Cutting Edge: IL-1α Is a Crucial Danger Signal Triggering Acute Myocardial Inflammation during Myocardial Infarction

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Myocardial infarction (MI) induces a sterile inflammatory response that contributes to adverse cardiac remodeling. The initiating mechanisms of this response remain incompletely defined. We found that necrotic cardiomyocytes released a heat-labile proinflammatory signal activating MAPKs and NF-κB in cardiac fibroblasts, with secondary production of cytokines. This response was abolished in Myd88−/− fibroblasts but was unaffected in nlrp3-deficient fibroblasts. Despite MyD88 dependency, the response was TLR independent, as explored in TLR reporter cells, pointing to a contribution of the IL-1 pathway. Indeed, necrotic cardiomyocytes released IL-1α, but not IL-1β, and the immune activation of cardiac fibroblasts was abrogated by an IL-1R antagonist and an IL-1α–blocking Ab. Moreover, immune responses triggered by necrotic Il1a−/− cardiomyocytes were markedly reduced. In vivo, mice exposed to MI released IL-1α in the plasma, and postischemic inflammation was attenuated in Il1a−/− mice. Thus, our findings identify IL-1α as a crucial early danger signal triggering post-MI inflammation. The Journal of Immunology, 2015, 194: 499–503.

Myocardial infarction (MI) triggers an inflammatory response aimed at healing the infarct, but it also may foster the process of ventricular remodeling, leading to cardiac dysfunction (1). It is acknowledged that such inflammation is triggered by danger signals (damage-associated molecular patterns [DAMPs]) released by necrotic myocardium and sensed by pattern receptors from the TLR and the NLR families in neighboring cells (2). Although some contribution to these processes has been proposed for TLR2, TLR3, TLR4 (reviewed in Ref. 3), and NLRP3 (4), as well as for DAMPs, such as HMGBl and S-100 proteins (5), the very proximal signal triggering inflammation in the ischemic heart has not been established. Therefore, the current study was designed to identify the nature of this signal and its sensing mechanisms.

Materials and Methods

Animal experiments were approved by our institutional review board (authorizations 2477, 2484, 2669).

Mice, cells, and treatments

Wild-type (WT) mice in this study were C57BL6/J (Janvier Labs, Le Genest-Saint-Ilie, France), Myd88−/− mice (a gift from Dr. Thierry Roger, Lausanne University), Nlrp3−/− mice (obtained from Prof. Pascal Schneider, Lausanne University), and Il1a−/− mice (obtained from Prof. Yoichiro Iwakura, Tokyo University of Science, Chiba, Japan) were backcrossed to C57BL6/J. Mouse neonatal ventricular cardiomyocytes (CMCs) and cardiac fibroblasts (CFs) were isolated by differential plating and cultured as described (6). Some experiments were done using cells isolated from adult mouse hearts immediately after differential plating and before culture, to separate CMCS from all nonmyocyte cells (NMCs; containing CFs, vascular cells, and some resident macrophages). For all experiments, cells were maintained in modified PBS-glucose buffer (7). Necrosis of cells was done by two cycles of freeze-thaw lysis or by exposure to H2O2 (250 μM, 20 min) and confirmed by propidium iodide staining and troponin release (Troponin T Immunoassay; Roche Diagnostics) (8). Conditioned media (2 ml) were obtained by necrosis of 8 × 10⁷ cells and cleared by centrifugation (10 min, 13,000 rpm, 4°C). Anakrin (Swedish Orphan Biovitrum) and IL-1α–blocking Ab (eBioscience) were used at 0.01–10 μg/ml. Heat inactivation of conditioned media was done by heating for 1 h at 95°C. DNase I (Roche) was used at 10 μg/ml. Salmonella LPS (Sigma) was used at 1 μg/ml.

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Abbreviations used in this article: AAR, area at risk; CF, cardiac fibroblast; CK, creatine kinase; CMC, cardiomyocyte; DAMP, danger-associated molecular pattern; IV, left ventricle; MI, myocardial infarction; MIR, myocardial ischemia and reperfusion; MPO, myeloperoxidase; NMC, nonmyocyte cell; WT, wild-type.

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Cytokines, creatine kinase, and troponin

CCL2/MCP-1, IL-6, and IL-1α were measured by commercial ELISAs (R&D Systems and BioLegend). Plasma creatine kinase (CK) and troponin were assayed using a Cobas 8000 automated analyzer (Roche).

Western immunoblotting

Nuclear and cytosolic proteins were immunoblotted as described (7) using Abs against ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK, phospho-JNK, phospho-c-JUN (Cell Signaling Technology), NF-κB p65 (Santa Cruz Biotechnology), IL-1β, TATA-binding protein (Abcam), and tubulin (Sigma).

Cardiac myeloperoxidase activity

Myeloperoxidase (MPO) was measured in myocardial extracts using tetramethylbenzidine and H2O2, as described (9), and was expressed in mU/mg proteins.

EMSA

Nuclear proteins (10 μg) were incubated with an [α-32P]dATP–labeled NF-κB probe (5′-GGCAGTTGAAGGGGACTTTCCCAGG-3′), and EMSA was performed as described (7).

TLR assay

HEK-293 cell lines overexpressing a given murine TLR protein and an inducible NF-κB reporter gene (HEK-Blue TLR cells assay; InvivoGen, Toulose, France) were treated with conditioned media from necrotic or living CMCs for 4 h. Specific TLR ligands were used as positive controls. TLR+, TNF-sensitive cells expressing the reporter gene only were used as negative controls.

Mouse MI

Myocardial ischemia (30 min) and reperfusion (2 h) were performed as described (8). At the end of reperfusion, blood and heart were obtained for measurement of cytokines. Area at risk (AAR; in percentage of left ventricle [LV]) and infarct size (in percentage of AAR or LV) were determined using standard Evans blue/triphenyl tetrazolium chloride staining (8).

RNA analyses by real-time PCR

RNA was reversed transcribed, and real-time PCR was performed using standard procedures. Gene expressions were normalized to endogenous controls (Rpl19, Gapdh, or Rps18). The list of oligonucleotides is given in Supplemental Table I.

Results and Discussion

In a first series of experiments, we determined whether dying CMCs release one or more factors able to trigger innate immune responses in CFs, which may represent “sentinel cells” to detect CMCs’ damage (10). Mouse neonatal CMCs were killed by H2O2 (8) or by freeze–thaw lysis (11), and necrosis was confirmed by propidium iodide staining and troponin release (Fig. 1A, 1B). CFs exposed to conditioned medium obtained from these necrotic CMCs displayed strong transcriptional activation and release of the chemokine CCL2 and the cytokine IL-6 (Fig. 1C–E), associated with the activation of ERK, JNK, p38, NF-κB, and AP-1 (Fig. 1F–H). Because nucleic acids and proteins are two major families of DAMPs (5), we evaluated whether the proinflammatory activity of CMCs would be altered by treatment with DNase or by heating to denature proteins. The stimulated transcription of Ccl2 and Il6 was unaffected by DNase but was eliminated by heating (Fig. 2A, 2B), supporting a protein origin of the DAMP in the medium. These findings indicate that one or more soluble, heat-sensitive DAMPs released by necrotic CMCs promote an immediate, reflex, innate immune response in CFs that could represent a primary source of inflammatory cytokines during MI.

Recent studies indicated that TLR2, TLR3, TLR4 (3), and NLRP3 (4) participate in the cardiac response to ischemia. To explore the role of TLRs or NLRP3 in our model, we examined the response of CFs deficient either in the TLR adapter MyD88 or in Nlrp3 to necrotic CMCs. Transcriptional activation of Ccl2 and Il6 was suppressed in Myd88−/− CFs (Fig. 2C), suggesting an instrumental role for a TLR-

FIGURE 1. Conditioned medium from necrotic CMCs triggers innate immune responses in CFs in vitro. CMC necrosis [(A) nuclear PI staining, (B) troponin T release] induced by H2O2 (250 μM, 20 min) or freeze–thaw (FT). CMCs in culture medium or in PBS-glucose (PBS-G) served as controls (n = 3–5 per conditions). mRNA of Ccl2 and Il6 in CFs exposed to conditioned medium from CMCs killed by H2O2 (CM-H2O2) (C) or conditioned medium obtained by freeze–thaw (CM-FT) (D) (n = 3–7). CFs in PBS-G for 4 h served as controls. (E) MCP-1 and IL-6 release by CFs exposed (8 h) to CM-FT or PBS-G (n = 4). (F) Phosphorylation of ERK, JNK, and p38. (G) Cytoplasmic/nuclear NF-κB p65 and nuclear phospho-c-Jun. (H) NF-κB DNA binding activity (EMSA) in CFs exposed to CM-FT. Tubulin and TBP (TATA-binding protein) were used as loading control in (F) and (G). LPS (1 μg/ml, 60 min) was a positive control in (G) and (H). Data are representative of at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. U, untreated.

Data analyses

Statistical analyses were done using GraphPad Prism 6.0 software. The Student t test was used for simple comparisons, and multiple comparisons were done with ANOVA, followed by the Dunnett or Bonferroni post test. A p value < 0.05 was considered statistically significant.
mediated process, consistent with previous findings implicating MyD88 in post-MI inflammation (12). In contrast, Nlrp3 deficiency did not have a significant influence (Fig. 2D), arguing against a role for this receptor in the early detection of necrotic CMCs by CFs. This is at variance with a recent study reporting NLRP3 activation in CFs 3 d after MI (4), suggesting that NLRP3 engagement is a delayed, but not immediate, event in the post-MI immune response.

To explore further the role of TLRs, we examined the proinflammatory activity of conditioned medium in TLR-deficient HEK 293 reporter cells selectively transfected with several TLR family members (with the exception of TLR1 and TLR6, which are expressed at low levels in native HEK 293 cells). Unexpectedly, conditioned medium did not activate any of the TLR-transfected cells (Fig. 2E), implicating the participation of an alternative MyD88-dependent signaling cas-

FIGURE 2. Necrotic CMCs release a heat-sensitive DAMP, triggering MyD88-dependent and NLRP3- and TLR-independent signaling pathways in CFs. Ccl2 and Il6 mRNA in CFs exposed (4 h) to conditioned medium obtained by freeze–thaw (CM-FT) (A), with or without DNase I or heat inactivation (HI) (B). CFs in PBS-glucose (PBS-G, 4 h) served as controls (n = 3). Ccl2 and Il6 mRNA in WT and Myd88−/− CFs (C) or WT and Nlrp3−/− CFs (D) stimulated for 1–4 h with CM-FT (n = 3–4). (E) HEK-TLR assay in response to TLR agonists or to conditioned medium obtained from live (CM-Ctl) or necrotic (CM-FT) CMCs (n = 2). *p < 0.05, **p < 0.01, ***p < 0.001.

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FIGURE 3. IL-1α is a major alarmin specifically released by necrotic CMCs. Il1a mRNA (A) and IL-1α protein (B) in neonatal CMCs (n = 3) and CFs (n = 3). (C) IL-1α in conditioned medium obtained by freeze–thaw (CM-FT) obtained from necrotic CMCs or necrotic CFs (n = 3). (D) IL-1α in adult CMCs and cardiac NMCs (n = 4). (E) Il6 mRNA in CMCs and CFs (n = 3). (F) Pro–IL-1β and mature IL-1β (p17) in total cell lysates from CMCs that were either left untreated (Ctrl) or stimulated with LPS (100 ng/ml, 18 h), and in conditioned medium from necrotic CMCs obtained by freeze–thaw (CM-FT). Supernatants of bone marrow macrophages (BMMs) stimulated for (18 h) with LPS (100 ng/ml) and nigericin (Nig, 5 μM) were used as positive control (n = 3). (G) Pro–IL-1β and mature IL-1β in total cell lysates from CFs that were either left untreated (Ctrl) or stimulated with LPS (100 ng/ml) or LPS and Nig (5 μM) and in the supernatants of CFs that were left untreated (Untr.) or exposed to CM-FT from necrotic CMCs (1 or 4 h). IL-1β in supernatants from CFs stimulated with LPS and Nig was used as a positive control (n = 3). (H) MCP-1 and IL-6 release by CFs stimulated (4 h) with CM-FT in the presence or absence of anakintra or IL-1α–blocking Ab (0.01–10 μg/ml) (n = 3). Levels of MCP-1 and IL-6 in CM-FT were used as control. (I) Ccl2 and Il6 mRNA in CFs exposed (1, 4 h) to conditioned media (CM-FT) from necrotic CMCs (CMCs→CFs) or necrotic CFs (CFs→CFs) (n = 9). *p < 0.05, **p < 0.01, ***p < 0.001.
neither cell type displayed detectable levels of mature IL-1β. IL-1β was present in the neutrophilic inflammation elicited in the tissues (15, 16). Such a role for IL-1β would take place during MI in vivo, as a murine model of myocardial ischemia and reperfusion (MIR). The expression of IL-1α in the myocardium was detected at similar levels under physiological conditions after sham surgery or after MIR (Fig. 4B), implying that IL-1α is not upregulated by MIR. In contrast, we found that IL-1α was detectable in plasma only after MIR, suggesting its passive release from the necrotic myocardium (Fig. 4G). When MIR was induced in Il1a-null mice, the degree of myocardial injury was not different from WT mice (comparable infarct size and circulating levels of CK and troponin, Fig. 4D–F). Notwithstanding this comparable tissue damage, Il1a−/− mice developed significantly less inflammation following MIR, as shown by a marked reduction in plasma and myocardial cytokines (Fig. 4G–I), as well as suppressed myocardial neutrophil infiltration (evaluated by MPO activity, Fig. 4J).

Our results identify IL-1α, released by dead CMCs and sensed by CFs, as a crucial danger signal in the initiation of sterile inflammation in the infarcted heart. In contrast, our studies do not support a role for TLR- or NLR-dependent mechanisms in such an initiating process, although it must be underscored that our experiments using TLR-expressing HEK 293 cells may be criticized because this represents an artificial in vitro system that cannot rule out a role for potential TLR ligands in the in vivo setting. It is also worth mentioning that our results do not exclude the involvement of additional signals that may be active in vivo, such as the autonomous nervous system, the renin–angiotensin system, and possibly other unidentified signals. Taking these limitations into account, our observations have potential clinical implications, because they provide a conceptual framework for the development of novel pharmacological compounds with a highly specific target in patients with acute MI. Such compounds could prevent adverse ventricular remodeling by interrupting a proximal trigger of excessive inflammatory responses, while leaving innate immune defenses essential for antimicrobial resistance unaltered.

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