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Cells Exposed to Sublethal Oxidative Stress Selectively Attract Monocytes/Macrophages via Scavenger Receptors and MyD88-Mediated Signaling

Anat Geiger-Maor,* Inbar Levi,* Sharona Even-Ram,† Yoav Smith,‡ Dawn M. Bowdish,§ Gabriel Nussbaum,¶ and Jacob Rachmilewitz*

The innate immune system responds to endogenous molecules released during cellular stress or those that have undergone modifications normally absent in healthy tissue. These structures are detected by pattern-recognition receptors, alerting the immune system to “danger.” In this study, we looked for early signals that direct immune cells to cells undergoing stress before irreversible damage takes place. To avoid detecting signals emanating from apoptotic or necrotic cells we exposed fibroblasts to sublethal oxidative stress. Our results indicate that both nonenzymatic chemical reactions and aldehyde dehydrogenase-2–mediated enzymatic activity released signals from fibroblasts that selectively attracted CD14+ monocytes but not T, NK, and NKT cells or granulocytes. Splenocytes from MyD88−/− mice did not migrate, and treatment with an inhibitory peptide that blocks MyD88 dimerization abrogated human monocyte migration. Monocyte migration was accompanied by downmodulation of CD14 expression and by the phosphorylation of IL-1R–associated kinase 1, a well-known MyD88-dependent signaling molecule. The scavenger receptor inhibitors, dextran sulfate and fucoidan, attenuated monocyte migration toward stressed cells and IL-1R–associated kinase 1 phosphorylation. Surprisingly, although monocyte migration was MyD88 dependent, it was not accompanied by inflammatory cytokine secretion. Taken together, these results establish a novel link between scavenger receptors and MyD88 that together function as sensors of oxidation-associated molecular patterns and induce monocyte motility. Furthermore, the data indicate that MyD88 independently regulates monocyte activation and motility. The Journal of Immunology, 2012, 188: 000–000.

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ince the introduction of the “Danger Model” (1), it is clear that innate immunity can be stimulated not only by viral or bacterial (nonself) components but also by nonmicrobial endogenous “danger signals.” The identity of the danger signals, and how the immune system senses stress or damage, is the focus of numerous studies that primarily center on the way the immune system discriminates between modes of cell death (2–4).

According to this proposal, trauma is associated with tissue damage that is recognized through the detection of “prepacked” intracellular molecules (5). These prepacked molecules include nucleic acids, lipids, and molecules such as HGMB1, ATP, and uric acid, which are normally hidden within the cell and are released from cells killed by acute abnormal death (3, 6), serving as ligands to an array of receptors primarily of the TLR family (7). Recognition by TLRs activates several signaling pathways primarily through the common MyD88 adaptor that in turn triggers an inflammatory response. Hence, the prevailing view is that immune cells are both attracted to wounds and are activated by danger signals released from damaged or necrotic cells.

In contrast to necrotic cell death resulting from trauma, programmed cell death is a continuous physiological process occurring in healthy tissue. Apoptotic cells are rapidly engulfed by phagocytes without triggering an inflammatory response, and in fact, apoptotic cell phagocytosis tends to be an anti-inflammatory trigger that may even promote tolerance (5). Interestingly, cells dying by physiological apoptotic death take an active role in their own engulfment by phagocytic cells (8). They have been shown to signal to immune cells through a variety of secreted “find-me” and exposed “eat-me” signals. Viable cells, in contrast, lack these signals and even express “do-not-eat-me” signals. Accordingly, the common features of putative signaling components that alert the immune system to danger is that they should not be sent by healthy cells or by cells undergoing normal physiological death and should fulfill two critical activities; first, they should attract immune cells to the site of damage, and second, they should activate the recruited cells that in turn will contribute to the clearance of cell debris and coordinate inflammation and wound repair (5).

Although the outcome of many insults is cell death, in the daily course of life, tissues and cells are constantly exposed to sublethal stressful conditions that do not necessarily end in physical damage or cause a noticeable effect. One such prevailing insult is oxidative stress. The sources of oxidants can be extrinsic or cell intrinsic, resulting from an imbalance in the oxidation/reduction process that generates reactive oxygen species. These highly reactive free
radicals can cause damage to lipids, proteins, and nucleic acids (9–11). In turn, these oxidatively modified cellular components can act as endogenous ligands of pattern recognition receptors, in particular, members of the scavenger receptor (SR) family (12–14). Whereas the mechanisms and signals involved in the way the immune system deciphers between modes of cell death are well studied, the immune response to stress before potential damage takes place have received little attention. The failure to assist cells suffering from sublethal stress and to remove toxic substances and counteract their harmful effects may often lead to tissue dysfunction and disease. Therefore, insights into the mechanisms that allow immune cells to respond to early signs of stress is fundamental to our understanding of how immune cells maintain homeostasis. To study this pivotal issue of how the immune system senses and responds to stress and to determine the danger signals expressed by cells undergoing sublethal stress, we exposed primary cultures of human fibroblasts to oxidative stress and tested their ability to attract immune cells. Most previous studies dealing with immune responses to oxidative stress involved experimental conditions that induce tissue damage or result in cell death. In the current study, we applied sublethal conditions to detect stress signals as such and to avoid signals released from dying cells (either apoptotic or necrotic cell death).

In this article, we present evidence that normal healthy cells exposed to sublethal oxidative stress release chemotactic signals that selectively attract monocytes. Nonenzymatic chemical reactions and specifically, the derivative of lipid peroxidation malondialdehyde (MDA), play a role in the generation of attractive signals. We further identified the mitochondrial enzyme ALDH-2 as an additional component of the chemotactic signal released by the cells. We conclude that the danger signal expressed by sublethal oxidative stress is monocyte-specific and can be detected using the sublethal conditions we established.

Materials and Methods

Cells

Primary human fibroblasts were provided by Dr. G. Zamir from the Department of Surgery, Hadassah Hebrew-University Medical Center (Jerusalem, Israel). Cells were maintained (not more than eight passages) in a high-glucose DMEM (Life Technologies) supplemented with 10% heat-inactivated FCS, 1% sodium pyruvate, and 1% penicillin/streptomycin. Cells were maintained (not more than eight passages) in a high-glucose DMEM (Life Technologies) supplemented with 10% heat-inactivated FCS, 1% sodium pyruvate, and 1% penicillin/streptomycin.

Transwell migration assay

A total of $0.5 \times 10^6$ PBMC were incubated with either mock-treated or peroxide-treated fibroblasts or medium alone for 10 min. In experiments using the MyD88 inhibitor peptide and its scrambled control, monocytes were pretreated for 30 min prior to placing them on the Transwell membranes. At the end of the incubation time, Transwell membranes were removed and washed with ice-cold PBS, and then, the cells were fixed with 4% PFA for 20 min, at room temperature, followed by permeabilization with 0.5% Triton X-100 and 4% PFA in PBS containing 5% sucrose for 5 min. Cells were then stained with Alexa Fluor 488-conjugated-phalloidin (Invitrogen, Eugene, OR) and DAPI (Calbiochem, Darmstadt, Germany). Transwell membranes were mounted on cover slides with FluoroGel mounting buffer (EMS). Images were obtained by a laser scanning confocal microscope system (Fluoview-1000; Olympus) with a ×60 Uplan-SAPO objective.

RT-PCR analysis for SRs expression. Total RNA was isolated from isolated monocytes using PerfectPure RNA Cultured Cell Kit (5 PRIME, Gaithersburg, MD). Specific primers for SR-AI, SR-AII, macrophage receptor with collagenous structure (MARCO) and scavenger receptor class A (SCARA)-5, SCARA-3, and SCARA-4, and RT-PCR protocols were according to DeWitte-Orr et al. (18).

Cytokine production

Cultures containing $0.15 \times 10^6$ fibroblasts, mock or peroxide treated, with or without $0.6 \times 10^6$ monocytes were plated in 24-well plates (Corning, Corning, NY). As positive controls, monocytes were incubated with 10 ng/ml LPS (Sigma-Aldrich) or with $0.15 \times 10^6$ necrotic fibroblasts. Cells were incubated for 24 h, and conditioned media were collected. Media was also collected from monocytes cultured alone. TNF-α and IL-1β levels in the conditioned media were assayed by ELISA (R&D Systems).

Gene array

Cultures containing $0.15 \times 10^6$ fibroblasts, mock or peroxide treated, with or without $0.6 \times 10^6$ monocytes were plated in 24-well plates...
(Corning). As control, monocytes were incubated alone. After 24 h, RNA was extracted using the PerfectPure RNA cultured Cell Kit (5 PRIME, Hamburg, Germany). Amplified and biotinylated sense-strand DNA were prepared according to the standard Affymetrix protocol from 200 ng total RNA (Expression Analysis Technical Manual; Affymetrix).

Following fragmentation, 5.5 μg biotinylated ssDNA was hybridized for 17 h at 45°C on GeneChip Human Gene 1.0 ST Array. GeneChips were washed and stained in the Affymetrix Fluidic Station 450. GeneChips were scanned using Affymetrix GeneChip Scanner 3000. Expression data were deposited at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33631; access number GSE33631).

Results

Cell motility is essential to the function of immune cells, which circulate throughout the body, seeking out signs of infection or tissue damage. After detecting find-me signals, immune cells can quickly and efficiently localize at site of damage or stress. To look for early find-me signals that alert immune cells to stress, we exposed fibroblasts to sublethal oxidative stress (Supplemental Fig. 1). This approach enabled us to detect signals emanating from stressed cells per se and to avoid any signals released from apoptotic or necrotic dying cells. We chose oxidative stress as a model because generation of reactive oxygen species is a downstream effect common to many biochemical processes and cellular stressors (19). A Transwell assay was established in which human PBMCs were allowed to migrate toward fibroblasts that were pretreated with hydrogen peroxide. The pretreatment strategy enabled us to look for soluble find-me signals emanating from fibroblasts that were exposed to stress and to avoid any direct effect of the peroxide on the immune cells that may directly induce their motility (specifically given that hydrogen peroxide gradients have been shown to induce leukocyte migration (20)).

Using the Transwell assay described above, we found that PBMCs migrated much more avidly toward stressed fibroblasts than toward control fibroblasts or fresh media (Fig. 1A). Cell type analysis revealed that a relatively high percentage of CD14+ cells migrated specifically toward the stressed cells (Fig. 1B), whereas only ~2–5% of CD3+ T cells, NKT cells, NK cells, or dendritic cells migrated nonspecifically to the lower chamber (no difference between migration toward stressed versus mock-treated cells). Interestingly, no granulocytes were observed in the lower chamber in experiments where whole blood was used instead of PBMCs (data not shown). These results demonstrate selective mobilization of CD14+ cells toward sublethally stressed cells. The extent of monocyte migration correlated with the concentration of peroxide added (Fig. 1C), and interestingly, monocyte migration toward stressed cells was evident as early as 15 min after placing the PBMC in the Transwell (Fig. 1D) and increased with time. The monocytes accounted for 60–70% of migrating leukocytes with maximal accumulation at 1–2 h. This selective migration contrasts to PBMC migration toward the chemokine stromal cell-derived factor 1 (SDF-1), which is characterized by migration of both monocyte and lymphocyte populations (data not shown). Of note, similar results were obtained whether migration assays were performed in the presence of serum in the media or in its absence (data not shown). In humans, circulating monocytes are divided into two major subsets on the basis of CD14 and CD16 expression (21). The two subsets are CD14highCD16− monocytes and CD14lowCD16+ monocytes that represent ~80–90 and ~10–20% of circulating monocytes, respectively. Subset analysis of monocytes migrating in response to fibroblasts treated with peroxide revealed that the CD14highCD16− but not CD14lowCD16+ subset migrated toward stressed cells (Fig. 1E).

A closer look at CD14 staining reveals that surface CD14 expression was downregulated on monocytes that migrated toward oxidized fibroblasts (i.e., those found in the lower chamber). Moreover, this downregulation was observed only in cells that migrated toward oxidized fibroblasts and not in monocytes that remained in the upper chamber or cells that migrated toward mock-treated fibroblasts or to SDF-1 (Supplemental Fig. 2A), pointing to a possible role for CD14 in monocyte migration toward sublethally stressed fibroblasts. Because CD14 is a coreceptor for several TLRs and was shown to directly bind several pathogen-associated molecular patterns as well as danger-associated molecular patterns, we first tested whether CD14 is involved in the recognition of attractive signals by adding a soluble form of CD14 to the Transwell system. Soluble CD14 has been shown to inhibit LPS-mediated responses (22–25). However, addition of soluble CD14 did not change the percentage of monocytes in the lower chamber of both mock-treated and stressed fibroblasts, suggesting it is not involved in ligand recognition and monocyte attraction (Supplemental Fig. 2B).

To further study the role of MyD88 in monocyte motility, we blocked MyD88-mediated signaling using a peptide that inhibits MyD88 homodimerization (15). Pretreatment of PBMC with the MyD88–homodimerization inhibitory peptide strongly reduced monocyte migration induced by oxidized fibroblasts, whereas a scrambled peptide with the same amino acid composition used as control was inactive (Fig. 2A). This indicates that MyD88-mediated signaling is an essential step for induction of monocyte migration by signals emanating from cells exposed to oxidative stress.

To further study the role of MyD88 in monocyte motility in response to stress, we turned to a murine cell system. Spleenocytes isolated from C57BL/6 mice migrated in a Transwell system toward oxidized human fibroblasts, albeit at a lower efficiency when compared with human monocytes (Fig. 2B, upper panel). In contrast to human PBMC, the cells that migrated to the lower chamber consisted of both F4/80+ cells and lymphocytes (data not shown). In contrast, spleenocytes derived from MYD88−/− mice failed to migrate toward oxidized fibroblasts (Fig. 2B, lower panel), again supporting the role of this signaling adaptor in monocyte migration.

We also tested the ability of oxidized fibroblasts to recruit macrophages in a peritoneal in vivo infiltration model. To this end, peroxide- and mock-treated fibroblasts were injected directly into the peritoneal cavity, and 4 d later, the peritoneum was washed with PBS, and cells were collected and counted. Supporting our previous in vitro results, there was considerably more recruitment of cells after injection of oxidized fibroblasts compared with mock-treated cells (~3-fold and higher) or to PBS alone or peroxide diluted in PBS (Fig. 2C). The chemotactic activity of oxidized fibroblasts was comparable (although lower) to that seen after injection of thioglycollate used as a positive control because of its potent ability to recruit macrophages. Furthermore, flow cytometric analysis revealed that, like thioglycollate-stimulated peritoneal cells, the recovered cells were mostly F4/80+. We repeated the experiment above, this time counting specifically F4/80+ cells using flow cytometry. These experiments corroborated the results above whereby oxidized fibroblasts recruited significantly more...
versus H₂O₂-treated fibroblasts in the lower chamber. After 10 h, the cells were washed and fresh culture media was added; then PBMC were placed over a Transwell (5.0 μm) and allowed to migrate for 1 h. Then Transwells were removed and cells were collected from both the upper and lower chambers. Cells were immunostained and then analyzed and counted by flow cytometry. The data are presented as percentage of migrating cells. A, The average of four separate experiments is graphed as percentage of migrating PBMC. B, The average of four separate experiments is graphed as percentage of CD14⁺ cells in the lower chamber. C, Migration of CD14⁺ cells toward fibroblasts treated with increasing concentrations of H₂O₂ is presented. One of four representative experiments is shown. D, CD14⁺ cell migration can be observed as early as 15 min. Similar results were obtained in three independent experiments. E, Peroxide-treated fibroblasts selectively attract CD14⁺CD16⁺ human monocyte subset. Dot plot flow cytometric analysis of monocyte subset distribution based on CD16 and CD14 expression in PBMCs (input) and in cells collected from the lower chamber following migration toward peroxide-treated fibroblasts (migrating). Similar results were obtained in four independent experiments. *p < 0.05, **p < 0.005.

F4/80 cells as compared with mock-treated cells or PBS alone (Fig. 2D). In contrast, injection of peroxide-treated fibroblasts to the peritoneum of MyD88−/− mice did not attract significant numbers of macrophages (Fig. 2D). Thus, these data underline the biological relevance of our findings and suggest that oxidative stress-related attraction signals play a role in MyD88-dependent macrophage recruitment in vivo.

To gain further insight into MyD88-mediated monocyte motility in response to oxidized cells, we examined the morphology of monocytes exposed to the various treatments. Monocytes were placed on a Transwell membrane over either media or mock-treated versus H₂O₂-treated fibroblasts in the lower chamber. After 10 min, monocytes were fixed and stained. Confocal microscopy images of actin and nuclei (phalloidin/DAPI)-stained monocytes placed over stressed fibroblasts demonstrated a rounded shape with cells protruding into the pores in contrast to a more flattened morphology of control cells (i.e., placed over media or mock-treated cells) (Fig. 3A, 3C). Monocytes placed over stressed fibroblasts and treated with MyD88 inhibitory peptide acquired a more rounded shape, as compared with cells treated with scrambled control peptide, with fewer membrane projections (Fig. 3B, 3C). These morphological changes are illustrated in Fig. 3D. MyD88 inhibitory peptide had no significant effect on the morphology of the control cells (data not shown). These data clearly demonstrate specific and MyD88-dependent changes in monocyte morphology in response to soluble factors released from peroxide-treated cells. The unique morphology acquired by cells treated with MyD88 inhibitory peptide may be an outcome of the partial response of these cells to the attractive signal emanating from stressed fibroblasts.

To further establish the link between MyD88 and monocyte motility, we investigated the induction of a downstream MyD88 signaling event in response to signals emanating from stressed cells. Interaction of MyD88 with the intracellular domain of an activated TLR initiates a signaling cascade. Once recruited by the receptor's TIR domain, MyD88 associates with the IRAK4. This receptor's TIR domain, MyD88 associates with the IRAK4. This event leads to recruitment and phosphorylation of IRAK1 (28). IRAK1 phosphorylation was evaluated in cell extracts from monocytes that were either exposed to mock- or H₂O₂-treated fibroblasts. To detect signaling events within the monocytes that are the consequence of the soluble find-me signal released by the fibroblasts, the monocytes were placed on a 0.4-μm Transwell membrane, and either mock-treated or H₂O₂-treated fibroblasts were placed in the lower chamber. At various time points, monocytes were collected and lysed, and cell extracts were fractionated on SDS-PAGE and immunoblotted using anti–phospho-IRAK1 Ab. Monocytes placed over peroxide-treated fibroblasts displayed a bimodal pattern starting with rapid IRAK1 phosphorylation at 1–2 min, followed by a slight decrease that increased again at 10–15 min after stimulation. In contrast, only a faint signal was observed in monocytes that were placed over mock-treated fibroblasts (Fig. 4A). In monocytes stimulated with LPS or necrotic fibroblasts, used as positive controls for MyD88-mediated signaling, a strong and gradual increase in IRAK1 phosphorylation was detected, peaking at 5–10 min following stimulation (Fig. 4B). We further tested the phosphorylation of downstream targets,
FIGURE 2. Human CD14+ cells and mouse macrophage migration toward H2O2-treated fibroblasts is MyD88 dependent. A, PBMC were pretreated for 30 min with either MyD88 inhibitory peptide (gray bars) or scrambled peptide (black bars) and then were placed in a Transwell over either mock (0)- or peroxide-treated fibroblasts and allowed to migrate for 1 h. Cells were analyzed as in Fig. 1. The percentage of CD14+ cell migration is shown. One of five representative experiments is shown. B, Cell migration was performed as above using splenocytes derived from either WT or MyD88−/− mice instead of human PBMCs. The percentage of macrophage migration is shown. Similar results were obtained in three independent experiments. C, In vivo cell recruitment into the peritoneal cavity in response to oxidized fibroblasts was tested. Mice were injected as described in Materials and Methods. The average number of cells in the peritoneal cavity of each group of mice is shown. One of three representative experiments is shown. The majority of cells recovered from the peritoneal cavity of mice injected with peroxide-treated fibroblasts was identified as F4/80+ and are similar to those recovered from mice injected with thioglycollate used as a positive control. D, Either PBS or peroxide- and mock-treated fibroblasts were injected directly into the peritoneal cavity of either WT or MyD88−/− mice and 4 d later, the cells were collected as described and the number of F4/80+ cells in each group was counted by flow cytometry. Comparable results were obtained in three independent experiments.

We therefore assessed MDA and 4-HNE as potential find-me signals in a migration assay. Because these compounds are extremely unstable, we used in the case of MDA a stable analog called MDA-Bis (dimethyl acetal). MDA-bis is used as standard in the thiobarbituric acid reactive substance assay used to measure MDA levels in the serum. MDA-bis added to the lower chamber induced selective migration of monocytes similar to that observed with oxidized fibroblasts (Fig. 5A). Interestingly, 4-HNE failed to induce monocyte migration at all concentrations tested, possibly because of the instability of this compound. Furthermore, although macrophages from splenocytes isolated from WT mice migrated in response to MDA-bis, macrophages derived from MYD88−/− mice failed to do so (Fig. 5B). In addition, treatment of human monocytes with MDA-bis induced weak but rapid and significant IRAK1 phosphorylation in a dose- and time-dependent manner (Fig. 5C). Taken together, these data demonstrate that MDA-bis selectively induces monocyte migration in a MyD88-dependent manner, replicating, at least partially, the effects seen with oxidized cells. Although in these experiments, we have used an analog of MDA, the data establish that compounds such as MDA or one of its derivatives, may represent at least part of the attractive signal released from oxidized cells.

Although our data established that monocyte motility in response to signals released from cells undergoing oxidative stress is dependent on MyD88 activity, the search using blocking Ab or cells from knockout mice for specific TLRs that mediate binding of find-me signals originating from oxidized cells did not yield conclusive results. It seems likely that this is due to some level of redundancy in the receptors and/or the ligands. It is noteworthy that, in general,
ligands of >TLRs require presentation via a coreceptor. The coreceptor CD14 has been implicated in facilitating TLR-mediated responses to bacterial lipopeptides by enhancing the physical proximity of the ligand to the TLR heterodimers, without binding directly to the receptor complex (30–32). However, as demonstrated above, soluble CD14 did not inhibit cell migration in our system (Supplemental Fig. 2B), suggesting that CD14 is not likely to play a role as coreceptor in our system.

SRs are another group of receptors that may bind ligands in our system. SRs bind a range of ligands of endogenous and exogenous origin with relatively low affinity. These ligands include, among others, oxidized lipids. By scavenging oxidized lipids from the environment, these receptors protect against oxidants and decrease inflammation (33). In addition to their role in clearance of these altered endogenous molecules, SRs have been recently identified as components that coordinate and enhance the response to TLR ligands (18, 34, 35). These observations led us to investigate whether SRs may be involved in the attraction of monocytes toward stressed cells.

Monocyte migration toward stressed cells was abrogated by treatment with SR-specific competitive ligands, fucoidan and dextran sulfate (DxSO4), but not their noncompetitive counterparts, fetuin, and chondroitin sulfate (ChSO4) (Fig. 6A, 6B). Monocyte migration in response to SDF-1, used as control, was not attenuated by the addition of either DxSO4 or fetuin (data not shown). In addition, both DxSO4 and fucoidan inhibited IRAK1 phosphorylation induced by stressed cells (Fig. 4A), thus linking SRs to MyD88 signaling. Given that these competitive inhibitors are specific for SR class A receptors (36), we next profiled class A SR expression in human monocytes using RT-PCR with previously described gene-specific primers (18). Human monocytes were found to express SR-AI, SR-AII, MARCO, and SCARA-5. SCARA-3 and SCARA-4 transcripts were undetectable (Fig. 6C). Treatment of monocytes with anti–SR-AI and AII-neutralizing Abs did not attenuate migration toward stressed fibroblasts (data not shown), suggesting the involvement of the other SR-A members (i.e., either MARCO or SCARA-5) to which specific blocking Abs are not available. Taken together, these results indicate that the class A SRs play an important role in the ligand binding event that induces MyD88-dependent signaling and monocyte migration.

Finally, we examined whether another type of stress, hypoxia, can induce monocyte migration in a similar manner. To that end, we evaluated PBMC migration toward fibroblast cultures exposed to...
continuous hypoxia for 2–24 h, as described previously. Similar to oxidative stress, we observed selective mobilization of CD14+ cells toward fibroblasts that were exposed to hypoxia. The number of monocytes increased with the duration of hypoxia, peaking at 18 h (Fig. 7A). Surprisingly, in contrast to monocyte migration toward peroxide-treated fibroblasts, treatment of PBMC with the MyD88 inhibitory peptide did not inhibit monocyte migration toward fibroblasts exposed to hypoxia (Fig. 7B), suggesting that different attraction signals/receptors are being used.

Inflammatory cytokine secretion is a predictable response following monocyte and macrophage recruitment to sites of damage. Moreover, MyD88 is a well-characterized signal transducer of innate immune responses that lead to monocyte and macrophage release of proinflammatory mediators. Next, we asked whether following the migration of monocytes toward oxidized fibroblasts they interact with the latter and respond by proinflammatory cytokine secretion. For that end, monocytes were incubated either alone or were cocultured with mock- or H2O2-treated fibroblasts for 24 h, and the levels of cytokines were determined in the conditioned media. No secretion of either TNF-\(\alpha\) or IL-1\(\beta\) was detected in conditioned media of monocytes alone or their coculture with either oxidized or mock-treated cells. In contrast, exposure of monocytes to LPS or necrotic fibroblasts resulted in significant TNF-\(\alpha\) and IL-1\(\beta\) release (Fig. 8A). Gene array analysis corroborated these findings by revealing that there is no significant change in the expression of genes associated with inflammation or phagocytosis in cocultures of monocytes and peroxide-treated fibroblasts as compared with monocytes cocultured with mock-treated fibroblasts (Fig. 8B), suggesting that overall, inflammation is not induced by exposure to sublethally stressed fibroblasts, despite the attraction of monocytes.

Whereas most studies attribute a proinflammatory role to reactive oxygen species and specifically to oxidized phospholipids (37, 38), more recent studies demonstrate that some products of phospho-

![Figure 5](image-url) **FIGURE 5.** MDA-bis induces monocyte motility and MyD88 signaling. A, PBMCs were placed in a 5.0-μm Transwell in the absence or presence of the indicated concentrations of either 4-HNE or MDA-bis in the lower chamber. After 1 h, cells were analyzed as in Fig. 1. The percentage of migrating CD14+ cells is shown. No other cell type analyzed including T, NK, and NKT cells and granulocytes significantly migrated in response to MDA-bis. One of three representative experiments is shown. B, Cell migration was performed as above using splenocytes derived from either WT or MyD88 \({}\sim\sim\) mice instead of human PBMCs. The percentage of migrating F4/80+ macrophages in the absence (black bars) or presence of 10 μM MDA-bis (gray bars) is shown. Similar results were obtained in three independent experiments. C, Monocytes were incubated for various time points with 10 or 100 μM MDA-bis. At the indicated time point, monocytes were washed with cold PBS and lysed. Cell extracts were subjected to SDS-PAGE and immunoblotted with anti-phosphorylated IRAK1 (upper panels). Anti-\(\beta\)-actin immunoblotting reveals the relative amounts of protein in each lane (lower panels). Similar results were obtained in three independent experiments.

![Figure 6](image-url) **FIGURE 6.** Class A SRs mediate MyD88 signaling and monocyte motility in response to peroxide-treated fibroblasts. Fibroblasts were either treated with PBS supplemented with the indicated concentrations of H2O2 (500 μM) or mock treated with PBS only (0). After 1 h, the cells were washed and fresh culture media was added; then PBMC were placed over a Transwell (5.0 μm) and allowed to migrate for 1 h as above in the presence of SR inhibitors or their controls: ChSO4 (black bars) or DxSO4 (gray bars) (A) and fetuin (black bars) or fucoidan (gray bars) (B). Transwells were then removed and cells were collected from both the upper and lower chambers. Cells were immunostained and then analyzed and counted by flow cytometry. The data are presented as the percentage of migrating CD14+ cells. C, Total RNA was isolated from monocytes and was subjected to RT-PCR using specific primers to SR-AI, SR-AII, MARCO, SCARA3, SCARA4, and SCARA5 transcripts. Similar results were obtained with monocytes from three different donors.
lipid oxidation may in fact exhibit anti-inflammatory properties (38–40). Specifically, these studies demonstrated that oxidized phospholipids inhibit TLR signaling in response to agonist stimulation (40–44). To test whether oxidized cells induce monocyte unresponsiveness, we stimulated monocytes with either LPS or necrotic fibroblasts in the absence or presence of oxidized fibroblasts. Apoptotic fibroblasts were used as a positive control for the ability to inhibit the monocyte proinflammatory response. As expected, apoptotic cells significantly inhibited TNF-α and IL-1β secretion in response to both LPS and necrotic cells; however, oxidized fibroblasts had no effect, ruling out any inhibitory activity (Fig. 8C). In aggregate, although MyD88 clearly plays a role in monocyte migration toward cells exposed to sublethal oxidative stress, it does not activate a downstream proinflammatory cytokine secretion in response to both LPS and necrotic cells; however, oxidized fibroblasts had no effect, ruling out any inhibitory activity. Hence, the role of MyD88 in monocyte/macrophage migration toward oxidized cells appears specific to cues emanating from oxidized cells. Furthermore, in contrast to the MyD88-dependent motility of monocytes in response to oxidatively stressed cells, activation of a MyD88-mediated pathway by either LPS or necrotic fibroblasts induced a strong inflammatory response but did not result in significant monocyte migration (data not shown). Hence, our data reveal a role for MyD88 in cell motility independent of its role in the inflammatory response to classic pathogen-associated molecular patterns or danger-associated molecular patterns. Furthermore, this MyD88-mediated monocyte response is specific to signals emanating from oxidized cells and does not participate in the response to other chemoattractants (SDF-1 or thioglycollate).

Several lines of evidence argue against the possibility that inhibition of MyD88 signaling causes a generalized defect in cell migration. First, thioglycollate-induced macrophage infiltration in MyD88−/− mice is comparable to that observed in wild-type mice. Second, human monocytes treated with MyD88-homodimerization inhibitory peptide migrated normally in response to SDF-1 or in response to cells exposed to hypoxia. Hence, the role of MyD88 in monocyte/macrophage migration toward oxidized cells appears specific to cues emanating from oxidized cells. Furthermore, in contrast to the MyD88-dependent motility of monocytes in response to oxidatively stressed cells, activation of a MyD88-mediated pathway by either LPS or necrotic fibroblasts induced a strong inflammatory response but did not result in significant monocyte migration (data not shown). Hence, our data reveal a role for MyD88 in cell motility independent of its role in the inflammatory response to classic pathogen-associated molecular patterns or danger-associated molecular patterns. Furthermore, this MyD88-mediated monocyte response is specific to signals emanating from oxidized cells and does not participate in the response to other chemoattractants (SDF-1 or thioglycollate).

Although the issue of how MyD88 signaling differentially regulates motility or inflammation is beyond the scope of the current study, it is tempting to speculate that the differences may be related to the duration and magnitude of IRAK1 phosphorylation. Significantly, within minutes of exposure to soluble factors released from oxidized fibroblasts but not mock-treated cells, monocytes specifically, the molecular nature of how dying cells alert the immune system to danger has been characterized (3). By subjecting normal fibroblasts to sublethal oxidative stress, in the current study, we were able to examine the response of immune cells to cellular stress per se, and the molecular identity of the signals emitted during stress. Our data show that fibroblasts exposed to sublethal doses of peroxide selectively attract CD14high CD16− human monocytes as well as mouse F4/80 macrophages. In addition to the attraction of monocytes and macrophages in vitro, oxidized fibroblasts also induced the attraction of macrophages upon i.p. injection in vivo. Chemotaxation of murine macrophages (both in vitro and in vivo) to oxidized human fibroblasts suggests that the signals and mechanism involved in this process are conserved across these species.

Interestingly, we observed that, following migration of monocytes in response to stressed fibroblasts, they expressed lower levels of CD14 on their cell surface. However, CD14 expression does not appear to be directly responsible for “sensing” signals released from stressed cells, because cell migration could not be competed by adding excess soluble CD14. Truman et al. (45) demonstrated that macrophages that had transmigrated in response to apoptotic Burkitt’s lymphoma cells showed increased CD14 expression. Despite the altered surface expression, CD14 in their study was also not involved in detecting the chemotactic signal. The authors suggested that this change in CD14 expression levels might represent readiness of the migrating monocytes for apoptotic cell clearance, as demonstrated previously (46).

We found that blocking MyD88-dependent signaling abrogated monocyte migration both in vitro and in vivo, demonstrating that signals released from stressed cells do not act through conventional chemotactic receptors but rather initiate signal transduction for migration through a yet-to-be defined sensor that signals through IRAK1. Hence, infiltration of monocytes/macrophages to the CNS was significantly lower as compared with WT mice. It is noteworthy that MPTP can lead to mitochondrial dysfunction and increased oxidative stress (48).
demonstrated increased IRAK1 phosphorylation, indicating activation of the MyD88 signaling pathway. However, in contrast to LPS and necrotic cells that induced a gradual and sustained MyD88-dependent signaling, the response to oxidized cells was biphasic and transient. In contrast, LPS and necrotic cells induced a strong proinflammatory response measured by cytokine production, whereas oxidized fibroblasts failed to induce any detectable response. Divergent roles have also been shown for MAPK signaling, which regulates diverse physiological functions including cell proliferation, differentiation, transformation, and survival. It is now clear that the duration and magnitude of signaling through the MAPK pathway, as well as the spatial restriction of MAPK activity, play a key role in determining the diverse physiological outcomes (49). Differences in IRAK1 phosphorylation kinetics or other signaling differences may skew the MyD88 response toward the Src family kinase/focal adhesion kinase axis, which has been shown to induce cytoskeleton reorganization and cell motility (50–52). A more detailed study into the signaling pathways and kinetics will be required to resolve this issue. Whatever the explanation for this phenomenon, the data suggest that ligands released from oxidized cells signal through MyD88-linked receptors in a “nonconventional” way that induces cell migration but not inflammation.

Two different mechanisms concerning the generation of factors able to trigger monocyte motility have been revealed in the current study. First, the local release of chemotactic factors seems to be mediated by oxidation of phospholipids and other cellular components that is initiated by reactive oxygen species and propagated via the classical mechanism of lipid peroxidation chain reaction (29). One such potential candidate for a danger signal has been

FIGURE 8. Peroxide-treated fibroblasts do not induce or inhibit monocyte inflammatory responses. A, Monocytes were either cultured alone or cocultured with mock- or peroxide-treated fibroblasts. As a control, monocytes were cocultured with apoptotic fibroblasts (induced by staurosporin), necrotic fibroblasts, or stimulated with LPS (10 ng/ml). After 24 h, conditioned media were collected and the levels of TNF-α and IL-1β secreted to the media were determined by ELISA. The data represent the mean values of triplicate samples and SDs. Data represent one of three independent experiments. B, Heat map of 98 genes associated with inflammation and phagocytosis showing gene expression levels in monocytes cocultured with either mock-treated fibroblasts (right column) or peroxide-treated fibroblasts (left column). In all genes, there was no significant change that exceeded 1.5-fold in the level of expression. C, To test whether oxidized fibroblasts inhibit monocyte response to stimuli, monocytes were either left untreated or were stimulated with either LPS or necrotic fibroblasts in the absence (black bars) or presence of either peroxide-treated fibroblasts (white bars) or apoptotic fibroblasts (gray bars). After 24 h, conditioned media were collected and the levels of TNF-α and IL-1β secreted to the media were determined by ELISA. The data represent the mean values of triplicate samples and SDs. Data represent one of three independent experiments.
tested, namely MDA, a major product of lipid peroxidation. Specifically, our data demonstrated that addition of a stable analog of MDA by itself recapitulated, although to a lesser extent, some of the effects seen with oxidized fibroblasts; namely, it selectively attracted monocytes in a MyD88-dependent manner and induced IRAK1 phosphorylation. Second, chemotactic factors are generated by the enzymatic activity of ALDH-2. Our initial hypothesis was that activation of ALDH-2, an enzyme that acts to convert and detoxify reactive lipid peroxidation products, such as MDA, will decrease the level of these products and thus will reduce cell migration. In contrast, we found that ALDH-2 enzymatic activity enhanced cell migration, suggesting that the enzymatic metabolites generated by ALDH-2 also contribute to monocyte motility. In a similar fashion, the enzymatic activity of caspase-3 in apoptosis also releases the phospholipid lysophosphatidylcholine, that in turn acts as a putative attraction signal to induce the migration of macrophages (53).

Detection of stress signals, based on altered cellular components, is advantageous because the signals are generated immediately. Thus, immune cells can respond rapidly to enzymatic and non-enzymatic changes that occur during oxidative stress, without the delay required for gene transcription or increased protein production (1, 5). In accord with this proposal, monocytes have evolved to directly detect a complex series of modified molecules that behave like a “beacon” of environmental stress, all of which can be released immediately from stressed cells and have some capacity for chemoattraction of monocytes.

The SR family consists of structurally unrelated receptors with potential functions in binding and clearance of apoptotic cells and endogenous molecules (54). SRs are known to bind lipids and therefore may serve as the sensing receptors for monocytes to respond to newly generated lipid moieties released from oxidized cells. Indeed, our data suggest monocytes detect and respond to signals emanating from oxidized cells through class A SRs that also directly or indirectly transmit their intracellular signal through MyD88.

It is important to note that although our data support a role for oxidation metabolites and SR in recruitment of macrophages, a previous report demonstrated that oxidized low-density lipoprotein (LDL), but not native LDL, inhibited macrophage migration, a process that was suggested to contribute to macrophage trapping in the arterial intima. Interestingly, this inhibition was associated with generation of reactive oxygen species and the enhancement of focal adhesion kinase phosphorylation and actin polymerization that was dependent on signaling through a member of the SR B family, CD36 (55). The extent and type of oxidative alteration to native molecules may determine the outcome of monocyte response (i.e., whether monocytes migrate toward the insult) or remain in close proximity to the damaged cells.

The demonstration that SR-As play a role in monocyte attraction, and the ability of SR-A inhibitors to inhibit MyD88-dependent signaling does not exclude the involvement of TLRs in this system. Indeed, recent studies have shown that particular TLR ligands also depend on SRs. For example, CD36, a class B SR, has been shown to signal via a TLR2/6 heterodimer upon binding of microbial diacylglycerides (56). Furthermore, altered endogenous CD36 ligands, oxidized LDL and amyloid-β trigger sterile inflammation through TLR4/6 heterodimerization and MyD88-dependent signaling (35). In addition, MARCO, a class A SR, triggers macrophage cytokine responses via TLR2 and CD14 in response to a mycobacterial cell wall component, trehalose dimycolate. Because CD14 was shown to bind not only LPS but also phospholipids (57), a function shared with SRs (14), it was suggested to be involved in extracting and presenting MARCO-bound ligand to TLR2 (34). SR, therefore, may represent a principle monocyte sensor of exogenous and endogenous signals. In our system, these receptors seem to specifically recognize oxidatively modified lipids and other yet unknown ligands and function as coreceptors (with or without CD14 used as a tethering receptor) that trigger monocyte migration via a MyD88 signaling pathway.

The lack of an inflammatory response or an inhibitory effect on monocyte activation observed upon coculturing monocytes with oxidized fibroblasts confirms the sublethal nature of the treatment used in this study (given that necrotic cells are strong monocyte activators, and apoptotic cells are strong inhibitors of monocyte activation) and also raises the question of what is the purpose of monocyte migration in this setting. On the basis of the preliminary data (A. Geiger-Maor and J. Rachmilewitz, unpublished observations), we suggest that monocytes resolve the oxidative cellular damage, thereby limiting more extensive tissue injury.

In conclusion, we believe that the experimental approach used in this study of testing various “intermediate” conditions within the full continuum of cellular conditions rather than the extreme conditions (i.e., cell death) will allow us to explore the potential enormous diversity of immune responses, far beyond the canonical inflammatory response.

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Disclosures

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References


Human fibroblasts were exposed to 500µM H₂O₂ in PBS or mock treated with PBS for an hour. Then the cells were washed and cultured with normal culture medium for recovery. The cells were analyzed for the short and long term effects of oxidative stress on cell viability and proliferation. In these experiments cells were analyzed immediately after and at the indicated time points following stress. (A) To detect apoptotic or necrotic cell death that may result from peroxide treatment, cells were stained with Annexin-V-FITC and PI and analyzed by flow cytometry. The percentage of apoptotic cells (Annexin-V−/PI+, upper panel) and necrotic cells (Annexin-V+/PI−, lower panel) in mock- (black lines) and peroxide-treated cells (grey lines), are shown. Black bars represent cells that were treated with 500µM of H₂O₂ for 10hr and then incubated for 24hr, used as a positive control for peroxide lethal effect.

(B) To determine the viability of cells following peroxide treatment we used the cell viability indicator that makes use of the natural reducing power of living cells by converting resazurin (alamarBlue) to the fluorescent molecule, resorufin. The percent of reduced alamarBlue is shown for mock-treated (black lines) and peroxide-treated (grey lines) fibroblasts. The small differences between mock and peroxide treated cells are probably due to a difference in cell numbers as a result of proliferation arrest in the peroxide treated cultures. The data clearly demonstrate that the peroxide treatment used in this study is non-lethal. (C) Sub-lethal oxidative stress inhibits cell proliferation as demonstrated by CFSE dilution analysis. CFSE-labelled fibroblasts were either mock treated or exposed to oxidative stress for 1 hr using 500µM H₂O₂. After 24, 48 and 72 hours, cells were analyzed by flow cytometry for CFSE intensity. As shown, in contrast to mock treatment, peroxide treatment resulted in proliferative arrest that was not restored even 72h after exposure to stress. The observed cell division arrest is in agreement with reported responses associated with sub-lethal oxidative stress (1) validating the effectiveness of the treatment. Similar results were obtained in five independent experiments.

**Note:** In contrast to the observed resistance of the primary human fibroblasts used in this study, other cell lines including HEK293, HELA, Huh7 and MCF7 cells all died within hours of exposure to 500µM H₂O₂.
Figure S2. CD14 is downmodulated from the surface of migrating monocytes but is not responsible for the attraction of monocytes toward oxidized cells

(A) PBMCs were placed in a transwell over either mock- or peroxide-treated fibroblasts and were allowed to migrate for an hour. Cells in the lower and upper chambers were collected and stained with FITC-conjugated anti-CD14 Abs. The levels of CD14 staining on CD14-positive cells collected from the lower (thick line) and upper chambers (thin line) placed over mock- (upper panel) or H$_2$O$_2$-treated fibroblasts (lower panel), is shown. CD14 staining reveals that surface CD14 expression was down-regulated on monocytes that migrated toward oxidized fibroblasts. Moreover, this down regulation was observed only in cells that migrated toward oxidized fibroblasts and not in monocytes that remained in the upper chamber, or cells that migrated toward mock-treated fibroblasts or to SDF-1, pointing to a possible role for CD14 in monocyte migration toward sub-lethally stressed fibroblasts. Data represents one of six independent experiments.

(B) Since CD14 is a co-receptor for several Toll-like receptors (TLRs), and was shown to directly bind several PAMPs as well as DAMPs, we first tested whether CD14 is involved in the recognition of attractive signals by adding a soluble form of CD14 to the transwell system. Soluble CD14 has been shown to inhibit LPS-mediated responses (2-5). To test the effect of soluble CD14 on monocyte migration, PBMCs were placed in a transwell over either mock- or peroxide-treated fibroblasts in the presence or absence of soluble CD14 (1μg/ml) and were allowed to migrate for an hour. As can be seen, addition of soluble CD14 did not change the percentage of monocytes in the lower chamber of both mock-treated and stressed fibroblasts, suggesting it is not involved in ligand-recognition and monocyte attraction. Data presented as percentage of migrating CD14$^+$ cells. One of three independent experiments is shown.

Another component of the TLR4 complex is MD2. MD2 expression is required for cell surface expression of TLR4 and for optimal ligand binding. For example, MD2 extracts LPS from circulating CD14-LPS complexes and transfers the LPS into a ternary complex with TLR4 (6, 7). The addition of a soluble chimeric TLR-4/MD2 protein had no effect on monocyte migration, as well (data not shown). Thus, the
CD14/TLR-4/MD2 complex does not seem to play a role in driving monocyte migration toward stressed cells in our system.
Figure S3. Phosphorylation of the downstream targets of MyD88, p38 and AKT, is not induced in monocytes by oxidized fibroblasts

Fibroblasts were plated in a 24 well dish and were either mock- or peroxide-treated as above. Monocytes were then placed in the upper chamber of a 0.4μm transwell and incubated for the indicated time. Following incubation, the transwells were removed; the monocytes were collected, washed with cold PBS and lysed. Cell extracts were subjected to SDS-PAGE and anti-phosphorylated P38 (upper panels) and AKT (middle panels) immunoblotting. Anti-AKT immunoblotting used to detect the relative amounts of protein in each lane (lower panels). Similar results were obtained in three independent experiments. HF: human fibroblasts.
Figure S4. Enzymatic and non-enzymatic routes for the generation of attractive signals by oxidized cells

(A) We tested whether de novo synthesis or other metabolic and enzymatic activities in the oxidized fibroblasts are required for the generation and release of factors that attract monocytes, or whether the attractive factors are a consequence of chemical reactions per se in which the peroxide interacts with pre-existing cellular components. To that end, fibroblasts were either left untreated (live) or were fixed with 1% paraformaldehyde for 5 min at room temperature. This relatively light fixation procedure preserves the cellular structure while making the cells metabolically inactive (8). Fixed fibroblasts were then extensively washed and were either exposed to oxidative stress with 500μM H₂O₂ or to PBS and then PBMCs were placed in a transwell over either live or pre-fixed fibroblasts that were mock- or peroxide-treated, and were allowed to migrate for an hour. Data presented as percentage of migrating CD14⁺ cells. An average of three experiments is shown. Although the overall numbers of monocytes in the lower chamber were lower in both groups as compared to their respective live cells, fixed and oxidized cells attracted significantly more monocytes than fixed and mock-treated cells. This data suggest that at least some of the attractive signals originate from pre-existing molecules that are either hidden and then revealed or changed due to oxidation and then released through the action of non-enzymatic chemical reactions following exposure to the oxidative agent.

(B) In addition to the non-enzymatic route we also tested whether enzymatic processes may play a role in the generation and release of attractive signals. Mitochondrial aldehyde dehydrogenase 2 (ALDH2) is a key enzyme in the NAD⁺-dependent oxidation of various aldehydes produced during intermediary metabolisms. ALDH2 is also known to detoxify toxic lipid aldehydes generated during oxidative stress such as 4-HNE and MDA, which are reactive by-products of lipid peroxidation (9, 10). To test whether byproducts of ALDH2 enzymatic activity may act as an attractive signal, we have enhanced ALDH2 activity by treating fibroblasts with Alda-1. Alda-1 is a small molecule that acts as a selective pharmacological agonist through its activity as a chemical chaperone for human ALDH2 (11). Pharmacologic enhancement of ALDH2 activity by Alda-1 has been shown to reduce ischemic damage to the heart (12). To test the effect of Alda-1 on the generation of attractive signals, fibroblasts were incubated with or without 20μM Alda-1 for an hour and then PBMCs were placed in a transwell and were allowed to migrate for an additional hour. Data presented as percentage of migrating CD14⁺ cells. An average of three
experiments is shown. The data clearly demonstrate that activation of ALDH2 can produce a signal that attracts monocytes. Notably, incubation of healthy fibroblasts with Alda-1 during the migration step also induced significant and selective monocyte migration (data not shown). Adding Alda-1 in the lower chamber during the migration step in the absence of fibroblasts did not result in significant monocyte migration (data not shown), ruling out a direct effect on the monocytes. Interestingly, treatment of fibroblasts with Alda-1 also resulted in CD14 down regulation in migrating monocytes (data not shown). Hence, direct ALDH2 activation can replicate the effect of oxidized fibroblasts on monocyte migration and CD14 downregulation. These findings suggest that enzymatic-conversion of aldehydes by ALDH2 activation represents another possible mechanistic step, in addition to the non-enzymatic route, in the generation of attraction signals for monocytes. (*: P< 0.05; ** P<0.005)
References


