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Distinct Roles of Hepatocyte- and Myeloid Cell-Derived IL-1 Receptor Antagonist during Endotoxemia and Sterile Inflammation in Mice

Céline Lamacchia,*† Gaby Palmer,*† Loraine Bischoff,* Emiliana Rodriguez,*† Dominique Talabot-Ayer,*† and Cem Gabay*†

IL-1R antagonist (IL-1Ra) is a natural inhibitor of the pleiotropic proinflammatory activities of IL-1. Although several reports described the effects of complete IL-1Ra deficiency, no study has examined the consequences of cell type-specific IL-1Ra inactivation during systemic inflammation. Previous in vitro data demonstrated high IL-1Ra production by hepatocytes and myeloid cells after endotoxin stimulation. In addition, hepatocyte IL-1Ra production is regulated as an acute-phase protein in vitro. In this study, we analyzed the production and functional role of hepatocyte- and myeloid cell-derived IL-1Ra during endotoxin-induced septic shock and acute IL-1β–induced sterile inflammation. Using conditional IL-1Ra knockout mice, we showed that hepatocytes and myeloid cells are the two major cellular sources of circulating IL-1Ra in response to LPS. Interestingly, IL-1Ra production by myeloid cells, but not hepatocytes, is critical for survival during endotoxemia. Furthermore, we provide the first in vivo evidence demonstrating that IL-1Ra is produced as an acute-phase protein by hepatocytes during IL-1β–induced inflammation and that hepatocyte-derived IL-1Ra functions as an endogenous negative feedback downregulating the proinflammatory effects of IL-1.

Taken together, our observations define distinct roles for two major cellular sources of IL-1Ra in response to different types of systemic inflammatory stimuli in vivo. The Journal of Immunology, 2010, 185: 2516–2524.

Interleukin-1 (referring to IL-1α and IL-1β) is a prototypic proinflammatory cytokine that plays a critical role in acute and chronic inflammation. IL-1β is released by activated macrophages and neutrophils and contributes to systemic inflammatory manifestations, whereas IL-1α remains primarily cell associated. The biological effects of IL-1β are tightly controlled at the level of its production and, then, by different natural inhibitors, including membrane-bound and soluble receptors, and IL-1R antagonist (IL-1Ra) (1). IL-1Ra binds to IL-1Rs but does not induce any intracellular signals and competitively inhibits the biological activity of IL-1 with its receptors. Evidence derived from animal studies and human patients indicates that the balance between IL-1 and IL-1Ra is critical to avoid excessive inflammatory responses. Indeed, severe hereditary inflammatory conditions have been described in children with excessive IL-1β production or IL-1Ra deficiency (2–5). Similarly, knockout mice lacking IL-1Ra exhibit exaggerated inflammation in different disease models (6–11). However, although several reports describe the effects of complete IL-1Ra deficiency, no study has examined the consequences of cell type-specific IL-1Ra inactivation on inflammatory responses.

IL-1 has been proposed as an important mediator of sepsis and septic shock (12). Indeed, a significant increase in circulating IL-1β levels was detected in patients with septic shock and after administration of endotoxin to healthy volunteers (13–15). In addition, previous studies showed that mice lacking IL-1–converting enzyme (now termed caspase-1) or IL-1R type I were resistant to endotoxic shock (16, 17). Consistently, the physiological consequences of infection and shock can be ameliorated, particularly in animal models, by the administration of rIL-1Ra, a specific IL-1 inhibitor (18–23). The contribution of the IL-1/IL-1Ra balance during septic shock was confirmed by the high susceptibility of IL-1Ra–deficient mice to LPS-induced lethality (6).

Different cell types are able to produce IL-1Ra. In vitro studies showed that human hepatocytes secrete large amounts of IL-1Ra as an acute-phase protein (APP) in response to inflammatory stimuli, such as IL-1β (24). Furthermore, a strong increase in circulating IL-1Ra levels was observed in patients in whom recombinant human IL-1β was injected, suggesting that the production of this cytokine might also be regulated as an APP in vivo (25). During LPS-induced systemic inflammation, the hepatic production of IL-1Ra is strongly upregulated and correlates with the presence of elevated serum levels of IL-1Ra (26). In addition, the total amount of IL-1Ra in the liver after LPS injection is significantly higher than in the lung and spleen (26). Based on these different observations, hepatocytes have been considered the major source of hepatic and circulating IL-1Ra during endotoxemia. However, in our previous work, we also observed that myeloid cells, such as macrophages and dendritic cells, produce large amounts of IL-1Ra in response to LPS in vitro (27). In addition, neutrophils can contribute, to a large extent, to the production of secreted IL-1Ra after LPS stimulation (28). These observations...
suggest that myeloid cells might also represent an important source of tissue and circulating IL-1Ra in response to LPS. However, the relative contribution of hepatocytes and myeloid cells versus other cellular sources of IL-1Ra in the control of systemic inflammatory responses is still unknown in vivo.

By using conditional knockout mice in which IL-1Ra production has been specifically targeted in hepatocytes (IL-1Ra<sup>flx/flx</sup>), myeloid cells (IL-1Ra<sup>flx/flx</sup>), or in both cellular sources (IL-1Ra<sup>flx/flx</sup>), we showed in this study that hepatocytes and myeloid cells are the two major cellular sources of circulating IL-1Ra in response to LPS and that IL-1Ra production by myeloid cells, but not hepatocytes, is critical for survival during endotoxemia. In contrast, hepatocytes are the major source of circulating IL-1Ra during acute sterile inflammation induced by IL-1β injection, and hepatocyte-derived IL-1Ra regulates the inflammatory responses induced by IL-1.

### Materials and Methods

#### Materials

Media used for mouse primary hepatocyte isolation and cell culture were obtained from Invitrogen Life Technologies (Basel, Switzerland). Recombinant human IL-1β and human IL-6 were obtained from R&D Systems (Abingdon, U.K.), and purified LPS was obtained from Fluka (Escherichia coli 055:B5, Buchs, Switzerland).

**Mice**

Wild-type (WT) C57BL/6J mice were obtained from Janvier (Le Genest-St-Isle, France). Albcre mice (B6.Cg-Tg(Alb-cre)21Mgn/J) and LysMcre mice (B6.129P2-lyz2<sup>M1</sup>cre<sup>M1</sup>/J) were purchased from The Jackson Laboratory (Bar Harbor, ME) and routinely genotyped by PCR according to the protocol of the vendor. IL-1Ra–deficient mice (IL-1Ra<sup>−/−</sup>) backcrossed in a pure C57BL/6J genetic background were originally obtained from Dr. M.J. Nicklin (Division of Molecular and Genetic Medicine, University of Sheffield, Sheffield, U.K.).

Ten- to 16-wk-old mice were anesthetized, the peritoneal cavity was opened, and the portal vein was cannulated. The liver was perfused for 10 min at a flow rate of 4 ml/min with Liver Perfusion Medium (37°C), and the inferior vena cava was subsequently cut. The perfused liver was digested for 12 min with Liver Digest Medium containing collagenase (37°C). The liver was removed and transferred to a receptacle containing cold Leibovitz’s L-15 medium, supplemented with 5% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. After tissue dissociation by brief manual shaking and pipetting, the resulting cell suspension was filtered through a sterile 70-μm nylon cell strainer (BD Falcon, Le Pont-De-Clai, France), sedimented by centrifugation (5 min, 500 rpm at 4°C), and washed in cold Hepatocytes Wash Medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Hepatocytes were purified by centrifugation on a sterile Percoll gradient adjusted to a density of 0.678 g/ml for 5 min at 500 rpm, 4°C (Percoll Plus, Amersham Biosciences, Glattbrugg, Switzerland) and subsequently washed. The percentage of living cells with hepatocyte morphology (∼90%) was determined by staining with trypan blue.

Initial primary hepatocytes were used for genomic DNA extraction and for stimulation experiments.

#### Culture and stimulation of mouse primary hepatocytes

Primary hepatocytes isolated from the liver of IL-1ra<sup>−/−</sup> and WT mice were seeded onto 24-well plates (Multiwell Primaria 24 well; BD Falcon), at a concentration of 1 × 10<sup>5</sup> cells, in 1 ml Williams E Medium supplemented with 10% FCS, 1 μM dexamethasone, 25 ng/ml epidermal growth factor, 100 nM insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin. The following day, fresh medium was added, and cells were cultured in the absence or presence of purified LPS (1 μg/ml) or with a combination of recombinant human IL-1β (1 ng/ml) and IL-6 (10 ng/ml). After 48 h, culture supernatants were collected, and IL-1Ra levels were analyzed by ELISA using commercial DuoSet ELISA Development Systems from R&D Systems (Minneapolis, MN). The detection limit for this test was 78 pg/ml.

#### Determination of il-1rn exon 2 excision efficiency by quantitative PCR

Genomic DNA was extracted from isolated primary hepatocytes or skin (control) with the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. Excision of il-1rn exon 2 by the Cre-recombinase was examined by quantitative PCR by using two pairs of primers (Fig. 2A): one pair specific for the floxed fragment containing il-1rn exon 2 (forward primer 5‘-CCAGCAAGCTTAGTAGTAATTTG-3’ and reverse primer 5‘-GTTGATTTTGGTTGATATTAGG-3’), or 0.9% sterile saline solution. Blood was collected by cardiac puncture on the day of administration. Systemic inflammation was induced by i.p. injection of 2 or 10 mg/kg LPS in 8–20-wk-old mice (females and males). LPS-induced lethality was determined by injecting mice i.p. with LPS (10 mg/kg) or with a combination of recombinant human IL-1β (1 ng/ml) and IL-6 (10 ng/ml). After 48 h, culture supernatants were collected, and IL-1Ra levels were analyzed by ELISA using standard protocol (40 cycles, annealing temperature 60°C). The number of copies of il-1rn exon 2 was normalized to that of il-1rn exon 3. Quantitative PCR analysis for each sample was performed in triplicates.

### LPS-induced systemic inflammation and lethality

Pure LPS was dissolved in saline solution (NaCl 0.9%) at 0.5 mg/ml. LPS solutions were freshly prepared on the day of administration. Systemic inflammation was induced by i.p. injection of 2 or 10 mg/kg LPS in 8–20-wk-old mice (females and males). LPS-induced lethality was determined by injecting mice i.p. with LPS (10 mg/kg) and monitoring survival every 12 h for 6 d. No additional mortality was seen after 6 d. Twelve- to 20-wk-old mice (females and males) were used for the survival experiment.

### IL-1β-induced nonseptic inflammation

Recombinant human IL-1β (R&D Systems) was dissolved in saline solution (NaCl 0.9%) at 2.5 μg/ml. IL-1β solution was freshly prepared on the day of administration. Sterile inflammation was induced by i.p. injection of 10 μg/kg IL-1β in 12–24-wk-old mice (females and males). Mice were injected i.p. with 10 mg/kg LPS, 10 μg/kg recombinant human IL-1β, or 0.9% sterile saline solution. Blood was collected by cardiac...
puncture under anesthesia 4 or 18 h after injection, and the animals were immediately killed by cervical dislocation. Sera were obtained after blood coagulation and subsequent centrifugation. Different organs (liver, spleen, lung, and skin) were rapidly dissected and frozen in liquid nitrogen. Total-protein extracts were prepared from different tissues by homogenization in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and protease inhibitor mixture [Complete, EDTA-free; Roche Diagnostics, Rotkreuz, Switzerland]), and lysates were cleared by centrifugation. Total protein concentrations were determined by DC Protein Assay (Bio-Rad). Serum and tissue concentrations of IL-1Ra, IL-6, IL-1b, CXCL1, and CXCL2 were quantified by ELISA using commercial Duoset ELISA Development Systems from R&D Systems. The detection limits for these tests were 16 pg/ml (for IL-1b, IL-6, and CXCL1), 78 pg/ml (for IL-1Ra), and 7.8 pg/ml (for CXCL2).

RNA extraction
Total RNA was extracted from different organs with the use of TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer’s protocol.

Quantitative real-time PCR
Total RNA (1 μg) was reverse transcribed, and total IL-1Ra mRNA levels were examined by quantitative PCR using the Power SYBR Green PCR kit (Applied Biosystems, Foster City, CA) and the following primers: 5′-GGGATACTAACCAGAAGACC-3′ (forward) and 5′-GAGCGGCAACGGCTTGCCCC-3′ (reverse). Raw threshold cycle values obtained with the SDS 2.2 (Applied Biosystems) were imported in Microsoft Excel (Redmond, WA). IL-1Ra expression was normalized to expression of the housekeeping gene GAPDH using the following primers: 5′-AGGCCGAATGGGAAAGCTTG-3′ (forward) and 5′-TACTGCAGCGCCCTACCC-3′ (reverse). The annealing temperature was 60°C. Nonreverse-transcribed RNA samples and water were included as negative controls.

Immunohistochemistry
Paraffin-embedded liver and spleen sections were deparaffinized in xylol and rehydrated through graded concentrations of ethanol. Endogenous peroxidase was blocked using 0.6% H2O2 for 10 min, and tissue sections were boiled in citrate-based Ag-unmasking solution (10 mM [pH 6]) for 3 min. To evaluate local expression of IL-1Ra, tissue sections were incubated with 14.4 μg/ml (for liver sections) or 7.2 μg/ml (for spleen sections) polyclonal goat anti-mouse IL-1Ra Ab (capture Ab of mouse IL-1Ra Duoset ELISA kit; R&D Systems) for 2 h. The sections were rinsed and incubated for 1 h with 1.6 μg/ml donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Heidelberg, Germany). Color was developed with diaminobenzidine, and tissues were counterstained with hematoxylin. In addition, sections were incubated with 6.6 μg/μl rabbit anti-human myeloperoxidase (DakoCytomation, Trappes, France) for 2 h and then incubated for 1 h with biotinylated swine anti-rabbit Ig diluted to 1:250 (DakoCytomation). The slides were incubated for an additional 15 min with alkaline phosphatase-labeled streptavidin diluted to 1:100 (Vector Laboratories, Sercion, Switzerland) and then reacted with alkaline phosphatase substrate solution (Vector Laboratories). Finally, the sections were counterstained with hematoxylin.

Statistical analysis
One-way ANOVA and the two-tailed t test were used for statistical analysis. A comparison between two groups using the two-tailed t test was made only when one-way ANOVA yielded statistically significant results. Survival curves after LPS injection were compared using the Kaplan–Meier statistic; p values ≤0.05 were considered significant.

Results
Characterization of floxed il-1rn mice
Three isoforms of IL-1Ra have been described in the mouse, one of which is secreted, whereas three others are intracellular (icIL-1Ra1, icIL-1Ra2, and icIL-1Ra3) (3, 33). These isoforms are generated from the il-1rn gene by the use of different first exons, alternative mRNA splicing, and differential translation initiation. We generated floxed il-1rn mice in which il-1rn exon 2, which is common to all IL-1Ra isoforms, is flanked by two loxP sites (27). To ascertain that the presence of loxP sites did not interfere with expression of the different IL-1Ra isoforms, the sera and different organs were collected from IL-1Raβlox/lox, IL-1Raβlox/lox, and WT littermate mice 4 h after i.p. injection of LPS (2 mg/kg) or saline solution. As previously described (26, 33), circulating levels of IL-1Ra were elevated in mice after LPS challenge, whereas IL-1Ra remained undetectable in the serum of mice injected with saline solution (Fig. 1A, data not shown). Serum IL-1Ra levels were not significantly different among IL-1Raβlox/lox, IL-1Raβlox/lox, and WT mice after LPS administration. In addition, IL-1Ra concentrations in the spleen, liver, and lung were increased upon LPS stimulation, but they did not differ significantly among the three genotypes (Fig. 1B–E). Consistent with previous data, Western blot analysis showed that the skin constitutively expresses high amounts of icIL-1Ra1 without further change after LPS challenge in vivo, whereas all IL-1Ra isoforms were present in the spleen, liver, and lung following in vivo stimulation by LPS (data not shown) (26, 34). Taken together, these results indicate that the incorporation of loxP sites had no influence on constitutive and LPS-induced production of IL-1Ra isoforms in floxed il-1rn mice.

Characterization of hepatocyte-specific IL-1Ra–deficient mice
The specificity of il-1rn targeting was examined by quantitative PCR in primary hepatocytes of IL-1RaβH mice as well as in skin used as a control tissue. The quantitative PCR results showed that il-1rn exon 2 was excised specifically and efficiently (91%) in hepatocytes of IL-1RaβH mice compared with WT mice. In contrast, exon 2 copy number in skin of IL-1RaβH mice was similar to that of WT mice (Fig. 2B).

Previous reports demonstrated that human primary hepatocytes stimulated with IL-6 and IL-1β produced elevated levels of IL-1Ra (24). Consistent with these data, our in vitro stimulation experiments showed that mouse primary hepatocytes were also able to secrete high levels of IL-1Ra in response to IL-1β/IL-6 challenge (23.3 ± 0.3 ng IL-1Ra/10⁶ cells) (Fig. 2C). Elevated IL-1Ra levels were also detected in the supernatants of LPS-stimulated mouse primary hepatocytes (15.2 ± 0.5 ng IL-1Ra/10⁶ cells) but remained undetectable in unstimulated cells (Fig. 2C, data not shown). We observed that IL-1Ra production by primary hepatocytes of IL-1RaβH mice was decreased by 90% and 98% compared with control mice after LPS and IL-1β/IL-6 stimulation, respectively (Fig. 2C). Taken together, these results show specific and highly efficient Cre recombinase-mediated deletion of il-1rn exon 2 in hepatocytes of IL-1RaβH mice. IL-1Raβlox/lox and LysM-Cre were crossed to obtain IL-1RaβM mice, as recently described (27). These mice were subsequently crossed with IL-1RaβH mice to obtain IL-1RaβH/M mice.

Gross examination of IL-1RaβH mice, IL-1RaβM mice, and IL-1RaβH/M mice
Previously, it was observed that IL-1Ra−/− mice had abnormal development, including postnatal runting, reduced fertility, pronounced neutrophilia, piloerection, growth deficit, and, in some cases, severe respiratory distress (6, 9, 16). Consistent with published data, we observed that female and male IL-1Ra−/− mice had significantly lower body weights than WT mice, confirming the critical role of endogenously produced IL-1Ra in normal growth (Supplemental Fig. 1, data not shown). The weight difference was apparent at 6 wk of age and continued into adult life. In contrast, IL-1RaβH, IL-1RaβM, and IL-1RaβH/M mice had similar weight and appearance upon gross examination as WT mice (Supplemental Fig. 1), suggesting that hepatocyte- and myeloid cell-derived IL-1Ra are not required for normal growth and health in the absence of a specific pathogenic stimulus. Furthermore, as opposed to IL-1Ra−/− mice, all of the conditional IL-1Ra–deficient mouse lines had normal fertility.
Hepatocytes and myeloid cells are the two major sources of circulating IL-1Ra in response to LPS

To define the relative contribution of hepatocytes and myeloid cells as cellular sources of circulating IL-1Ra in LPS-induced systemic inflammation, IL-1Ra<sup>dH</sup>, IL-1Ra<sup>dM</sup>, IL-1Ra<sup>dH+M</sup>, and WT mice were injected with LPS (10 mg/kg), and blood and different organs were collected after 4 and 18 h. The levels of circulating IL-1Ra were decreased by 15% and 57% in IL-1Ra<sup>dH</sup> and IL-1Ra<sup>dM</sup> mice, respectively, 4 h after LPS administration compared with WT mice (Fig. 3<sub>A</sub>). IL-1Ra levels were markedly decreased in the circulation of double-mutant mice following LPS injection (decrease of 92% and 75% 4 and 18 h after injection, respectively) (Fig. 3). In contrast, circulating IL-6 and IL-1β levels were not significantly different in conditional IL-1Ra–deficient mouse lines compared with WT mice (data not shown). These results suggest that hepatocytes and myeloid cells represent the two major cellular sources of circulating IL-1Ra during LPS-induced systemic inflammation.

Cellular sources of IL-1Ra in different organs in response to LPS

In our previous work, we showed that the liver, spleen, and lung constitute the three major sources of IL-1Ra in response to LPS (26). Thus, we decided to examine the cellular sources of IL-1Ra in these organs. The specific disruption of il-1rn gene expression in hepatocytes led to a partial reduction (56%) of hepatic IL-1Ra levels in response to LPS, indicating the presence of another source of IL-1Ra in the liver (Fig. 4<sub>A</sub>). Indeed, liver IL-1Ra levels were decreased by 20% in LPS-treated IL-1Ra<sup>dM</sup> mice (Fig. 4<sub>A</sub>), and the immunohistochemical localization of IL-1Ra in liver of LPS-treated IL-1Ra<sup>dH</sup> mice showed the presence of other IL-1Ra<sup>e</sup> cells (Fig. 4<sub>B</sub>), which were also positive for myeloperoxidase (Supplemental Fig. 2), a specific marker of myeloid cells. Most importantly, the immunostaining of these cells was virtually absent in IL-1Ra<sup>dM</sup> mice (Fig. 4<sub>B</sub>). Based on these different observations, it is likely that myeloid cells, including Kupffer cells and circulating neutrophils, represent another important source of IL-1Ra in the liver in response to LPS. Consistent with these findings, IL-1Ra levels were markedly decreased (~76%) in liver extracts of IL-1Ra<sup>dH+M</sup> mice (Fig. 4<sub>A</sub>). These results were supported by quantitative real-time PCR analysis showing a significant decrease in total IL-1Ra mRNA levels in liver extracts of IL-1Ra<sup>dH+M</sup> mice (Supplemental Fig. 3). Taken together, these observations indicate that hepatocytes and myeloid cells are important sources of IL-1Ra in the liver during LPS-induced systemic inflammation.

We observed a strong decrease in IL-1Ra levels in the spleen (~70%) and lung extracts (~50%) of IL-1Ra<sup>dH</sup> and IL-1Ra<sup>dH+M</sup> mice compared with WT mice (Fig. 4<sub>C</sub>), whereas IL-1Ra<sup>dH</sup> did not significantly differ from WT mice. Production of total IL-1Ra mRNA levels in spleen and lung were also markedly decreased (Supplemental Fig. 3). Consistent with these results, immunohistochemical analysis showed that myeloid cells represented an important source of IL-1Ra in the spleen (Fig. 4<sub>D</sub>, Supplemental Fig. 2) and lung (data not shown) during endotoxemia.
FIGURE 2. Characterization of conditional hepatocyte-specific IL-1Ra–deficient mice. A, Alb-Cre–mediated deletion of il-1rn exon 2. Exons are depicted as boxes and numbered. The vector-derived sequences and the mouse il-1rn genomic sequences are represented by bold and thin lines, respectively. LoxP sites are shown as black triangles, and FRT sites are shown as double gray triangles. Diagram is not depicted to scale. B, IL-1rn exon 2 copies were quantified in mouse primary hepatocytes and skin by quantitative PCR on genomic DNA. The number of copies of il-1rn exon 2 was normalized to that of il-1rn exon 3. Location of PCR primers are indicated in A (arrows a and b for exon 2, arrows c and d around exon 3). Results represent exon2/exon3 ratio × 100. Data are mean ± SEM for hepatocyte-specific IL-1Ra–deficient mice (IL-1RaH; n = 3) and control mice (WT; n = 3). ***p < 0.001, by Student t test. C, Production of IL-1Ra by mouse primary hepatocytes. Hepatocytes were cultured in the presence of LPS (1 μg/ml) or with a combination of IL-1β (1 ng/ml) and IL-6 (10 ng/ml). After 48 h, supernatants were collected, and IL-1Ra levels were measured by ELISA. Values represent the mean ± SEM of IL-1Ra production by cells isolated from hepatocyte-specific IL-1Ra–deficient mice (IL-1RaH, n = 3) and control mice (WT, n = 3). **p < 0.01, versus cells isolated from WT mice with similar stimulation assessed by ANOVA, two-tailed t test, ic, intracellular; s, secreted.

Myeloid cell-derived IL-1Ra is critical for survival during endotoxemia

It was previously demonstrated that IL-1Ra plays a critical role in survival during endotoxemia (6). To further define the functional role of hepatocyte- and myeloid cell-derived IL-1Ra in survival in response to a high dose of endotoxin (Fig. 5), conditional IL-1Ra–deficient mice (IL-1RaH, IL-1RaD, IL-1RaHH+M), constitutively IL-1Ra–deficient mice (IL-1RaH), and WT mice were infected i.p. with 10 mg/kg LPS, and lethality was determined by monitoring survival for 6 d. As expected, IL-1Ra−/− mice were more susceptible than WT mice to the lethal effects of endotoxin. The survival curves of IL-1RaH, IL-1RaHH+M, and IL-1Ra−/− mice had similar profiles, showing no significant difference. In contrast, IL-1RaD mice exhibited significantly better survival than IL-1RaH mice, as well as a lethality profile similar to WT mice. These results indicate that myeloid cells, but not hepatocytes, play a predominant role in the IL-1Ra–mediated control of survival during endotoxemia.

Hepatocytes represent the major source of circulating IL-1Ra during IL-1β–induced sterile inflammation

We also examined the effects of cell type-specific IL-1Ra inactivation in a model of nonseptic inflammation. IL-1β is a principal mediator of the acute inflammatory response and is known to induce IL-1Ra production by hepatocytes in vitro. To determine the contribution and the biological role of hepatocytes as a cellular source of IL-1Ra in a model of sterile inflammation, the production of IL-1Ra was examined following the administration of IL-1β. We supposed that the contribution of hepatocytes to the circulating levels of IL-1Ra might be more important in this nonseptic inflammatory model compared with endotoxemia because previous results from our laboratory showed that myeloid cells exhibit a modest response to IL-1 in vitro (C. Lamacchia, G. Palmer, and C. Gabay, unpublished observations). We first analyzed circulating IL-1Ra levels at different time points after IL-1β challenge in control mice. We observed that plasma levels of IL-1Ra were increased 4 h after 10 μg/kg recombinant human IL-1β injection and gradually decreased until 18 h after injection (data not shown). Thus, we collected blood and various organs of conditional IL-1Ra–deficient mice and WT mice 4 h after IL-1β challenge. We observed a strong decrease in IL-1Ra mRNA levels in the liver extracts of IL-1RaH and IL-1RaHH+M mice compared with WT mice (Supplemental Fig. 4A). In contrast, there was no significant difference between IL-1RaD and WT mice, suggesting that hepatocytes represent the major source of IL-1Ra in the liver in response to IL-1β–induced systemic inflammation. These results were supported by the immunohistochemical analysis showing that only hepatocytes produce IL-1Ra in the liver of IL-1β–treated WT mice (Supplemental Fig. 5).
IL-1Ra expression was also analyzed in the spleen of conditional IL-1Ra–deficient and WT mice. We observed that the IL-1Ra mRNA levels in the spleen were lower than those in the liver of IL-1β–treated WT mice (Supplemental Fig. 4). There was a significant decrease in IL-1Ra mRNA levels in the spleen extracts of IL-1RaΔM and IL-1RaΔH+M mice versus WT mice after IL-1β challenge (Supplemental Fig. 4B). In contrast, there was no significant difference between IL-1RaΔH and WT mice. These results indicate that spleen myeloid cells produce IL-1Ra in response to IL-1.

After IL-1β administration, the levels of circulating IL-1Ra were significantly decreased in IL-1RaΔH and IL-1RaΔH+M mice compared with WT mice (Fig. 6A). In contrast, there was no significant difference between WT and IL-1RaΔM mice, suggesting that hepatocytes represent the major source of circulating IL-1Ra in response to IL-1β–induced systemic inflammation. Most importantly, the levels of different inflammatory mediators produced in response to IL-1β, such as IL-6, CXCL1, and CXCL2, were significantly enhanced in the serum of IL-1RaΔH and IL-1RaΔH+M mice compared with control and IL-1RaΔM mice, indicating that hepatocyte-derived IL-1Ra exerts a negative feedback effect on IL-1–induced inflammation (Fig. 6B–D). In the serum of WT mice injected with saline solution, IL-6 and CXCL2 remained undetectable, and CXCL1 levels were low (112 ± 58 pg/ml) (data not shown).

Discussion

Although several reports described the effects of complete IL-1Ra deficiency, no study has examined the effects of cell type-specific inactivation of IL-1Ra during systemic inflammation. In this study, by using conditional IL-1Ra knockout mice, we provide new insights regarding IL-1Ra production by different cellular sources. Indeed, we show that hepatocytes and myeloid cells are the major producers of circulating IL-1Ra in response to LPS, whereas hepatocytes are the major cellular source of circulating IL-1Ra in response to systemic acute stimulation by IL-1β. In addition, myeloid cell-derived, but not hepatocyte-derived, IL-1Ra plays a critical role in survival during endotoxemia.

Despite their low production levels in basal conditions, several reports showed that the balance between IL-1 and IL-1Ra plays an important role in the normal physiology of various organs and tissues (reviewed in Refs. 12 and 35). In fact, IL-1Ra–deficient mice exhibit...
FIGURE 6. Contribution of hepatocytes and myeloid cells as sources of circulating IL-1Ra after systemic challenge with IL-1β. IL-1Ra (A), IL-6 (B), CXCL1 (C), and CXCL2 (D) levels were quantified by ELISA in sera of control mice (WT), IL-1RaΔH mice, IL-1RaΔM mice, and IL-1RaΔHΔM mice 4 h after 10 μg/kg IL-1β injection. Results are shown as individual values for each mouse (symbols) and mean values (lines). *p < 0.05; **p < 0.01; ***p < 0.001, versus WT mice by ANOVA, two-tailed t test.

decreased fertility and a general appearance suggesting illness, as well as a decreased body mass, compared with WT mice, due to growth retardation after weaning, a phenotype not caused by feeding suppression but rather by impaired lipid accumulation due to low plasma levels of insulin and low lipase activity (6, 36, 37). Interestingly, we observed that neither hepatocyte- nor myeloid cell-derived IL-1Ra deficiency influenced growth and health in normal condition. This finding suggests that basal IL-1Ra levels produced by other cell types, such as fibroblasts, adipocytes, epithelial cells, and endothelial cells, play an important role in the maintenance of a normal physiological state, whereas hepatocyte- and myeloid cell-derived IL-1Ra may be more relevant in an inflammatory context.

Previous data showed that the liver represents one of the main sources of IL-1Ra following LPS injection (26, 33). We showed that mouse primary hepatocytes secrete high levels of IL-1Ra following LPS or IL-1β/IL-6 stimulations in vitro. These results suggest that after systemic LPS injection, the in vivo production of IL-1Ra by hepatocytes may be due to a direct effect of LPS and/or to indirect stimulation mediated by IL-1β and IL-6, two proinflammatory cytokines known to play a major role in the induction of APP by the liver (38, 39). Accordingly, it was shown that IL-1Ra production by human primary hepatocytes and hepatoma cells is enhanced by IL-1β and that the combination of IL-1β and IL-6 has an even stronger effect (24). Several cell types, particularly myeloid cells, are known to produce IL-1β and IL-6 in response to LPS. For example, it was reported that liver Kupffer cells are stimulated to produce the IL-6 signal to hepatocytes during endotoxemia (40). Consistent with these different observations, we observed elevated levels of IL-1β and IL-6 in the circulation and liver of LPS-treated mice (data not shown).

In addition to hepatocytes, our results showed that liver myeloid cells, including circulating neutrophils and resident macrophages, represent an important source of hepatic IL-1Ra in response to LPS. Consistent with these in vivo data, we recently observed that primary mouse myeloid cells, such as macrophages and dendritic cells, are able to secrete large amounts of IL-1Ra in response to LPS in vitro (27). The residual production of IL-1Ra in liver extracts of IL-1RaΔHΔM mice (~25%) is most likely related to the presence of other cellular sources of IL-1Ra, such as stellate cells (Ito cells) and endothelial cells, and it may also reflect an incomplete Cre-mediated excision of il-1rn exon 2 in hepatocytes and myeloid cells.

Myeloid cells are known to be critical in the initiation, maintenance, and resolution of inflammation. In the presence of inflammatory signals, such as LPS, they initiate nonspecific defense mechanisms by overexpressing proinflammatory cytokines, including IL-1. However, these cells also express anti-inflammatory or regulatory cytokines, thus participating in the regulatory loop involved in the termination of the inflammatory process. Indeed, the production of IL-1Ra by myeloid cells is critically important to maintain an adequate IL-1/IL-1Ra balance and prevent the development of excessive IL-1 signaling. Consistent with this hypothesis, we observed that myeloid cell-derived IL-1Ra deficiency destabilized the regulation of the innate immune response during endotoxic shock, which, in turn, led to an increased lethality. In addition to the modulation of the inflammatory response to LPS, we recently demonstrated that myeloid cell-derived IL-1Ra plays a crucial role in the regulation of the adaptive immune response by controlling the development of Th1 and Th17 responses and the development of severe arthritis (27).

Myeloid cell-derived IL-1Ra deficiency results in a decrease ~50% of circulating IL-1Ra levels in response to LPS. Despite the presence of significant amounts of residual serum IL-1Ra, the mortality in IL-1RaΔM mice corresponded to that of mice completely deficient in IL-1Ra. Previously, it was reported that heterozygous IL-1Ra–deficient mice, in which total IL-1Ra production is decreased by half, exhibited an intermediate rate of LPS-induced lethality (6). These results suggest that local production of IL-1Ra by myeloid cells plays a key role in the control of tissue inflammation and in the prevention of multiple organ failure during endotoxia. Previously, we observed that the early production of IL-1Ra in some tissues preceded the detection of IL-1Ra in the circulation (33). Thus, it is conceivable that local IL-1Ra may constitute the major regulatory mechanism by which subsequent inflammatory responses are controlled, whereas the magnitude of circulating levels of IL-1Ra may reflect the severity of the inflammatory process.

Interestingly, we observed that circulating IL-1Ra levels are dependent on different cellular sources according to the type of
inflammatory stimulation used, thus emphasizing the complexity of the cellular system involved in the production of IL-1Ra. Indeed, in contrast to LPS, which triggers the production of IL-1Ra by hepatocytes and myeloid cells, hepatocytes are the major source of circulating IL-1Ra in response to a proinflammatory stimulation induced by IL-1β in vivo. This result is consistent with the recognition of IL-1Ra as an APP. Indeed, IL-1 is able to induce IL-6 production by many other cell types, which, in combination with IL-1, can stimulate the production of IL-1Ra by hepatocytes. In addition, our results showed that myeloid cells can produce IL-1Ra mRNA in the spleen in response to a systemic injection of IL-1. Although previous results from our laboratory showed that myeloid cells, such as bone marrow-derived macrophages, do not respond directly to IL-1β in vitro (C. Lamaccia, G. Palmer, and C. Gabay, unpublished observations), it is still plausible that myeloid can respond indirectly to IL-1 stimulation in vivo. Nevertheless, this production of IL-1Ra by myeloid cells does not contribute significantly to circulating IL-1Ra in response to IL-1 in vivo.

The induction of specific inflammatory mediators and the recruitment of leukocytes to sites of inflammation are known to characterize inflammatory processes mediated by IL-1. Interestingly, we observed that circulating levels of IL-6, CXCL1, and CXCL2 were significantly enhanced in IL-1Ra−/− and IL-1RaH−/− mice compared with WT mice after IL-1β administration. Thus, our data provide the first in vivo demonstration that hepatocyte-derived IL-1Ra acts as an endogenous negative feedback mechanism to downregulate the proinflammatory effects of IL-1, thereby promoting the resolution of the inflammatory response.

In conclusion, our results show that the production of IL-1Ra by myeloid cells in response to LPS is critical for survival during endotoxemia, whereas hepatocyte-derived IL-1Ra is essential to downregulate IL-1-induced acute systemic inflammation. Taken together, these findings define distinct roles for two major cellular sources of IL-1Ra in the response to different types of systemic inflammatory stimuli in vivo.

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Disclosures
The authors have no financial conflicts of interest.

References


Figure S1. Normal growth in mice lacking hepatocyte- and/or myeloid cell-derived IL-1Ra

The results represent the mean body weight in grams (g; ± SEM) as a function of age in females. Control mice (WT, (■), n= 6 to 25 females per time point), IL-1RaΔH mice ((▲), n= 6 to 18 females per time point), IL-1RaΔM mice ((♦), n= 7 to 17 females per time point), IL-1RaΔH+M mice ((●), n= 6 to 16 females per time point) and IL-1Ra deficient mice (IL-1Ra−/−, (○), n= 5 to 10 females per time point). **p<0.01 IL-1Ra−/− vs. control mice, IL-1RaΔH mice, IL-1RaΔM mice and IL-1RaΔH+M mice for each time point, as assessed by ANOVA, two-tailed t-test.

Figure S2. Immunohistochemical localization of IL-1Ra and myeloperoxidase on liver and spleen sections from control mice at 4h after LPS injection (10 mg/kg)

The tissue sections were observed at x200 magnification. MPO: myeloperoxidase

Figure S3. Quantification of total IL-1Ra mRNA levels in different organs in response to LPS challenge

Total RNA was extracted from the liver, the spleen and the lung of IL-1RaΔH, IL-1RaΔM, IL-1RaΔH+M and wild-type (WT) mice 4h after LPS challenge (10mg/kg). The expression level of total IL-1Ra mRNA was determined by quantitative real-time (RT)-PCR. Results are expressed relative to GAPDH mRNA level. Each symbol represents a single mouse. Horizontal lines show the mean. **p<0.01, ***p<0.001 vs. WT mice, as assessed by ANOVA, two-tailed t-test.

Figure S4. Quantification of total IL-1Ra mRNA levels in different organs 4h after NaCl or IL-1β injection (10 μg/kg)

Total RNA was extracted from the liver (A) and the spleen (B) of IL-1RaΔH, IL-1RaΔM, IL-1RaΔH+M and wild-type (WT) mice 4h after IL-1β challenge. RNA extraction was also performed on both organs isolated from WT mice after saline solution (NaCl) injection. The expression level
of total IL-1Ra mRNA was determined by quantitative real-time (RT)-PCR. Results are expressed relative to GAPDH mRNA level. Each symbol represents a single mouse. Horizontal lines show the mean. ***p<0.001 vs. IL-1β-treated WT mice, as assessed by ANOVA, two-tailed t-test.

Figure S5. Immunohistochemical localization of IL-1Ra on liver section from wild-type mice at 4h after NaCl or IL-1β injection (10 μg/kg)

The tissue sections were observed at x200 magnification.
Figure S1

Females

Body weight (g) vs. weeks

- WT
- II-1Ra
- II-1Ra
- II-1Ra
- II-1Ra
- II-1Ra

Weeks 6 to 15
Figure S4

A  Liver

B  Spleen

Relative L. rRNA/18S rRNA

WT  L-15a HET  L-15a HET  L-15a DKO  WT

IL-1β, 4h  NaCl

WT  L-15a HET  L-15a HET  L-15a DKO  WT

L-1p, 4h  NaCl