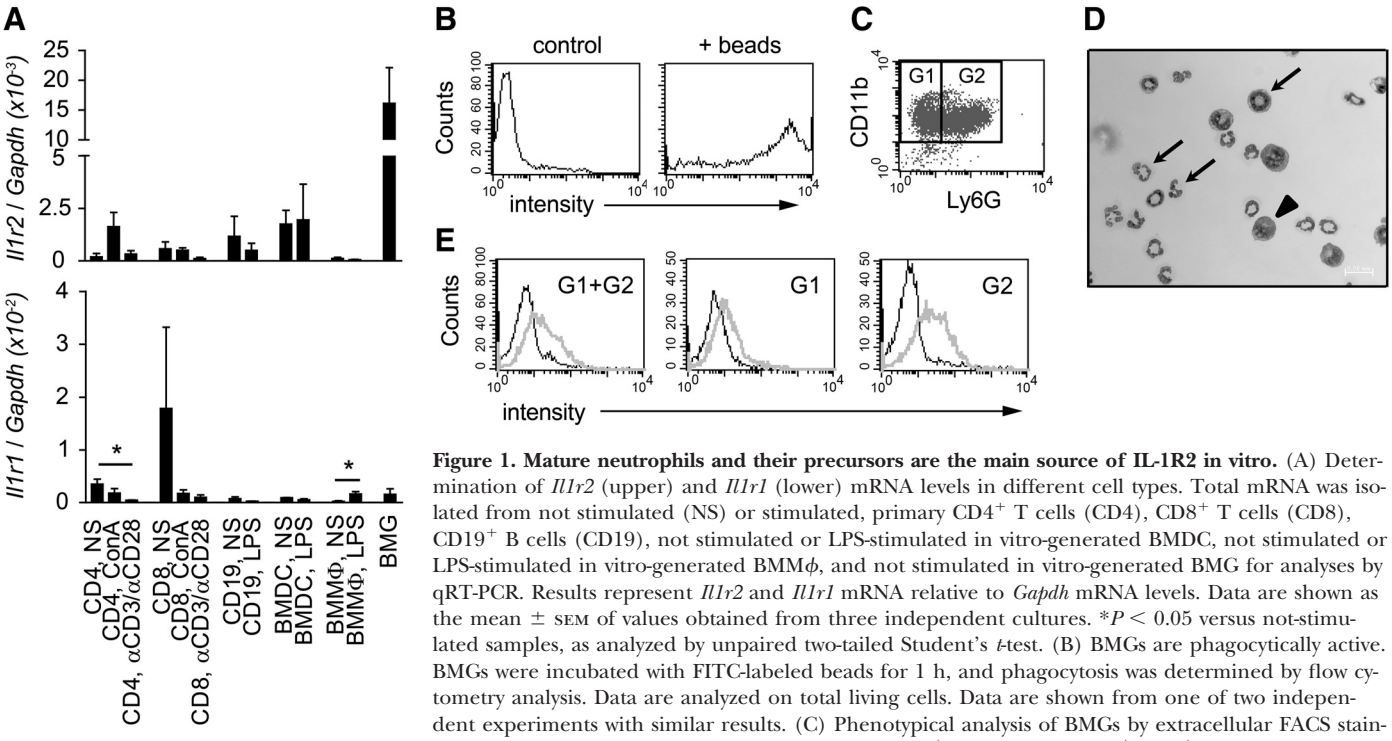

Figure 1. Mature neutrophils and their precursors are the main source of IL-1R2 in vitro. (A) Determination of *Il1r2* (upper) and *Il1r1* (lower) mRNA levels in different cell types. Total mRNA was isolated from not stimulated (NS) or stimulated, primary $CD4^+$ T cells (CD4), $CD8^+$ T cells (CD8), $CD19^+$ B cells (CD19), not stimulated or LPS-stimulated in vitro-generated BMDC, not stimulated or LPS-stimulated in vitro-generated BMMϕ, and not stimulated in vitro-generated BMG for analyses by qRT-PCR. Results represent *Il1r2* and *Il1r1* mRNA relative to *Gapdh* mRNA levels. Data are shown as the mean ± SEM of values obtained from three independent cultures. $*P < 0.05$ versus not-stimulated samples, as analyzed by unpaired two-tailed Student's *t*-test. (B) BMGs are phagocytically active. BMGs were incubated with FITC-labeled beads for 1 h, and phagocytosis was determined by flow cytometry analysis. Data are analyzed on total living cells. Data are shown from one of two independent experiments with similar results. (C) Phenotypical analysis of BMGs by extracellular FACS staining. BMGs were stained with mAb specific for CD11b and Ly6G. G1 and G2 correspond to $CD11b^+Ly6G^{neg}$ or $CD11b^+Ly6G^+$ BMGs, respectively. Data are analyzed on total living cells. Data are shown from one of three independent experiments with similar results. (D) BMGs were centrifuged on microscope slides and stained with May-Grünwald/Giemsa solutions. Arrows indicate mature PMN cells; arrowhead, neutrophil precursor. Data are shown from one of two independent experiments with similar results. (E) BMGs express IL-1R2 on their surface. BMGs were stained with mAb specific for CD11b and Ly6G as surface markers. IL-1R2 expression (gray lines) and the matched isotype control (black lines) were analyzed on the whole BMG population (G1+G2) or on $CD11b^+Ly6G^{neg}$ (G1) or $CD11b^+Ly6G^+$ (G2) cells. Data are analyzed on total living cells. Data are shown from one of three independent experiments with similar results.

their surface compared with mature $CD11b^+Ly6G^+$ neutrophils (G2). These data show that among in vitro-cultivated immune cells, BMGs express the highest levels of IL-1R2 transcripts and the protein on their surface. For the following experiment, the whole BMG population consisting of $CD11b^+Ly6G^{neg}$ and $CD11b^+Ly6G^+$ cells was used, if not specified otherwise.

HC up-regulates IL-1R2 expression on BMG

Glucocorticoids, such as dexamethasone or HC, up-regulate IL-1R2 transcripts and protein expression on human monocytes [42], human PMN cells [7, 22], and human B cells [43]. We wanted to investigate if this also applies to BMGs and other myeloid cells in the mouse. Mouse BMG, BMDC, and BMMϕ were stimulated with HC for 20 h. This resulted in a significant, tenfold increase of IL-1R2 transcripts in BMGs (**Fig. 2A**, upper), but no change in IL-1R1 mRNA levels (Fig. 2A, lower). The protein levels of IL-1R2 on the surface (Fig. 2B) or in cell lysates (Fig. 2C) of BMGs were strongly increased in HC-treated compared with unstimulated BMGs. A time-course analysis of IL-1R2 protein and *Il1r2* transcript expression in BMG demonstrates the fast up-regulation of IL-1R2 protein and transcript, which is stable over time, whereas *Il1r1* transcript levels are only altered marginally (Supplemental Fig. 1A and B). Although BMDC and BMMϕ also showed increased transcript levels for IL-1R2 upon HC treatment, expression remained much lower than in BMG (Fig. 2A, upper), and no protein could be detected by extracellular FACS staining (Fig. 2B). Of note, the stimulation of BMG with HC for 20 h induced a strong increase of IL-1R2 protein surface expression on $CD11b^+Ly6G^{neg}$ and $CD11b^+Ly6G^+$ cells (Supplemental Fig. 2A and B), meaning that the neutrophilic precursors are also able to up-regulate the decoy receptor. IL-1R2 protein in lysates of HC-treated BMG was detected at ~70 kDa by Western blot analysis (Fig. 2C). IL-1R2 protein levels in lysates of unstimulated BMGs were below the detection limits of this technique. The signal is a doublet of bands, which likely reflects different levels of glycosylation, as shown already for human IL-1R2 [44]. As *N*-glycosylation sites have been described for human and mouse IL-1R2 [8, 45, 46], lysates of BMG, pretreated with HC to increase IL-1R2 expression, were treated with PNGase F to remove *N*-linked sugars. This resulted in the loss of the 70-kDa bands and the detection of a new signal at ~55 kDa, corresponding to the expected size for this 410-aa protein (Fig. 2D).

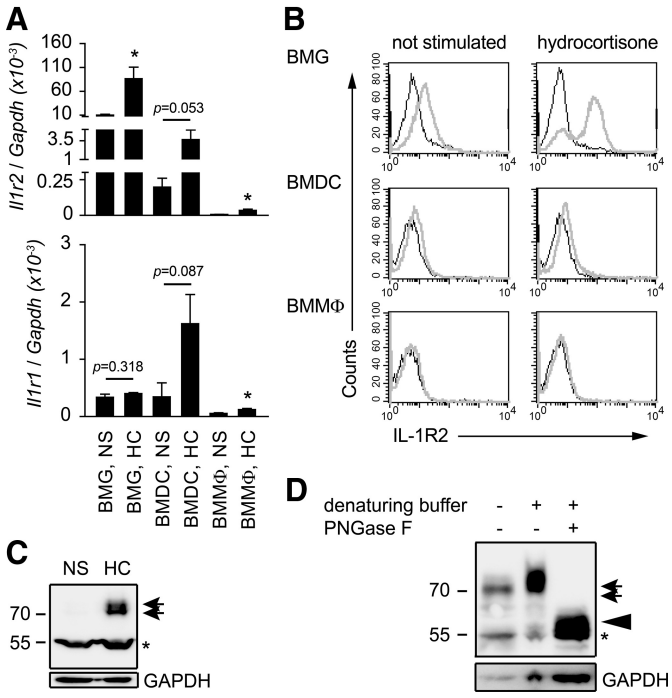

Figure 2. HC up-regulates IL-1R2 expression on BMG. (A) Determination of *Il1r2* (upper) and *Il1r1* (lower) mRNA levels in myeloid cells upon stimulation with HC. Total mRNA was isolated from not stimulated or HC-treated BMG, BMDC, and BMMϕ for analyses by qRT-PCR. Results represent *Il1r2* and *Il1r1* mRNA expression levels relative to *Gapdh* mRNA levels. Data are shown as the mean ± SEM of values obtained from three independent cultures. $*P < 0.05$ versus not-stimulated samples, as analyzed by unpaired two-tailed Student's *t*-test. (B) HC up-regulates the protein expression of IL-1R2 on neutrophils and their precursors but not on other myeloid cells. BMG, BMDC, and BMMϕ were left untreated or stimulated with HC and stained for IL-1R2 (gray lines) and the matched isotype control (black lines) by extracellular FACS staining. Data are analyzed on total living cells. Data are shown from one of three independent experiments with similar results. (C) HC up-regulates the expression of IL-1R2 in BMG. Western blot analysis of lysates of not stimulated and HC-treated BMGs (5×10^6 cell equivalents/lane) using anti-IL-1R2 polyclonal antibody and anti-GAPDH polyclonal antibody as loading control. Arrows indicate the specific signal for IL-1R2; *nonspecific band. Data are shown from one of three independent experiments with similar results. (D) IL-1R2 is a highly *N*-glycosylated protein. Western blot analysis of lysates of untreated or PNGase F-treated BMGs prestimulated with HC (2×10^6 cell equivalents/lane) using anti-IL-1R2 polyclonal antibody and anti-GAPDH polyclonal antibody as loading control. Arrows and arrowhead indicate the specific signal for glycosylated or *N*-deglycosylated IL-1R2, respectively. *Nonspecific band. Data are shown from one of three independent experiments with similar results.

IL-1β binds to IL-1R2, whereas binding of IL-1Ra is not observed

IL-1β binds with high affinity and specificity to IL-1R2; however, IL-1Ra binds poorly, if at all, to the decoy receptor [2]. **Figure 3** shows that IL-1β was coimmunoprecipitated together with IL-1R2 (Fig. 3A, lane 2), whereas IL-1Ra was not detected (Fig. 3B, lane 2). These results demonstrate that IL-1β binds to IL-1R2, naturally expressed on BMG, whereas binding of IL-1Ra could not be observed.

IL-1R2 is shed upon LPS stimulation in vitro and in vivo

IL-1R2 appears as a membrane-bound receptor, but it has been shown for human neutrophils and transfected cells that it can be shed from the plasma membrane and released into the supernatant in vitro upon treatment with different stimuli, such as LPS [9, 10], fMLP [11], IL-4 [7], or TNF-α [12]. Flow cytometric analysis demonstrated that LPS decreased IL-1R2

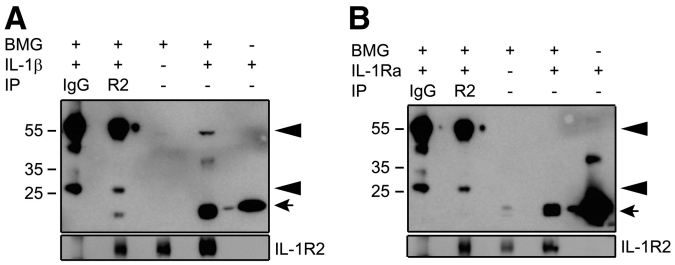

Figure 3. IL-1R2 expressed on BMG coprecipitates IL-1β but not IL-1Ra. Coimmunoprecipitation of HC-stimulated BMGs incubated with mouse rIL-1β (A) or mouse rIL-1Ra (B), respectively, using an anti-IL-1R2 pull-down antibody (R2) or the matched isotype control (IgG). Western blot analysis of BMG lysates (5×10^6 cell equivalents/lane) or 10 ng mouse rIL-1β (A) or mouse rIL-1Ra (B) using anti-IL-1β polyclonal antibody (A) or anti-IL-1Ra polyclonal antibody (B) and anti-IL-1R2 polyclonal antibody (A and B) as control for the efficiency of the immunoprecipitation (IP). Arrows indicate IL-1β (A) or IL-1Ra (B). Arrowheads indicate heavy (upper band)- or light (lower band)-chain of the pull-down antibody. Data are shown from one of two independent experiments with similar results.

cell-surface expression from unstimulated and HC-stimulated BMGs (**Fig. 4A**). Cellular IL-1R2 protein levels, analyzed by Western blot, were also diminished upon LPS treatment of HC-stimulated BMGs (Fig. 4B). fMLP or IL-4 induced neither shedding nor up-regulation (flow cytometry; data not shown).

We investigated further whether shed IL-1R2 could be found in the supernatant. Therefore, a polyclonal antibody to mouse IL-1R2 (R2) and the matched isotype control (IgG) were used for immunoprecipitation of IL-1R2 from cell lysates and conditioned media of HC-stimulated BMGs, without or with LPS treatment (Fig. 4C). A band of 70 kDa was immunoprecipitated from lysates by the IL-1R2-specific antibody from untreated BMG (Fig. 4C, lane 3) but not after LPS treatment (Fig. 4C, lane 5), as expected. In agreement with this observation, shed IL-1R2, slightly smaller than membrane-bound IL-1R2 (Fig. 4C, lane 9 vs. lane 3), could be immunoprecipitated with the antibody against IL-1R2 (R2) from LPS-conditioned supernatant. No soluble IL-1R2 was present in untreated control BMG supernatants.

We further investigated by flow cytometric analysis the surface expression of IL-1R2 over time after LPS treatment in HC-treated BMG (Fig. 4D, upper). One hour, 6 h, and 12 h after induction of shedding by addition of LPS, the GMFI of IL-1R2 expression on HC- and LPS-treated BMGs averaged 9.11 ± 0.60, 50.66 ± 2.03, and 71.08 ± 8.84, respectively, compared with IL-1R2 expression on BMGs treated with HC alone (GMFI of 112.0±5.29, 94.11±2.40, and 76.69±1.54, respectively). Twenty-four hours after LPS treatment, the GMFI of IL-1R2 even exceeded expression levels observed on BMG treated with HC only (160.8±21.16 vs. 85.60±5.81). However, *Il1r2* mRNA levels were altered only marginally by the addition of LPS to HC-treated BMG (Fig. 4D, lower). There was no effect of LPS on the expression of *Il1r1* transcripts (data not shown). In contrast to HC-treated BMG, unstimulated BMG did not re-express IL-1R2 protein on their surface after LPS-induced shedding (Supplemental Fig. 3A). Furthermore, LPS induced a significant decrease of *Il1r2* transcripts on unstimulated BMG (Supplemental Fig. 3B). The stimulation with LPS for more than 12 h led to massive cell death. Thus, IL-1R2 is re-expressed on the surface of HC-treated BMG upon LPS treatment in vitro. Under these conditions, shedding and re-expression of IL-1R2 protein are processes that take place independently of changes in steady-state *Il1r2* mRNA expression.

To study which protease is responsible for the proteolytic ectodomain shedding of membrane-bound IL-1R2, we analyzed shedding from BMGs isolated from ADAM17$^{ex/ex}$ mice [32] and their WT littermates. Recent in vitro studies with transfected cells identified human IL-1R2 as a new substrate of ADAM17 [13, 14], which is known to induce shedding of TNF-α, TNF-RI/II, IL-6R, L-selectin, and other membrane-bound proteins also [47]. Flow cytometric analysis demonstrated that LPS decreased IL-1R2 cell-surface expression from WT BMGs, whereas IL-1R2 shedding was clearly inhibited on BMG isolated from ADAM17$^{ex/ex}$ mice (Fig. 4E, upper). The ADAM10-selective inhibitor GI had no effect on the release of IL-1R2, whereas the ADAM10-/ADAM17-selective inhibitor GW inhibited shedding of IL-1R2 to a comparable extent, as shown with BMGs from ADAM17$^{ex/ex}$ mice (Fig. 4E, lower).

As all of these experiments were performed with in vitro-generated BMGs, which present a heterogeneous population consisting of neutrophilic precursors and mature neutrophils, we wanted to know whether mature neutrophils, freshly isolated ex vivo, also expressed IL-1R2. We therefore investigated the expression of the receptor on peripheral blood cells of naïve C57BL/6J mice by extracellular flow cytometry staining. Indeed, CD11b$^+$Ly6G$^+$ cells (G2) corresponding to mature neutrophils but not CD11b$^+$Ly6G^{neg} cells corresponding to monocytes (G1) expressed IL-1R2 on their surface (**Fig. 5A**). To investigate if HC and LPS have the same impact on IL-1R2 surface expression on neutrophils in vivo as seen in vitro, we monitored surface IL-1R2 expression on peripheral blood neutrophils following in vivo HC and LPS administration by flow cytometric analysis. Cell surface expression of IL-1R2 on Ly6G$^+$ neutrophils increased significantly over baseline at 12 h after HC injection (Fig. 5B). The GMFI of IL-1R2 averaged 8.865 ± 2.713 for six mice at baseline, 8.433 ± 1.104 at 2 h, 19.35 ± 1.617 at 6 h, 41.84 ± 2.479 at 12 h, and 19.72 ± 1.871 at 24 h after HC injection (Fig. 5B). Cell surface expression of IL-1R2 on Ly6G$^+$ neutrophils was decreased over baseline, 2 h after LPS injection, and increased over baseline at 6 h and 24 h after LPS injection (Fig. 5C). The GMFI of IL-1R2 averaged 5.717 ± 0.656 for the 12 mice at baseline, 0.922 ± 0.394 at 2 h, 9.958 ± 1.920 at 6 h, and 31.58 ± 7.837 at 24 h after LPS injection (Fig. 5C). To investigate if the impact of LPS on IL-1R2 was selective, we checked the expression of the neutrophil-specific marker Ly6G, a GPI-anchored protein. Indeed, several GPI-anchored substrates for ADAM17, such as CD14 [48] and metastasis-associated C4.4A [49], have been identified. Ly6G expression increased over baseline and peaked at 2 h after LPS injection and then declined to the baseline level (Supplemental Fig, 4A, left). Thus, LPS does not induce nonspecific shedding of cell surface proteins. Furthermore, LPS treatment led to an overall increase of Ly6G$^+$ neu-

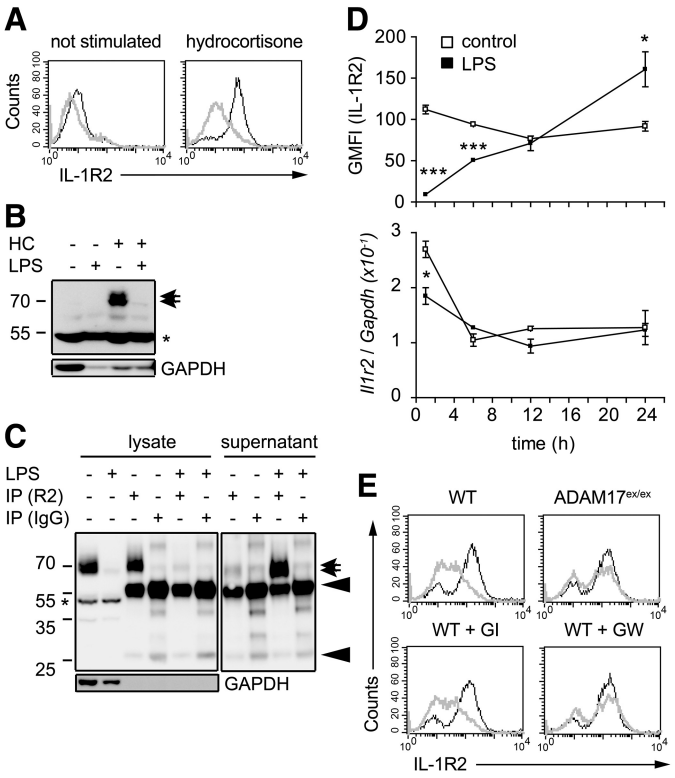

Figure 4. IL-1R2 is shed upon LPS stimulation in vitro. (A and B) BMGs were prestimulated with HC or left untreated before shedding was induced by the addition of LPS for 1 h. (A) Samples were stained for IL-1R2 by extracellular FACS staining. Black or gray lines show IL-1R2 expression, without or after LPS treatment, respectively. Data are analyzed on total living cells. Data are shown from one of three independent experiments with similar results. (B) Western blot analysis of BMG lysates (4×10^6 cell equivalents/lane) using anti-IL-1R2 polyclonal antibody and anti-GAPDH polyclonal antibody as a loading control. Arrows indicate the specific signal for IL-1R2. *Nonspecific band. Data are shown from one of three independent experiments with similar results. (C) Shed IL-1R2 is found in the supernatant. HC-treated BMGs were stimulated with LPS for 1 h or left untreated. IL-1R2 was immunoprecipitated in lysates or supernatants using an anti-IL-1R2 pull-down antibody (R2) or the matched isotype control (IgG). Western blot analysis of BMG lysates (lane 1+2; 5×10^6 cell equivalents/lane) or immunoprecipitated samples of lysates (5×10^6 cell equivalents/lane) and supernatant using anti-IL-1R2 polyclonal antibody and anti-GAPDH polyclonal antibody as loading control. Arrows indicate the specific signal for IL-1R2. Arrowheads indicate heavy (upper band)- or light (lower band)-chain of the pull-down antibody. *Nonspecific band. Data are shown from one of three independent experiments with similar results. (D) BMGs were prestimulated with HC for 20 h, and shedding was induced by the addition of LPS (■), or cells were left untreated (□). At indicated time-points, the surface IL-1R2 expression levels were assessed by flow cytometry using mAb for IL-1R2, and its matched isotype control (upper) or *Il1r2* transcripts were analyzed (lower). For protein expression analysis, GMFI was calculated by subtracting the GMFI of the isotype control-stained BMGs from the GMFI of the corresponding IL-1R2-stained BMGs. Data are shown as the mean ± SEM of values obtained from three independent cultures. Data are analyzed on 7-AAD^{neg} cells. $^*P < 0.05$; $^{***}P < 0.001$ versus untreated BMG samples, as analyzed by unpaired two-tailed Student's *t*-test. For transcript expression analysis, cells were collected, and total mRNA was isolated for analyses by qRT-PCR. Results represent *Il1r2* mRNA expression levels relative to *Gapdh* mRNA levels. Data are shown as the mean ± SEM of values obtained from three independent cultures. $^*P < 0.05$ versus control samples, as analyzed by unpaired two-tailed Student's *t*-test. (E) BMGs from WT or ADAM17$^{ex/ex}$ mice were prestimulated with HC for 20 h, and shedding was induced by the addition of LPS for 1 h. Metalloprotease inhibitors GI or GW were added 15 min before the induction of shedding. Samples were stained for IL-1R2 by extracellular FACS staining. Black or gray lines show IL-1R2 expression without or upon LPS treatment, respectively. Data are analyzed on total living cells. Data are shown from one of two independent cultures with similar results.

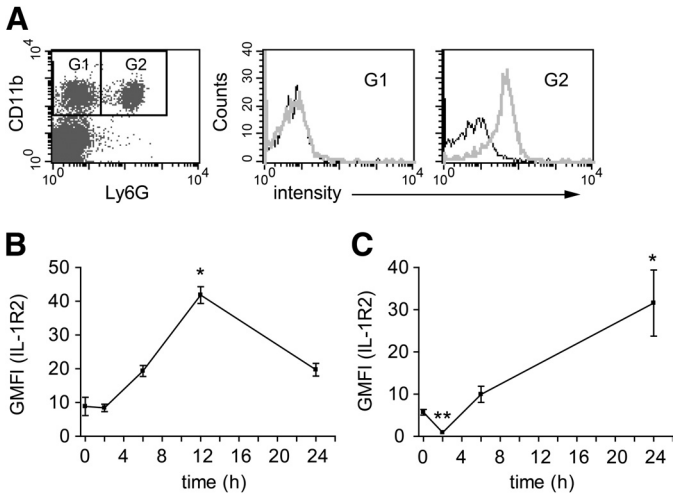

Figure 5. IL-1R2 surface expression is regulated by HC and LPS in vivo. (A) IL-1R2 is expressed by CD11b$^+$Ly6G$^+$ peripheral blood cells. Freshly isolated peripheral blood cells were stained by extracellular FACS staining with mAb specific for CD11b and Ly6G as surface markers for neutrophils (dot plot). IL-1R2 expression (gray lines) compared with its matched isotype control (black lines) was analyzed on CD11b$^+$Ly6G^{neg} (G1) and on CD11b$^+$Ly6G$^+$ (G2) cells (histogram plot). Data are analyzed on total living cells. Data are shown from one of three independent experiments with similar results. (B and C) IL-1R2 is up-regulated on or shed from the surface of neutrophils in vivo. HC sodium succinate (125 mg/kg body weight) (B) or LPS (2 mg/kg body weight) C) was injected i.p. into C57BL/6J mice (n=6, or n=12), respectively. At baseline and after indicated time-points postHC or LPS administration, blood was taken, and the surface IL-1R2 expression levels on Ly6G$^+$ cells were assessed by flow cytometry using mAb for IL-1R2 and its matched isotype control. GMFI for IL-1R2 was calculated by subtracting the GMFI of the isotype control-stained Ly6G$^+$ neutrophils from the GMFI of the corresponding IL-1R2-stained Ly6G$^+$ neutrophils. Data are shown as the mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$ versus baseline samples, as analyzed by paired two-tailed Student's *t*-test. Data are analyzed on total living cells.

trophils in the peripheral blood (Supplemental Fig. 4A, right). HC had only a marginal effect on the expression of Ly6G and on the percentage of Ly6G$^+$ neutrophils (Supplemental Fig. 4B).

These data demonstrate that IL-1R2 is not only expressed on in vitro-generated BMGs but also on freshly isolated ex vivo CD11b$^+$Ly6G$^+$ neutrophils from naïve mice. Furthermore, LPS induces shedding of naturally expressed IL-1R2 from the surface of BMG in vitro and peripheral blood neutrophils in

vivo, whereas HC up-regulates naturally expressed IL-1R2 on the surface of peripheral blood neutrophils in vivo.

Expression of anti-inflammatory IL-1R2 and of inflammatory IL-1 is differentially regulated in BMGs

We have demonstrated that HC up-regulates IL-1R2 mRNA (Fig. 2A) and protein (Fig. 2B and C) significantly on BMGs, whereas LPS induces IL-1R2 protein shedding from the surface (Fig. 4). To widen our understanding of the effects of LPS, HC, or HC followed by LPS stimulation on the regulation of the IL-1 system in BMGs, we analyzed transcript and protein levels of the proinflammatory cytokines IL-1α, IL-1β, and the antagonists sIL-1Ra and icIL-1Ra1 (**Fig. 6A**, transcripts, and B, proteins). LPS alone potently induced IL-1α and IL-1β mRNA levels (Fig. 6A), as well as IL-1α and IL-1β proteins (Fig. 6B). However, in BMGs pretreated with HC, the stimulatory effect of LPS on IL-1α and IL-1β protein levels was attenuated. In contrast to its stimulatory effect on IL-1R2 expression, HC alone was devoid of any effect on IL-1α and IL-1Ra mRNA and protein levels. Moreover, HC tended to suppress IL-1β mRNA levels in untreated BMGs. These data demonstrate that HC and LPS differently regulate the transcript and protein expression for IL-1R2, IL-1α, IL-1β, and IL-1Ra in BMGs.

IL-1R2 expression in vivo in pathological conditions

To investigate IL-1R2 expression in different pathological conditions, which involve the recruitment of neutrophils to the site of inflammation, thioglycolate-induced peritonitis and endotoxin-induced acute lung injury were used as in vivo models. The peritoneal exudate of thioglycolate-treated mice consisted of ~75% $Ly6G^+$ neutrophils (data not shown). IL-1R2 mRNA levels in peritoneal cells increased significantly after thioglycolate injection, whereas no significant change in IL-1R1 mRNA could be detected (**Fig. 7A**). The increase of IL-1R2 mRNA likely resulted from the massive influx of $Ly6G^+$ neutrophils into the peritoneal cavity, which also express IL-1R2 protein on their surface (Fig. 7A, histogram plot).

In the in vivo model of acute lung injury, LPS instillation caused a significant increase of IL-1R2 mRNA in BAL cells, whereas IL-1R1 transcript levels remained unchanged (Fig. 7B). $Ly6G^+$ neutrophils, present in the BAL, which was collected from LPS-instillated mice, showed IL-1R2 surface expression (Fig. 7B, histogram plot). Histological assessment of lung sections demonstrated that LPS instillation elicited infiltration of neutrophils into the lung parenchyma and alveolar space (Fig. 7C, H&E staining, upper). Immunohistochemistry, using antibodies against IL-1R2 and the neutrophil marker Ly6G, showed that neutrophils were stained positive with both markers (Fig. 7C, lower). These data demonstrate that membrane-bound IL-1R2 is found on neutrophils in vivo at the site of inflammation.

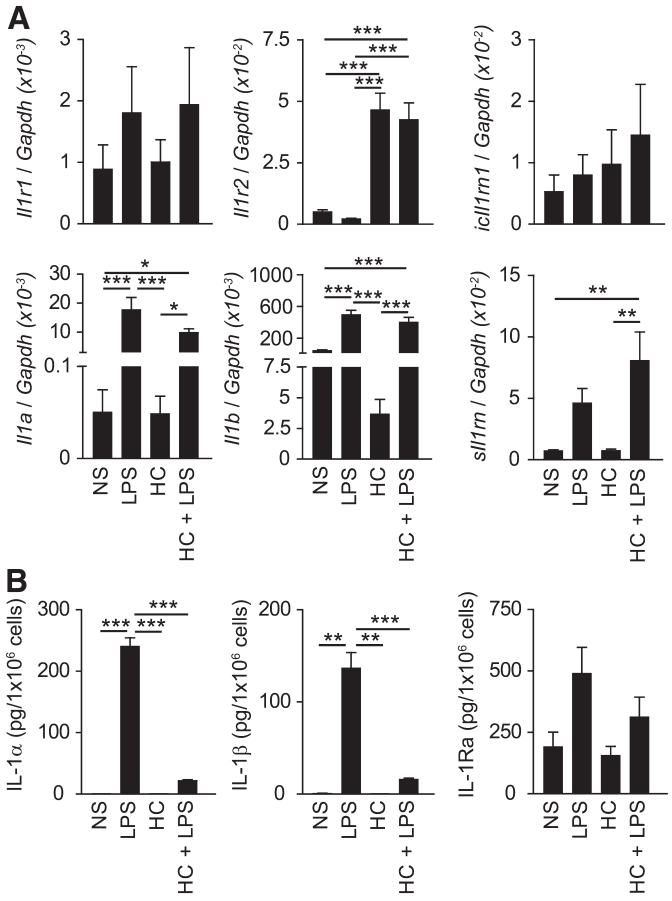

Figure 6. Effect of HC and LPS on IL-1 ligand and receptor expression in BMG. (A) Determination of *Il1r1* (upper left), *Il1r2* (upper middle), *icIlrn1* (upper right), *Il1a* (lower left), *Il1b* (lower middle), and *sIl1rn* (lower right) mRNA levels in BMGs, which were left untreated, treated for 1 h with LPS, for 20 h with HC, or for 20 h with HC and subsequently for 1 h with LPS. Total mRNA was isolated from not stimulated, LPS- or HC-treated, or HC- and LPS-treated BMN samples for analyses by qRT-PCR. Results represent *Il1r1*, *Il1r2*, *icIl1rn1*, *Il1a*, *Il1b*, and *sIl1rn* mRNA expression levels relative to *Gapdh* mRNA levels. Data are shown as the mean ± SEM of values obtained from five independent cultures. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, as analyzed by one-way ANOVA with Tukey's post-test for pairwise comparison when the ANOVA was statistically significant. (B) Determination of IL-1α, IL-1β, and IL-1Ra cytokine levels in lysates of BMG. BMG were left untreated, treated for 6 h with LPS, for 20 h with HC, or for 20 h with HC and subsequently for 6 h with LPS. Not stimulated, LPS- or HC-treated, or HC- and LPS-treated BMG samples were lysed, and cytokine levels were measured by ELISA. Data are shown as the mean ± SEM of values obtained from three independent cultures. $**P < 0.01$; $***P < 0.001$, as analyzed by one-way ANOVA with Tukey's post-test for pairwise comparison when the ANOVA was statistically significant.

DISCUSSION

The proinflammatory activities of IL-1 are tightly controlled at different levels. With the use of human cells or artificial mouse systems based on the overexpression of IL-1R2, such as cell lines or transfected cells, IL-1R2 has been shown to act as a decoy receptor and to regulate the biological effects of IL-1 in vitro and in vivo. However, only little is known about its natural expression and regulation in physiologic and pathologic conditions in the mouse. The results presented herein provide a comprehensive characterization of the natural expression of

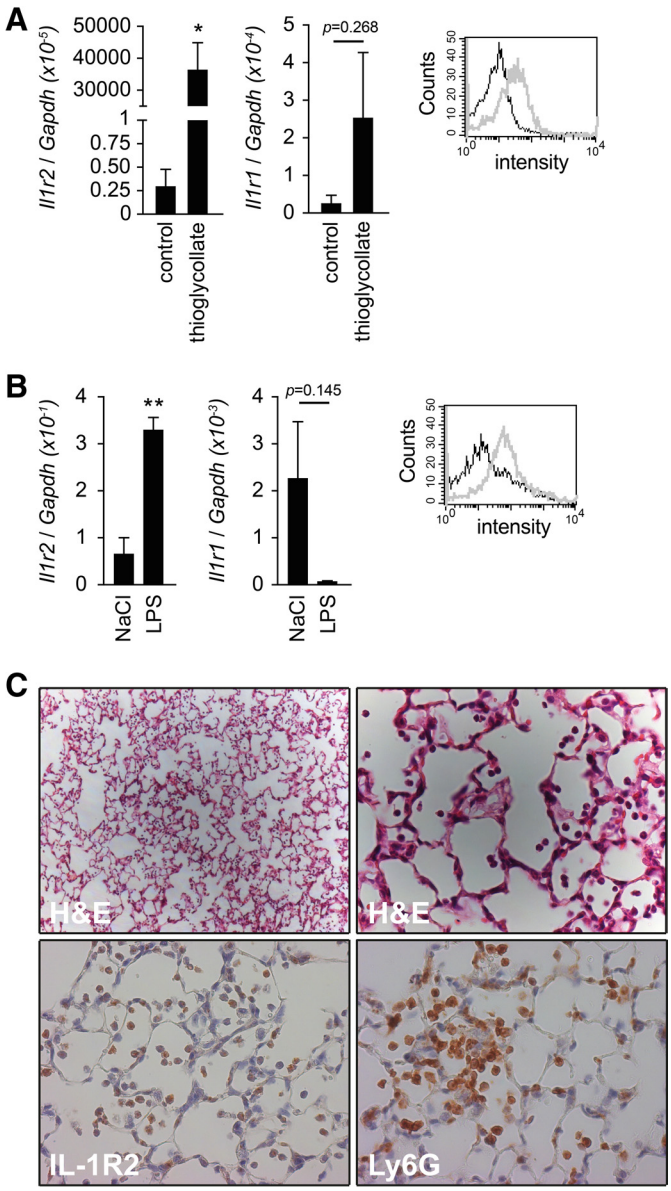

Figure 7. IL-1R2 expression in vivo in acute inflammatory conditions. IL-1R2-expressing neutrophils are recruited to the site of inflammation during thioglycolate-induced peritonitis (A) and endotoxin-induced acute lung injury (B and C). (A) Peritonitis was induced in C57BL/6J mice by injecting thioglycollate i.p. (B and C) Acute lung injury was induced in C57BL/6J mice by intratracheal instillation of LPS. Determination of *Il1r2* (left) and *Il1r1* (right) mRNA levels in peritoneal cells, 4 h postinjection (A), or in BAL cells, 6 h after LPS instillation (B). Total mRNA was isolated from peritoneal cells of control (n=3) and thioglycollate-treated mice (n=3; A) or from BAL samples of saline (NaCl; n=3)- and LPS (n=3)-instillated mice (B) for analyses by qRT-PCR. Results represent *Il1r2* and *Il1r1* mRNA expression levels relative to *Gapdh* mRNA levels. Data are shown as the mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$ versus control samples, as analyzed by unpaired two-tailed Student's t-test. Expression of IL-1R2, analyzed on Ly6G$^+$ cells of the peritoneal fluid (A) or Ly6G$^+$ BAL cells (B), was analyzed, 4 h postinjection or 6 h postinstillation, respectively, using anti-IL-1R2 mAb (gray lines) and the matched isotype control (black lines). Data are analyzed on total living cells. (C) Representative H&E-stained sections (upper) and immunohistochemical localization of IL-1R2 and Ly6G on sections of lungs of LPS-instillated C57BL/6J mice, 6 h after induction of acute lung injury (original magnification: upper left, 100×; upper right and lower panels, 400×).

IL-1R2 in the mouse, which is regulated by anti- and proinflammatory molecules. This knowledge is the essential background to design future animal models concerning the role of IL-1R2 in vivo.

Our data indicate that among immune cells, neutrophils are the major source of constitutively expressed IL-1R2. HC augments IL-1R2 surface expression, has no effect on IL-1Ra, but at the same time decreases the expression of LPS-induced IL-1α and IL-1β. LPS induces rapid shedding of the receptor from the membrane in vitro and in vivo, mainly mediated by the protease ADAM17. Thereafter, IL-1R2 is re-expressed on the cell surface of HC-treated BMG in vitro. Moreover, we showed that naturally expressed IL-1R2 binds IL-1β, whereas binding of IL-1Ra was not observed. Finally, IL-1R2 expression is detected on neutrophils under inflammatory conditions, such as thioglycolate-induced peritonitis and acute lung injury in vivo.

In vitro, the decoy receptor is found on the surface of mature CD11b$^+$Ly6G$^+$ neutrophils and to a lower extent, on CD11b$^+$Ly6G^{neg} metamyelocytes, which are neutrophilic precursors. This suggests that IL-1R2 is already present on premature neutrophils, which is consistent with previous findings [50]. Ex vivo, IL-1R2 is expressed on CD11b$^+$Ly6G$^+$ peripheral blood neutrophils from naïve mice, as well as on Ly6G$^+$ neutrophils, which have been recruited to the site of inflammation upon the induction of different pathological situations. These findings are in line with data obtained in the human system, indicating that IL-1R2 is expressed by peripheral PMN cells [7, 19, 21, 22], but they differ from other human data, demonstrating that also B and T cells [2], monocytes [19], natural Tregs [23, 24], and M2 macrophages [41] are a source of IL-1R2. It is known that there are discrepancies in protein expressions between mouse and man. For instance, the receptors FcαR1 and FcγRIIA/C are only present on human cells, and the surface protein CD33 is found on neutrophils in the mouse but on monocytes in humans (reviewed in ref. [51]). Although IL-1R2 mRNA levels were enhanced in BMDC and BMMϕ upon stimulation with HC, the protein was not detected, suggesting that it was not translated or expressed at levels remaining below the detection limit of the techniques used here. Our findings are also different from some mouse data [2, 25–29], describing IL-1R2 expression on B and T cell lines, BMDCs, ex vivo CD4$^+$ T cells in a senescent mouse model, and monocytes after *L. monocytogenes* infection. These discrepant observations might be explained by the fact that in these studies, the cell lines were investigated and known to express a different panel of proteins than primary cells, or different in vivo mouse models, stimulation protocols and analysis techniques were used. Thus, we cannot exclude the possibility that IL-1R2 is expressed on cells other than neutrophils in the mouse during different in vivo situations.

The function of IL-1R2 is the regulation of IL-1-mediated inflammatory and immune responses by trapping IL-1 [7]. We observed that IL-1R2 expressed on BMGs indeed bound IL-1β, whereas we could not detect binding of IL-1Ra. IL-1 and its antagonist IL-1Ra have similar affinities for IL-1R1 [52, 53], whereas the affinity of IL-1Ra for IL-1R2 is much lower than that of IL-1 [53]. Thus, IL-1R2 represents an additional mech-

anism to IL-1Ra to antagonize the potential, harmful effects of IL-1.

Transcript and protein levels on the surface of BMGs are highly elevated by the glucocorticoid HC. This is consistent with data demonstrating that dexamethasone, a synthetic member of the glucocorticoid group, up-regulates IL-1R2 surface expression on human neutrophils [7, 22] and monocytes [36]. As a result of their anti-inflammatory effect, glucocorticoids are also applied as drugs to treat inflammatory diseases. We demonstrated that HC up-regulated IL-1R2 on BMG and $Ly6G^+$ peripheral neutrophils and suppressed the production of proinflammatory IL-1α and IL-1β in BMGs. These findings might explain how stress-induced or exogenously administered HC exerts its anti-inflammatory action by regulating the expression of IL-1R2 and IL-1.

It has been described that IL-1R2 is rapidly released within 4 h or less from the membrane of the human PMN cell upon LPS [9] or fMLP [11] treatment, whereas IL-4 [7] and dexamethasone [22] exert their induction of shedding activity only after 18 h or longer. We showed that under naïve conditions, IL-1R2 is constitutively expressed on mouse neutrophils. It is membrane-bound and rapidly shed (1 h) from the surface upon LPS treatment in vitro, whereas IL-4 and fMLP had no impact on IL-1R2 expression on BMGs. The latter finding differs from the results published by Colotta et al. [7], who demonstrated a regulatory function of IL-4 on the expression and release of IL-1R2 on human PMN cells. Systemic endotoxin induces the release of IL-1β into the circulation to start an inflammatory host response against endotoxin-producing bacteria. The cleavage of the extracellular part of IL-1R2 by LPS might be a regulatory mechanism, in addition to the induction of IL-1Ra to counter-regulate IL-1β-mediated immune responses. In contrast to its membrane-bound form, shed IL-1R2 is released into the circulation and can bind and trap circulating IL-1β more easily.

Rapid shedding of IL-1R2 was also seen in vivo, 2 h after LPS injection. At later time-points (6 h and 24 h), IL-1R2 expression on $Ly6G^+$ peripheral blood neutrophils returned to or even exceeded baseline levels. A similar finding has been described for CD163 upon LPS treatment of human PBMCs [54]. This phenomenon can be explained by the re-expression of IL-1R2 on the surface of neutrophils in vivo, as demonstrated in vitro, and by the recruitment of neutrophils from the bone marrow upon LPS injection. The rapid release of constitutively expressed IL-1R2 and its re-expression on the surface suggest a critical role of neutrophils during IL-1-mediated immune responses.

Our observation that the metalloproteinase ADAM17—the sheddase activity of which is rapidly activated in response to a variety of different stimuli, such as PKC activation by phorbol esters or TLR agonists—induced ectodomain shedding of IL-1R2 from the surface of BMG is supported by recent in vitro data using transfected cell lines, which identified human IL-1R2 as a substrate of ADAM17 [13, 14]. However, we showed for the first time that ADAM17 is the major protease that cleaves endogenously expressed IL-1R2 in response to LPS. As IL-1R2 shedding is not blocked completely—neither in BMG from $ADAM17^{ex/ex}$ mice nor by using the ADAM10/17-specific inhibitor GW—it is possible that other proteases also contribute to LPS-mediated IL-1R2 release. In vitro studies identified further enzymes that could release IL-1R2 from the membrane, such as the aminopeptidase regulator of TNFR1 shedding [15] and the β-site amyloid precursor protein-cleaving enzymes 1 and 2 [44].

Neutrophils are indispensable for immune defense but can also play a pathological role by causing tissue damage in the host. Several reports extend the role of neutrophils to the release of pro- and anti-inflammatory cytokines, immunoregulatory cytokines, chemokines, and effector molecules of the innate and adaptive immune system [55]. We showed in vitro that neutrophils produce IL-1 and anti-inflammatory molecules, such as IL-1Ra and IL-1R2, to limit the proinflammatory effects of IL-1. Furthermore, in the course of our experiments, we detected in vivo a constitutive expression of IL-1R2 on neutrophils that have been recruited to the site of acute inflammation. Furthermore, the injection of HC increased the expression of IL-1R2 on the surface of peripheral blood neutrophils. These findings lead us to speculate that neutrophil-derived IL-1R2 dampens IL-1 activities at the site of inflammation and that the application of an anti-inflammatory drug helps to increase the amount of IL-1R2 on the surface.

AUTHORSHIP

P.M. designed the study, performed experiments, analyzed data, and wrote the manuscript. G.P. analyzed the data and wrote the manuscript. S.V., C.L., E.R., and D.T-A. performed experiments and analyzed the data. S.R-J. and A.C. analyzed data and wrote the manuscript. C.G. supervised the project, designed the study, analyzed the data, and wrote the manuscript.

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DISCLOSURES

The authors declare no conflict of interest.

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