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IL-1α and IL-1β Recruit Different Myeloid Cells and Promote Different Stages of Sterile Inflammation

Peleg Rider,*1 Yaron Carmi,*1 Ofer Gutman,* Alex Braiman,* Idan Cohen,* Elena Voronov,* Malka R. White,* Charles A. Dinarello,† and Ron N. Apte*

The immune system evolved to protect the host from invading pathogens and to restore tissue homeostasis after injury or damage. The immune response against pathogens occurs through tissue-associated phagocytes, which discriminate self-from nonself through pattern-recognition receptors. Such recognition is subsequently followed by maturation to macrophages, which in turn initiate and drive the inflammatory/immune response against nonself molecules (1). In contrast, the response to tissue injury requires recognition of alarm signals in a sterile environment by cells of the innate immune system. As a result, myeloid cells are recruited into damaged tissue. Initially, neutrophils are recruited (2), followed by phagocytes of the mononuclear lineage, which differentiate and redefine their transcriptome to express genes involved in tissue repair (3). The identity of cell components that are released exclusively during necrosis and how the subsequent inflammatory response is orchestrated to restore tissue homeostasis are under current extensive investigations.

Most cells are vulnerable to hypoxic conditions due to the loss of mitochondrial respiration and acidosis (4). Because tissue injury is usually associated with insufficient blood supply and ischemia, the microenvironment of various pathologies involves anaerobic and lactic acid metabolism, which results in necrosis and not apoptosis (8). Most alarmins are constitutively expressed under homeostatic conditions and released upon necrosis (9–14), but not apoptosis (13, 15, 16). Surprisingly, endogenous alarmins can interact with TLR (heat shock proteins, high mobility group box 1 [HMGB1]) or type I IL-1R (IL-1R1), which are also key receptors in recognizing and directing the immune response against microbial pathogens. In a key study by Chen et al. (9), it was demonstrated that products of necrotic cells stimulate the immune system in an IL-1R1–dependent manner rather than through TLR signaling.

The IL-1 molecules comprise a major proinflammatory family of cytokines, which act mainly through the induction of a complex network of proinflammatory cytokines/mediators and via expression of integrins on leukocytes and endothelial cells (17–20). Of the 11 members of the IL-1 family of ligands, IL-1α and IL-1β are the two major agonistic molecules, whereas the IL-1R antagonist (IL-1Ra) is a physiological inhibitor of preformed IL-1. IL-1α, IL-1β, and the IL-1Ra bind to IL-1R1 signaling receptor. Although both IL-1α and IL-1β trigger inflammation in a pathway initiated through Myd88 activation and culminated in NF-κB–induced transcription of inflammatory genes, several findings suggest that

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both IL-1 agonists may have different activities. IL-1β is not expressed under normal homeostatic conditions by bone marrow-derived myeloid cells and is active only upon cleavage of its precursor by caspase-1 on the inflammasome, which also leads to its secretion (20, 21). Clinical and experimental observations have correlated IL-1β to pathologies in which inflammatory manifestations are observed. IL-1α, unlike IL-1β, can be found constitutively inside cells under normal homeostasis, and it is active in its precursor as well as calpain-processed mature form (21). IL-1α has a functional nuclear localization sequence and is active in the intracellular compartment, especially in the nucleus, as a transcription regulator, whereas it affects inflammation and immunity outside the cell. Thus, IL-1α belongs to a group of dual-function cytokines, together with IL-33, HMGB1, and IL-37 (12, 22–25).

Nevertheless, the possibility of differences in function of IL-1α and IL-1β during sterile inflammation is still hypothetical. Previous reports suggest that IL-1α serves as an alarm signal for initiating inflammation in response to tissue injury (9, 10). IL-1α is present in the skin, wherein injuries lead to a hypoxic state, resulting in massive cell necrosis (5–7). Indeed, we recently demonstrated that IL-1α is released from necrotic cells, whereas apoptotic cells retained IL-1α within the nucleus as a mechanism to preserve immune tolerance and prevent inflammation (26).

In this paper, we have studied the differential effects of IL-1α and IL-1β on the initiation and duration of inflammation induced by products of necrotic cells. By using the Matrigel plug (BD Biosciences) model of sterile inflammation, we have demonstrated that following hypoxia-mediated IL-1α release, IL-1α and IL-1β are further expressed at different phases of the inflammatory response by neutrophils and macrophages, respectively. Furthermore, different subsets of myeloid cells were found to be recruited following stimulation by cell-derived IL-1α or IL-1β, suggesting that each of these IL-1R agonists induces a distinct and unique response in inflammation. Better understanding these functions may enable future approaches to more accurately attenuate inflammation in different pathologies.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 mice were obtained from Harlan Laboratories. Other strains used were: IL-1α and IL-1β knockout (KO) mice (27), caspase-1 KO mice (28), IL-1βBP transgenic mice (29), and IL-1R1 KO mice purchased from The Jackson Laboratory (Bar Harbor, ME). The KO mice are homozygous for the relevant mutations and were backcrossed with WT C57BL/6 mice as described to maintain identical genetic background (27, 28). Animal studies were approved by the Animal Care Committee of Ben-Gurion University of the Negev (Beer Sheva, Israel). Male 6–8–wk-old mice were used in all experiments.

Cell culture

BD7 cells were cultured in a sealed anaerobic workstation (Concept 400; Ruskinn Technology/Joan) providing hypoxic conditions (O2 0.3%, 5% CO2, 95% N2, and 37°C). After 24 h incubation, the cells were collected and analyzed by Annexin V/propidium iodide (PI) staining (Bender MedSystems). The cell medium was collected and analyzed for IL-1α and IL-1β by ELISA (R&D Systems and BD Pharmingen).

HEK-T293 cells were transfected using the calcium phosphate technique in 24-well plates. Transfection was carried out with 400 μl CaCl2 (250 mM), which was mixed with 8 μg plasmid DNA, and then was dropped gently into 400 μl HEPES-buffered saline (×2 concentrated) during gentle agitation to produce aggregates. Next, the solution was added to the cell culture media. The plasmids added to the transfection solution are the IL-1α and IL-1β pEGFP-N1 constructs described below. After 24 h, cells were washed with PBS, lysed by four cycles of freeze-thawing, centrifuged, and supernatants were collected.

To obtain cells from different organs, mice were sacrificed, and heart, kidney, liver, and lungs were removed, digested with collagenase II (heart) or collagenase V at 37°C for 1 h, with stirring. Cells were separated using a 70-μm cell strainer (BD Pharmingen), and lysed by four cycles of freeze-thawing.

Constructs of IL-1–GFP

IL-1β cDNA was amplified by PCR using forward (5′-CAAGCTTGGCACCATGCTAAGG-3′) and reverse (5′-CGGTACCGAAGAACAGG-3′) primers cloned into the PGEM-T easy vector (Promega). Next, inserts were sequenced and cloned into pEGFP-N1 (Clontech) using HindIII and KpnI restriction sites. The IL-1α precursor was cloned with GFP as described (26).

The Matrigel plug assay of inflammation

Hyposic or normoxic cell medium (40 μl) or, alternatively, 5 μg HEK-T293 transfected lysate or 20 μg total lysate from cells obtained from mouse tissues, was mixed with liquid Matrigel (500 μl; BD Biosciences) and injected subcutaneously into IL-1α KO, IL-1β KO, or WT mice. For IL-1 neutralization, 1 μg/mouse IL-1Ra (Amgen) or neutralizing Abs to IL-1α (AF-400-NA; R&D Systems), anti-IL-1β (AF-401-NA; R&D Systems), or goat IgG (R&D Systems) were mixed with the Matrigel (10 μg/mouse).

To isolate infiltrating cells, Matrigel plugs (BD Biosciences) were removed from mice and incubated with an enzyme mixture containing 1 g/100 ml collagenase type IV, 20,000 units/100 ml DNase type IV, and 1 mg/ml hyaluronidase type V (Sigma-Aldrich, Relovot, Israel) in 2 ml HBSS (Life Technologies), at 37°C for 1 h, as described (18). Cells were counted using an automated cell counter (Digital Bio).

Detection of IL-1α and IL-1β

Lysates of BD7 cells were separated on PAGE, transferred to nitrocellulose membranes (Whatman), and blotted with goat anti-C-terminal IL-1α Abs (AF-400-NA; R&D Systems) or with polyclonal rabbit anti–IL-1α N-terminal peptide Abs raised against the 21-aa sequence CYSENE-DYSSAIDHLSNLNQKS. To measure the total protein load, mouse anti–β-actin (691001; MP Biomedical) was used. Additionally, transfected HEK-T293 cell lysates were blotted with hamster anti–IL-1β (MAB4102; R&D Systems) and mouse anti–GFP (MMS-118P; Covance).

Total RNA was extracted from the cells (RNeasy; Qiagen, Valencia, CA) and quantified using a NanoDrop (ND-1000 spectrophotometer; NanoDrop Technologies). cDNA Reverse-Transcription was performed with 1 μg total RNA as a template using random hexamers and MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). Subsequently, IL-1α expression was monitored using the Real-Time PCR ABI Prism 7500 sequence detection system (Applied Biosystems). Primers used for real-time PCR were β-actin: forward, 5′-GGTCTCACAATCATGCTCGGG-3′ and reverse, 5′-GGGTCAAGATAGGCTCCTATG-3′; and IL-1α: forward, 5′-CCGAGTTTCAATGCTCTTT-3′ and reverse, 5′-ACTGTGGAGATGATGC-TT-3′.

Confocal microscopy

BD7 cells and HEK-T293–transfected cells were plated and observed in Fluorodish plates (World Precision Instruments). BD7 cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with 1:200 diluted goat anti–IL-1α Ab (AF-400-NA; R&D Systems) and secondary donkey anti-goat Cy3 (Jackson ImmunoResearch Laboratories).

For frozen sections, immunofluorescent stained Matrigel plugs (BD Biosciences) were fixed in 4% paraformaldehyde for 6 h and equilibrated in a 20% sucrose solution for 24 h, embedded in frozen tissue matrix (Tissue-Tek OCT, Torrance, CA), and frozen at −80°C. The 12-μm-thick sections were blocked with 20% nonimmune goat serum and stained with 1:50 diluted goat anti–IL-1β Ab (AF-401-NA; R&D Systems), goat anti–IL-1α (AF-400-NA; R&D Systems), or 1:100 diluted rat anti–CD11b (M1/70; eBioscience) Abs. Specimens were subsequently stained with secondary Abs conjugated with either Cy2 or Cy3 (Jackson ImmunoResearch Laboratories).

Cells were examined using a 405-nm excitation laser line for either DAPI in fixed BD7 cells. Microscopy was performed with an Olympus Fluoview FV1000 confocal microscope (Olympus).

Flow cytometry

Single-cell suspensions obtained from Matrigel plugs (BD Biosciences) were analyzed using flow cytometry (FACSCanto; BD Biosciences). Datasets were analyzed using FlowJo software (Tree Star). mAbs conjugated to FITC, PE, PE-Cy7, PE-Cy5.5, allopurinolycyin, allopurinocyanin-Cy7, or Pacific Blue specific for the following Ags were used: CD11b...
levels (Fig. 1B) elevated under hypoxia, as indicated by mRNA transcripts and protein higher compared with Matrigel plugs (BD Biosciences) containing supernatants of hypoxic BD7 was removed from mice 24 h following injection, enzymatically digested, and supernatants from hypoxic or normoxic BD7 cells and injected s.c. as such, or with IL-1Ra, into WT mice (Supplemental Fig. 3C), indicate that the precursor of IL-1α is constitutively expressed in keratinocytes and other types of epithelial cells.

Because most tissue injuries are associated with decreased perfusion and ischemia resulting in cellular hypoxic stress, we cultured BD7 keratinocytes under normoxic and hypoxic conditions and examined their expression of IL-1α. As indicated by immunofluorescent staining, both normoxic and hypoxic BD7 keratinocytes express intracellular IL-1α, with similar localization of IL-1α in the cytoplasm and nucleus (Fig. 1A). In contrast, upon apoptotic stress, the nuclear localization of IL-1α in BD7 keratinocytes is increased (Supplemental Fig. 1). Interestingly, yet consistent with our previous reports (18, 31), IL-1α was upregulated under hypoxia, as indicated by mRNA transcripts and protein levels (Fig. 1B). Most importantly, by using novel Abs against both the N-terminal and C-terminal residues of IL-1α produced in our laboratory, Western blot analysis of BD7 lysates clearly indicated that the dominant form of IL-1α is a 31-kDa precursor rather than its processed 17-kDa form (Fig. 1B, middle panel).

Because alarmins are released from dying cells to signal danger, we next examined the effects of continuous hypoxic conditions on IL-1α release from BD7 keratinocytes. Initially, the level of necrosis was evaluated in BD7 keratinocytes exposed to hypoxia for 24 h. As indicated by PI staining, whereas only marginal levels of necrosis were observed in normoxic BD7 cells, ~95% of the hypoxic BD7 cells were necrotic (Fig. 1C). Moreover, the levels of IL-1α in supernatants from hypoxic BD7 cells were between four and five times higher compared with supernatants from normoxic BD7 cells (Fig. 1D, left panel). Significantly, IL-1β was not detected under hypoxic or normoxic conditions, as detected by ELISA (data not shown). We next assessed the capacity of supernatants from hypoxic and normoxic BD7 cells to recruit leukocytes in a Matrigel plug assay (BD Biosciences) of inflammation. Thus, Matrigel (BD Biosciences) was mixed with supernatants from hypoxic or normoxic BD7 cells and injected s.c. into WT mice. Matrigel plugs (BD Biosciences) were removed from mice 24 h following injection, enzymatically digested, and the infiltrating cells were counted. As shown in Fig. 1D, the number of leukocytes that infiltrate into Matrigel plugs (BD Biosciences) containing supernatants of hypoxic BD7 was ~4-fold higher compared with Matrigel plugs (BD Biosciences) containing supernatants of normoxic BD7 cells. Significantly, a considerable reduction in the number of infiltrating leukocytes into plugs was detected under hypoxic or normoxic conditions, as detected by ELISA (data not shown). We next assessed the capacity of

Statistical analyses
Each experiment was performed three times. In Matrigel plug assays (BD Biosciences), each experimental group consisted of at least three mice. Significance of results was determined using the nonparametric one-way ANOVA, when multiple groups are analyzed, or nonparametric Student t test.

Results
The precursor form of IL-1α is the active component that recruits leukocytes when released from hypoxic cells

Because alarmins are released from dying cells to signal danger, we next examined the effects of continuous hypoxic conditions on IL-1α release from BD7 keratinocytes. Initially, the level of necrosis was evaluated in BD7 keratinocytes exposed to hypoxia for 24 h. As indicated by PI staining, whereas only marginal levels of necrosis were observed in normoxic BD7 cells, ~95% of the hypoxic BD7 cells were necrotic (Fig. 1C). Moreover, the levels of IL-1α in supernatants from hypoxic BD7 cells were between four and five times higher compared with supernatants from normoxic BD7 cells (Fig. 1D, left panel). Significantly, IL-1β was not detected under hypoxic or normoxic conditions, as detected by ELISA (data not shown). We next assessed the capacity of supernatants from hypoxic and normoxic BD7 cells to recruit leukocytes in a Matrigel plug assay (BD Biosciences) of inflammation. Thus, Matrigel (BD Biosciences) was mixed with supernatants from hypoxic or normoxic BD7 cells and injected s.c. into WT mice. Matrigel plugs (BD Biosciences) were removed from mice 24 h following injection, enzymatically digested, and the infiltrating cells were counted. As shown in Fig. 1D, the number of leukocytes that infiltrate into Matrigel plugs (BD Biosciences) containing supernatants of hypoxic BD7 was ~4-fold higher compared with Matrigel plugs (BD Biosciences) containing supernatants of normoxic BD7 cells. Significantly, a considerable reduction in the number of infiltrating leukocytes into plugs was

**FIGURE 1.** The precursor form of IL-1α is released from hypoxic cells and recruits leukocytes into Matrigel plugs (BD Biosciences). A. Immunofluorescence staining of IL-1α (green) in BD7 keratinocytes cultured for 24 h under normoxic or hypoxic conditions (original magnification ×400). B. Left panel, Relative quantification (RQ) of IL-1α mRNA in BD7 keratinocytes cultured for 24 h under normoxic or hypoxic conditions. Results represent mean ± SEM of three biological repeats. *p < 0.05. Center panel shows Western blot analysis of extracts from BD7 keratinocytes using anti-N-terminal IL-1α (top panel) or anti-C-terminal IL-1α Abs (middle panel). Bottom panel shows β-actin as loading control. Mean ± SEM of IL-1α relative increase (measured by band densitometry) from three blots is represented in right panel. C. Flow cytometry analysis of Annexin V/PI staining in 24 h normoxic or hypoxic BD7 keratinocytes. D. Mean levels of IL-1α in supernatants of normoxic or hypoxic BD7 keratinocytes (left panel). Results represent average ± SEM. **p < 0.01. Supernatants of normoxic or hypoxic BD7 keratinocytes injected as such, or with IL-1Ra, into WT mice (right panel). Graphs indicate the total number of infiltrating cells in Matrigel plugs (BD Biosciences) after 24 h. Data shown are from a single experiment. Experiments were repeated three times, and each experimental group consisted of three mice. Results represent the average ± SEM. **p < 0.01 compared with the hypoxia subgroup.
observed upon addition of IL-1Ra to Matrigel (BD Biosciences) containing hypoxic supernatants (Fig. 1D, right panel).

**IL-1α and IL-1β affect different phases of sterile inflammation**

It was of interest to establish whether the initial recruitment of leukocytes depends only on IL-1α derived from necrotizing cells or whether IL-1 of host origin is also involved in it, as IL-1Ra blocks the binding of both IL-1β and IL-1α, whether from necrotizing cells or host origin. For this purpose, Matrigel (BD Biosciences) containing supernatants of hypoxic BD7 keratinocytes were injected as such or mixed with neutralizing Abs against IL-1α or IL-1β, into WT, IL-1α, or IL-1β KO mice. As shown in Fig. 2A, Matrigel (BD Biosciences) mixed with hypoxic supernatants induced a massive leukocyte infiltration 24 h following injection to WT mice. In contrast, the number of leukocytes was markedly reduced when anti–IL-1α Abs were added to the plugs (Fig. 2A). Neutralizing IL-1β Abs reduce the infiltrating cell numbers but to a much lesser extent (Fig. 2A). Interestingly, no significant reduction in the amount of the leukocyte infiltrate in Matrigel plugs (BD Biosciences) was observed in IL-1α or IL-1β KO mice, indicating that IL-1α of necrotic cell origin, rather than IL-1 of host origin, mediates the early leukocyte infiltrate (Fig. 2A). Because the inflammatory process requires a sustained influx of leukocytes for several days, we next assessed the number of infiltrating cells 5 d after injection. Indeed, in comparison with day 1, the number of infiltrating cells increased ∼2-fold in Matrigel plugs (BD Biosciences) containing hypoxic supernatants (Fig. 2B). Surprisingly, neutralization of IL-1α or injection of supernatants into IL-1α KO host mice resulted in a noticeable reduction in the number of infiltrating leukocytes. However, neutralizing IL-1β or injection of Matrigel plugs (BD Biosciences) into IL-1β KO mice resulted in a more significant reduction in the number of infiltrating cells (Fig. 2B). These results indicate that for initiation of inflammation, IL-1α is essential, whereas for propagation of the inflammatory response, IL-1β plays a dominant role.

Next, we characterized the cells that infiltrate into Matrigel plugs (BD Biosciences) containing hypoxic supernatants 24 h and 5 d following injection. The vast majority of infiltrating cells was of myeloid lineage with a phenotype of SSC<sup>high</sup>/CD11b<sup>+</sup>/GR1<sup>high</sup> neutrophils and or CD115<sup>+</sup>/CD11b<sup>+</sup>/Ly6C<sup>high</sup> monocytes. However, on day 5, the infiltrating myeloid cell populations were dramatically altered, and mainly CD11b<sup>+</sup>/Ly6C<sup>+</sup>/F4-80<sup>high</sup> mature macrophages, rather than monocytes, were observed, concomitant with neutrophil disappearance (Supplemental Fig. 2). Addition of anti–IL-1α Abs to Matrigel (BD Biosciences) containing hypoxic supernatants or injection into IL-1α KO mice largely reduced neutrophil recruitment on day 1 (Fig. 2C), yet only marginally affected the accumulation of monocytes and macrophages. For example, neutrophils consisted 46% of the total infiltrate in WT mice, 6.8% in IL-1α KO, and IL-1β KO mice 38.7%. Upon blocking IL-1β, either by addition of anti–IL-1β Abs to Matrigel (BD Biosciences) or by injection of supernatants to IL-1β KO mice, neutrophil recruitment did not change on day 1 (Fig. 2C), yet we observed significantly reduced macrophage recruitment into Matrigel plugs (BD Biosciences) on day 5 compared with WT mice (Fig. 2D). Taken together, these results suggest that IL-1α released from hypoxic cells initiates inflammation, whereas host-derived IL-1α and, more pronouncedly, IL-1β are needed to potentiate and maintain the inflammatory process.

**IL-1α and IL-1β are expressed in different myeloid subsets during inflammation**

We next sought to assess the contribution of host-infiltrating myeloid cells to IL-1α and IL-1β production in the inflamma-
tory process. For this purpose, infiltrating cells were recovered from Matrigel plugs (BD Biosciences) containing hypoxic super-
natants 24 h and 5 d following injection into WT mice and stained for intracellular IL-1 molecules and lineage markers. On day 1, IL-1α was expressed in 15% of the cells that were Gr1hi/CD11bhi neutrophils. Expression of IL-1β was only marginal in infiltrating cells into Matrigel plugs (BD Biosciences) on day 1; it was found only in ∼0.8% of infiltrating cells, all Gr1hi/CD11bhi neutrophils (Fig. 3B). On day 5, IL-1α in CD11b-positive myeloid cells was almost nonexistent. In contrast, the expression of IL-1β was markedly elevated in day 5 Matrigel plug (BD Biosciences)-infiltrating cells, reaching up to 3% of the cells, all macrophages (Fig. 3A). To corroborate these results, frozen sections of Matrigel plugs (BD Biosciences) containing hypoxic supernatants were costained for the IL-1 and CD11b molecules. On day 1, high levels of IL-1α, and also IL-1β, were found in CD11b-positive cells (Fig. 3B). Consistent with the flow cytometry results (Fig. 3A), low levels, if any, of IL-1α were found on day 5, whereas IL-1β was abundantly expressed in CD11b-positive myeloid cells at this time.

**Recruitment of macrophages during sterile inflammation is caspase-1 dependent**

In contrast to IL-1α, which is active as a precursor, IL-1β must be cleaved by caspase-1 (also known as IL-1–converting enzyme) on the inflammasome prior to its secretion (32). However, several studies suggested that IL-1β might also be activated and processed in a caspase-1–independent manner by extracellular proteases, particularly in cases in which neutrophils are abundant (33). We thus assessed the role of caspase-1 in sterile inflammation, relevant to the later phase of macrophage recruitment and the propagation of IL-1α–induced inflammation. Hence, Matrigel (BD Biosciences) containing supernatants of hypoxic BD7 cells were injected into WT and caspase-1 KO mice. Matrigel (BD Biosciences) was also injected into IL-18BP transgenic mice because caspase-1 is required for the processing of IL-18, which is known

**FIGURE 3.** IL-1α and IL-1β are differentially expressed by myeloid cells. A, Intracellular staining of IL-1α and IL-1β in total cell infiltrate in Matrigel plugs (BD Biosciences) containing supernatants of hypoxic BD7. B, Frozen sections of Matrigel plugs containing supernatants of hypoxic BD7 costained for CD11b (green) and IL-1α (yellow) or for CD11b (green) and IL-1β (yellow). Photomicrographs and FACS analysis, shown in this figure, are from a representative experiment out of three performed (original magnification ×400).

**FIGURE 4.** Recruitment of macrophages is caspase-1 dependent. A, Total infiltrating cells on day 1 in Matrigel plugs (BD Biosciences) containing supernatants of hypoxic BD7 cells. B, Total infiltrate cell number of Matrigel plugs, containing supernatants of hypoxic BD7 cells, on day 5. C, Flow cytometry analysis of infiltrating cells in Matrigel plugs on day 5. Numbers indicate the percentage of F4-80+CD11b+ cells. Results shown are from a three experiment (n = 9). *p < 0.05, **p < 0.001 versus WT group.
for its ability to mediate inflammation in ischemic disorders (34). As shown in Fig. 4A, in 24 h Matrigel plugs (BD Biosciences), the number of infiltrating cells was comparable in all experimental groups. However, on day 5, a marked reduction in inflammatory cell number was observed in Matrigel plugs (BD Biosciences) in caspase-1 KO mice. A less significant reduction was observed in plugs in IL-18BP transgenic mice, in which IL-18 activity is neutralized by the binding protein (Fig. 4A). Similar to the results obtained in IL-1β KO mice, reduced levels of macrophages were found in plugs from caspase-1 KO mice (Fig. 4C). Therefore, caspase-1–processed IL-1β plays a major role in the propagation of sterile inflammation, possibly also assisted by additional factors, such as IL-18 cleavage.

*IL-1α and IL-1β mediate different myeloid cell infiltrations: precursor IL-1α induces neutrophil infiltration, whereas mature IL-1β recruits macrophages*

Our data (Fig. 1B, middle panel, Supplemental Fig. 3B) as well as other reports strongly indicate that IL-1α is mainly present as a precursor (22) rather than in its processed form. In addition, even after processing to obtain mature IL-1α, most of the protein still exists in its precursor form (35). It has been suggested that mature IL-1α and IL-1β in their native or recombinant forms exert the same biological activities. However, it is not known whether the precursor of IL-1α, which is the abundant form of this cytokine in many cell types, has the same biological activities as the mature forms of IL-1α or IL-1β. Furthermore, as shown above, IL-1α and IL-1β are expressed at different phases of the inflammatory response and are derived from different infiltrating cells, suggesting that they may have distinct biological roles. To approach the latter, we decided to examine the activity of the IL-1 molecules in their native forms as produced by cells. Thus, HEK-293T cells were transfected with constructs of either the precursor form of IL-1α or the mature form of IL-1β fused to GFP. Confocal microscopic analysis indicated typical cellular compartmental localization of precursor IL-1α in the nucleus, whereas IL-1β was found in the cytosol (Fig. 5A). To verify that each clone expressed only one

**FIGURE 5.** IL-1α and IL-1β recruit different myeloid infiltrating cells into Matrigel plugs (BD Biosciences). A. Confocal microscopy of HEK-T293 cells transfected with either GFP alone, GFP fused to the precursor of IL-1α (precIL-1α GFP), or to mature IL-1β (matIL-1β GFP) (original magnification ×400). B. Western blot analysis of lysates from HEK-T293–transfected cells stained with anti–IL-1α, anti–IL-1β, or anti-GFP Abs. C. Flow cytometry analysis of cell infiltrate in Matrigel plugs containing lysates of transfected cells 24 h after injection. Numbers indicate the percentage of CD11b-gated cells. Mean ± SEM of neutrophil and macrophage recruitment from three different experiments is presented in right panels. *p < 0.05 versus GFP group.
type of the IL-1 molecules, lysates from the transfected cells were subjected to Western blot analysis using specific Abs. Indeed, immunoblotting of cell lysates using anti–IL-1α Abs resulted in two bands only in cells transfected with the IL-1α construct: one major band of 59 kDa, which represents the precursor form of IL-1α fused to GFP, and a weaker band at 45 kDa, representing the processed form of IL-1α fused to GFP (Fig. 5B, top panel). Blotting with anti–IL-1β Abs resulted in a single 45-kDa band only in cells transfected with the mature IL-1β construct, representing mature IL-1β fused to GFP (Fig. 5B, middle panel). The Western blot was also performed with anti-GFP Abs that verified the expression patterns of the vectors in control and IL-1–transfected cells (Fig. 5B, bottom panel).

Next, we assessed whether cell-derived IL-1α and IL-1β in the above-described experimental system recruit the same or different inflammatory cells. Thus, Matrigel (BD Biosciences) was mixed with lysates from either GFP-transfected cells, precursor IL-1α–transfected cells, or mature IL-1β–transfected cells and injected into WT mice. After 24 h, plugs were removed, and the infiltrating cell populations were analyzed by flow cytometry. Although GFP-containing lysates had a minute effect on cell recruitment, the lysates of both IL-1–GFP-containing Matrigels (BD Biosciences) displayed massive cell recruitment (Supplemental Fig. 4). As shown in Fig. 5C, Matrigel (BD Biosciences) mixed with lysates from IL-1α–transfected cells recruited significantly higher levels of SSC<sup>high</sup>/CD11b<sup>+</sup>/Gr1<sup>high</sup>/Ly6G<sup>high</sup> neutrophils compared with Matrigel (BD Biosciences) containing GFP- or IL-1β–transfected cells. In contrast, Matrigel (BD Biosciences) mixed with lysates from IL-1β–transfected cells induced massive recruitment of SSC<sup>high</sup>/CD11b<sup>+</sup>/CXCR4<sup>+</sup>/F4-80<sup>+</sup> inflammatory monocytes, whereas Matrigel (BD Biosciences) mixed with lysates from GFP- or IL-1α–transfected cells recruited only basal levels of these cells (Fig. 5C). We verified that the recruitment of these populations was dependent on IL-1 signaling by injecting into IL-1R KO mice (Supplemental Fig. 4). These results indicate that the different molecules of IL-1 recruit distinct populations of myeloid cells.

Cell lysates of lung, liver, heart, and kidney trigger neutrophil recruitment in an IL-1α–dependent manner

Finally, to confirm that IL-1α–mediated sterile inflammation, as induced by necrotic keratinocytes, is of physiologically relevance to tissue damage in vivo, cell lysates from different representative organs, including heart, kidney, liver, and lungs, were assessed for their inflammation-inducing potential. Thus, tissue cells were separated, isolated, and lysates were prepared from them. We chose this approach because IL-1α is mainly intracellularly located, and we aimed to assess the role of tissue cell-derived IL-1α in the induction of inflammation, trying to minimize the effects of extracellular proteins. Subsequently, equal amounts of lysates from single-tissue cell suspensions, as calibrated by protein levels, were mixed with Matrigel (BD Biosciences) and injected into WT mice, with anti–IL-1α–neutralizing Abs or with control IgG. After 24 h, Matrigel plugs (BD Biosciences) were removed, and infiltrating cells were counted and analyzed by flow cytometry. The results show that cell products originating from all tissues examined were able to recruit infiltrating cells into Matrigel plugs (BD Biosciences), and the infiltration was significantly reduced upon IL-1α neutralization (Fig. 6A). Furthermore, flow cytometry analysis showed a marked reduction in CD11b<sup>+</sup>/Gr1<sup>high</sup>/Ly6G<sup>high</sup> neutrophils following IL-1α neutralization compared with the isotype IgG control (Fig. 6B). Thus, these results show that IL-1α serves as an alarmin, initiating sterile inflammation in different tissues, such as skin, heart, kidney, liver, or lungs following necrosis.

Discussion

IL-1α has recently been described as a major alarmin molecule involved in the initiation of sterile inflammation, as evidenced by early neutrophil infiltration into affected sites. This has been shown in experimental models of peritonitis (9, 10, 36) and in a model of inflammation induced in Matrigel plugs (BD Biosciences) supplemented with products of damaged tissue, which we have established in our laboratory (26). IL-1α, IL-33, and HMGB1 belong to a family of dual-function cytokines, which can be located in the nucleus of cells where they perform regulatory functions, or they can be released into the microenvironment and act as alarmins, inducing inflammation (11–13). We have shown that intracellular IL-1α, which is constitutively expressed at low levels in epithelial or mesenchymal cells, is released upon cell necrosis, whereas in apoptotic cells, it avidly binds to the chromatin and is not released into the microenvironment and thus possibly acts to restrain inflammation. To induce necrotic cell death, hypoxia was chosen, as it represents one of the major forms of stress and is involved in ischemia-induced tissue damage.
which is followed by inflammation and repair. In addition to secretion of preformed IL-1α, its expression in cells under hypoxic stress is upregulated before cell death. Furthermore, we have shown that the uncleaved precursor of IL-1α, which can bind to IL-1R, induces an inflammatory response, whereas the propiece of the IL-1α molecule, which lacks the IL-1R-binding domain, is inactive (26). Both the precursor of IL-1α and the mature form, derived from cleavage of the precursor by calpain, can bind the signaling IL-1R. An in vitro study on IL-1 binding to its receptor showed that although IL-1β binds the receptor only as a processed cytokine, IL-1α can bind it both in its precursor or mature form (37). However, it has not been established whether these two IL-1α molecules have identical or distinct functions, and it is also not known whether they signal through the same or different signaling complexes.

Previous studies have emphasized the role of either IL-1α or IL-1β in sterile inflammation, but have not elaborated on their integrated role in this process. In this study, we have assessed in a comparative manner the role of IL-1α and IL-1β in initiation and propagation of the sterile inflammatory response. In the initiation of sterile inflammation, which involves neutrophil recruitment, IL-1α of dying cell origin not only acts alone, but also possibly induces IL-1α production by host cells, and IL-1α of both sources synergizes in neutrophil recruitment (38) (Supplemental Fig. 3A). In contrast, at later stages of the sterile inflammatory response (day 5), in which the major infiltrating cell is the macrophage, IL-1β is the dominant IL-1 molecule. Use of caspase-1 KO mice highlighted the requirement of the classical IL-1β processing/secretion pathway in sterile inflammatory responses. Similar results were reported on the involvement of the inflammasome, especially of the NLRP3 type, in processing and secretion of IL-1β in response to various agents that induce sterile inflammation, such as nanoparticles (39), uric acid crystals (40), silica crystals, and alumina salts (41) as well as products of necrosis that induce sterile inflammations (42). Indeed, our studies have shown that IL-18, which is also processed on the inflammasome, also plays some role in the inflammatory responses in our experimental system.

We exclude the possibility that the observed effects on cell recruitment are solely due to the differential appearance of the IL-1 molecules at distinct phases of the inflammatory response, emphasizing differentially distinct biological functions of IL-1α and IL-1β in inflammation. It has been suggested that rIL-1α and IL-1β, which represent the mature forms of the cytokines, have the same biological activities, as they bind to the same signaling receptor. However, the most abundant natural form of IL-1α is the precursor molecule, which is not present in rIL-1α preparations. Indeed, the processing of precursor IL-1α in cells by calpain is inefficient due to the presence of calpain inhibitors in cells and, in any case, is not essential for IL-1α function. The association between processing and secretion of IL-1α in myeloid cells, which are among the few cells that actively secrete this cytokine, has not been established as for IL-1β. Whereas most studies focused on the biological activity of mature IL-1α in inflammation, some reports also described activities of the precursor molecule. For example, in a mouse model of cerebrovascular inflammation, the precursor of IL-1α in platelets was shown to mediate infiltration of neutrophils (43), the effects of vitamin D on upregulation of the IL-1α precursor in skin keratinocytes (30), as well as the role of precursor IL-1α in collagen deposition in systemic sclerosis in fibroblasts (44). The exact pathway(s) of signaling of the different IL-1 molecules through IL-1RI and its biological consequences is not completely clear, as no comparative studies with the different IL-1 molecules derived from cells have been performed. It might also be possible that other products of damaged tissue contribute to the inflammatory response of cell-derived IL-1. However, the dominant role of the IL-1 molecules in induction and maintenance of sterile inflammation is demonstrated by the reduction of cell recruitment to background levels upon IL-1 neutralization or using IL-1 KO mice. We assume that the unique effects of the IL-1 molecules on the inflammatory response are through a cascade of cytokines/chemokines that they induce. As such, it may be that different cell-associated IL-1 molecules may induce a different array of molecules, which may fine-tune the inflammatory responses. Preliminary results in this direction demonstrate that cytokines are induced in a stronger manner by the precursor of IL-1α than by mature IL-1β and that G-CSF, which has been involved in neutrophil infiltration, is induced only by the precursor of IL-1α (P. Rider, unpublished observations). The role of cell-associated IL-1α in inducing sterile inflammation is a general phenomenon, because lysates from heart, kidney, liver, and lungs induced an inflammatory response in an IL-1α-dependent manner. These results are in accordance with our previous results in which necrotic cells lacking IL-1α (lysates of primary fibroblasts from IL-1α KO mice or from WT mice treated with anti–IL-1α Abs) failed to induce this early inflammatory response. It is notable that relatively small quantities of cell lysates of the different organs were used to induce inflammation, and we also assured that the cell content, rather than extracellular proteins, was used to induce inflammation. Our preliminary results also indicated the role of IL-1α in skin wound healing, which is retarded in IL-1α-/-KO mice (O. Guttman, unpublished observations). This further substantiates the results of other studies showing that cell-associated IL-1α is an alarming of sterile inflammation (9, 10, 36) and supports models of tissue damage to the liver (45, 46), heart (47), kidney (48), and lungs (49), showing dependence on IL-1 signaling. It has to be emphasized that the differential role of IL-1α and IL-1β described in this study is relevant to sterile inflammation, and in other models, the specific action of the IL-1 molecules may differ.

Overall, our results show a sequential action for IL-1α and IL-1β in sterile inflammation. Initially, the inflammatory response is induced by IL-1α derived from damaged cells that possibly induces host-derived IL-1α that helps to maintain the response. IL-1β is important for the propagation of the inflammatory response. In addition, we have delineated specific functions of cell-associated precursor IL-1α that are distinct from mature IL-1β; thus, precursor IL-1α recruits neutrophils, whereas mature IL-1β recruits macrophages. These results may lead to novel approaches to intervene in processes in which there is infiltration of specific myeloid cells in tissue damage and inflammation.

Disclosures
The authors have no financial conflicts of interest.

References


