Recruitment of macrophages to sites of cell death is critical for induction of an immunologic response. Calcium concentrations in extracellular fluids vary markedly, and are particularly high at sites of injury or infection. We hypothesized that extracellular calcium participates in modulating the immune response, perhaps acting via the seven-transmembrane calcium-sensing receptor (CaR) on mature monocytes/macrophages. We observed a dose-dependent increase in monocyte chemotaxis in response to extracellular calcium or the selective allosteric CaR activator NPS R-467. In contrast, monocytes derived from mice deficient in CaR lacked the normal chemotactic response to a calcium gradient. Notably, CaR activation of monocytes bearing the receptor synergistically augmented the transmigration response of monocytes to the chemokine MCP-1 in association with increased cell-surface expression of its cognate receptor, CCR2. Conversely, stimulation of monocytes with MCP-1 or SDF-1α reciprocally increased CaR expression, suggesting a dual-enhancing interaction of Ca^{2+} with chemokines in recruiting inflammatory cells. Subcutaneous administration in mice of Ca^{2+}, MCP-1, or (more potently) the combination of Ca^{2+} and MCP-1, elicited an inflammatory infiltrate consisting of monocytes/macrophages. Thus extracellular calcium functions as an ionic chemokinetic agent capable of modulating the innate immune response in vivo and in vitro by direct and indirect actions on monocyctic cells. Calcium deposition may be both consequence and cause of chronic inflammatory changes at sites of injury, infection, and atherosclerosis.
Extracellular calcium elicits a chemokinetic response from monocytes in vitro and in vivo

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Recruitment of macrophages to sites of cell death is critical for induction of an immunologic response. Calcium concentrations in extracellular fluids vary markedly, and are particularly high at sites of injury or infection. We hypothesized that extracellular calcium participates in modulating the immune response, perhaps acting via the seven-transmembrane calcium-sensing receptor (CaR) on mature monocytes/macrophages. We observed a dose-dependent increase in monocyte chemotaxis in response to extracellular calcium or the selective allosteric CaR activator NPS R-467. In contrast, monocytes derived from mice deficient in CaR lacked the normal chemotactic response to a calcium gradient. Notably, CaR activation of monocytes bearing the receptor synergistically augmented the transmigration response of monocytes to the chemokine MCP-1 in association with increased cell-surface expression of its cognate receptor, CCR2. Conversely, stimulation of monocytes with MCP-1 or SDF-1α reciprocally increased CaR expression, suggesting a dual-enhancing interaction of Ca2+ with chemokines in recruiting inflammatory cells. Subcutaneous administration in mice of Ca2+, MCP-1, or (more potently) the combination of Ca2+ and MCP-1, elicited an inflammatory infiltrate consisting of monocytes/macrophages. Thus extracellular calcium functions as an ionic chemokinetic agent capable of modulating the innate immune response in vivo and in vitro by direct and indirect actions on monocyte cells. Calcium deposition may be both consequence and cause of chronic inflammatory changes at sites of injury, infection, and atherosclerosis.


Introduction
Accumulation of immune cells at sites of injury or infection is a critical dimension of host defense that is achieved by highly conserved mediators of cell adhesion and cell motility. The large family of protein cytokines capable of inducing cell migration is collectively termed chemokines. Chemokines can be produced by virtually every cell type in mammals (1–3). Chemokines mediate their function via seven-transmembrane, G protein–coupled receptors (7-TMR); the absence of either chemokines or their receptors results in marked phenotypic alterations in mice (3–5). These include altered inflammatory responses to pathogenic or allergenic challenges, and mitigated atherosclerotic changes in models of vascular disease (6). Extracellular fluids at sites of injury or infection have been reported to contain high concentrations of calcium (7–9), and chronic inflammatory conditions and atherosclerosis are associated with deposition of calcium salts (6, 10, 11). The concentration of calcium in such settings can be substantially higher than in the serum (7–9). We hypothesized that such extracellular calcium gradients actively participate in modulating the immune response, acting via the calcium-sensing receptor (CaR).

The CaR is a member of the 7-TMR superfamily, and is responsive to Ca2+ concentrations within the millimolar range found in extracellular fluids (12). It was originally defined by its role in mediating systemic calcium homeostasis; however, it has since been shown to have pleiotropic effects including altering cellular proliferation, differentiation, and apoptosis (13–16). In hematopoietic cells, it is expressed on mature monocytes/macrophages and subsets of progenitor populations in the bone marrow (17, 18). Animals engineered to be deficient in this receptor appear normal at birth, but die with severely elevated blood calcium levels within the first few weeks of life (19, 20). Activation of the receptor is maximal at 5 mM Ca2+ (13), and selective CaR activators have been developed that efficiently mimic Ca2+-induced activation through an allosteric mechanism (e.g., NPS R-467 and its less active stereoisomer, S-467) (21). These agents are low molecular weight compounds known
as calcimimetics that interact with the CaR’s transmembrane domains and potentiate the actions of polycationic agonists (such as Ca²⁺ itself) that bind to the receptor’s amino-terminal extracellular domain. Calcimimetics are currently in clinical trials for treating primary hyperparathyroidism, a disorder in which the CaR is underactive, and represent useful pharmacological tools for assessing the CaR’s mediatory role in CaR-expressing cells in which high Ca²⁺ modulates cellular function. CaR signal transduction is mediated via a pertussis toxin–inhibitable Gαi pathway as well as a pertussis toxin–insensitive mechanism that probably involves Gαq/11 (22–24).

We have recently shown cell-surface expression of the CaR on adult human CD14⁺ PBMC (17, 18, 25). However, the physiological role of the CaR in mononuclear cells is unknown. We examined whether ionic calcium is a chemokinetic agent for human monocytes, whether this activity is mediated via the G protein–coupled CaR, and whether interactions with other chemokinetic agents are induced by CaR activation. We assessed the in vivo consequences of activating the CaR, and noted marked infiltration with monocytes. These data strongly support the role of extracellular calcium in modulating monocyte localization and suggest ionic calcium as a primitive mediator of immune function.

**Methods**

*Preparation of CD14⁺ PBMC.* Low-density cells were isolated from human and mouse peripheral blood using Ficoll-Hypaque (Pharmacia Biotech Inc., Piscataway, New Jersey, USA). CD14⁺ monocytes were purified by sorting on a FACSVantage (Becton Dickinson Immunocytometry Systems, San Jose, California, USA), based on CD14 expression. Purified cells were incubated in either calcium-free medium or with 1.5 mM CaCl₂ and either the selective calcium receptor activator NPS R-467 or its less active stereoisomer, NPS S-467, in 3 mM Ca²⁺. Cells were incubated for up to 24 hours in 5% CO₂ humidified medium was subsequently supplemented with 0.5 mM, 1 mM, 2 mM, 3 mM, or 5 mM CaCl₂ (Sigma Chemical Co., St. Louis, Missouri, USA) to achieve the desired level of extracellular calcium, or with 1 μM of the selective CaR activator NPS R-467 or its less active stereoisomer, NPS S-467, in 3 mM Ca²⁺. Cells were incubated for up to 24 hours in 5% CO₂ humidified air. Monocytes were then detached from the plate using a cell scraper. All calcium concentrations shown in the presented data represent the total Ca²⁺ in the basal medium plus any added Ca²⁺.

*Cytosolic calcium changes.* Purified monocytes were loaded with Indo-1 AM (Molecular Probes Inc., Eugene, Oregon, USA). Cells were collected on a FACSVantage for 30 seconds to establish a baseline emission value for Indo-1 AM. Chemokine ligands were then added to cells incubated in Ca²⁺-free media, or supplemented with 1.5 mM or 4.5 mM CaCl₂ and either the selective calcium receptor activator NPS R-467 or the less active NPS S-467 (1 μM) (a gift of E. Nemeth, NPS Pharmaceuticals Inc., Salt Lake City, Utah, USA). Data acquisition then continued for up to 5 minutes.

*Chemokine receptor expression and flow cytometric analysis.* Purified monocytes were incubated in Ca²⁺-free medium supplemented with 1 mM, 3 mM, or 5 mM CaCl₂, or with 1.5 mM CaCl₂ plus 1 μM NPS R-467 or NPS S-467. Cells were washed once in PBS with 1% FCS and then resuspended in 100 μL of Ca²⁺-free PBS. Monoclonal antibody against the CaR was added to the cells, and they were incubated alone or with the CaR peptide for 30 minutes at room temperature. Cells were washed with PBS containing 1% FCS, resuspended in PBS with 1% FCS, and incubated for 15 minutes at room temperature with FITC-conjugated anti-mouse IgG. The cells were washed again, and conjugated monoclonal antibody was added and incubated for 15 minutes at room temperature. Monoclonal antibodies used were anti-CXCR4, anti-CCR5 (PharMingen, San Diego, California, USA), anti-CCR2 (R&D Systems Inc., Minneapolis, Minnesota, USA), anti-CD14, and anti-CD4 (Becton Dickinson Immunocytometry Systems). Stained cells were fixed with 1% paraformaldehyde and were assayed within 24 hours. Flow cytometric analysis was performed using a dual laser FACSCalibur calibrated with 2-μm CaliBRITE beads (both from Becton Dickinson Immunocytometry Systems). Data acquisition and analysis were performed using CellQuest software (Becton Dickinson Immunocytometry Systems).

*Breeding and genotyping of CasR⁻/⁻ mice.* CasR⁻/⁻ mice were bred as described previously (19). To produce CaR⁻/⁻ mice for this study, heterozygotic mice were intercrossed. The mice studied were of either 129S6/SvEv or 129S6/SvEv/Swiss Webster mixed genetic background. (Mice homozygous for the CaR knockout allele do not live longer than 3 weeks.) Mice were genotyped using the following protocol. A sample
of DNA (1 μL) was obtained from a section (< 5 mm) of the tail of each mouse to be genotyped according to established techniques (19). In this way the genotypes of CaR–/–, CaR+/–, and CaR+/+ mice were obtained. Peripheral blood was obtained from mice of each genotype at the time of sacrifice on day 6–8 after birth.

Biologic activity of ionic calcium in vivo. C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were injected subcutaneously at a marked, shaved spot on the abdomen with 20 μL of 5 mM CaCl2 or 20 μL of MCP-1 at a concentration of 10 ng/mL, with a combination of either MCP-1 and 5 mM CaCl2, or 10 μM NPS R-467 in PBS. Control mice were injected subcutaneously with 20 μL PBS alone. All subcutaneously injected agents contained less than 0.004 ng/mL of LPS by the limulus assay (Sigma Chemical Co.), as described previously (25). Mice were sacrificed by CO2 asphyxiation 18 hours after injection, and injection sites were excised and snap frozen. From these, 4-μm cryosections were obtained and stained with hematoxylin and eosin. Immunocytochemistry was performed on the frozen sections using alkaline phosphatase–conjugated Mac-1 antibody (PharMingen) as described previously, and assessed by light microscopy (25).

Results
Calcium mobilization and chemotaxis. Combining flow cytometry results with our prior data showing the presence of CaR mRNA and protein in monocytes by RT-PCR and Western analysis, we demonstrated that the CaR is expressed on the cell surface of PBMC (Figure 1a). We next examined the CaR’s functional relevance in monocytes. Extracellular Ca2+ increased the cytosolic Ca2+ concentration in a dose-dependent manner, with a maximal response at 4.5 mM Ca2+ (Figure 1b), consistent with the receptor’s known capacity to elevate cytosolic calcium by activating phospholipase C.

A transmigration assay was then used to determine if calcium was capable of inducing monocyte chemotaxis. A dose-dependent effect was seen, with maximal chemotaxis observed at 4.5 mM Ca2+ in a checkerboard analysis of chemotactic response to a concentration gradient of ionic calcium. Chemotaxis was inhibitable by pertussis toxin and by the tyrosine kinase inhibitors herbimycin and genistein, but not by the PI-3 kinase inhibitor wortmannin (Figure 2, a and b). The role of the CaR in these responses was further assessed by exposure of the cells to spermine, a polycationic CaR agonist (26), and to the selective CaR activator NPS R-467 or its less active stereoisomer, NPS S-467 (21, 27). Stimulation of cytosolic calcium responses and transmigration were noted with each of these agents. The greater potency of R-467 than S-467 in enhancing the actions of high Ca2+ on these two biological responses is fully in accord with their known pharmacological actions on the cloned CaR, and strongly support the receptor’s role in mediating these actions of extracellular Ca2+ on monocytes (21) (Figure 2c).
To confirm the role of CaR in mediating chemotaxis induced by a calcium gradient, we examined mononuclear cells derived from neonatal CaR–/–, CaR+/–, and CaR+/+ mice. The cells were capable of chemotactic movement toward MCP-1; however, CaR–/– cells failed to transmigrate to the Ca2+ gradient (Figure 2d), definitively demonstrating the role of the CaR in the response.

**Chemokine receptor interactions.** We next evaluated whether calcium interacted with other regulators of monocyte migration by assessing the coexpression of chemokine receptors and the CaR on primary human adult mononuclear cells. CCR2 and CCR5 were present on 100% of CD14+ PBMC, and CXCR4 was present on 87%; coexpression of the CaR with CCR2 was noted in 97% of CD14+ PBMC, and coexpression with CXCR4 was seen in 83%. Functional responses of monocytes to activation of chemokine receptors were then measured in the presence or absence of CaR stimulation using...
transmigration assays. Chemotaxis of CD14+ PBMC to a positive concentration gradient of MCP-1 was dependent upon extracellular calcium, as has been reported by others (28, 29), whereas MIP-1α and SDF-1α induced migration independent of calcium (data not shown). The percentage of cells migrating in response to MCP-1 could be augmented by raising extracellular Ca2+ with peak activity noted at 4.5 mM Ca2+ (Figure 2e). The increase in transmigration elicited by a combination of MCP-1 and elevated Ca2+ concentrations was greater than the sum of transmigration evoked by either MCP-1 or Ca2+ alone, indicating the synergistic nature of the interaction. In contrast, no synergistic effect was noted when either MIP-1α or SDF-1α was used in conjunction with added Ca2+.

The mechanism by which Ca2+ enhanced responsiveness to MCP-1 was evaluated by assessing receptor expression kinetics after CaR stimulation. Exposure of cells to elevated Ca2+ resulted in upregulation of cell-surface CCR2 at 3 hours (Table 1). In contrast, antibodies directed against CCR5 failed to demonstrate any change in median fluorescence intensity, whereas CXCR4 expression increased but did not achieve statistical significance. Similarly, the fluorescence intensity of anti-CD4 antibody staining did not vary with Ca2+ concentration, thereby excluding a nonspecific enhancement of antibody staining in the presence of increased calcium. Inhibition of new protein synthesis by cycloheximide had no effect on the Ca2+-mediated upregulation of CCR2, indicating that Ca2+ altered receptor processing or trafficking (or both) between cell-surface and intracellular pools, rather than the generation of new receptor molecules.

To assess whether there was reciprocal regulation of the CaR by chemokine receptors, we evaluated CaR surface expression on cells before and after exposure to the chemokines SDF-1α or MCP-1. The mean fluorescence intensity of CaR expression was 361 ± 25 units (mean ± SEM) for untreated monocytes, and rose to 673 ± 59 after MCP-1 stimulation. Monocytic expression of CaR rose to 519 ± 48 with SDF-1α treatment.

**Table 1**

<table>
<thead>
<tr>
<th>Ca2+ concentration (mM)</th>
<th>Mean fluorescence intensity of CCR2 (mean ± SEM)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM Ca2+</td>
<td>204 ± 16</td>
<td>0.0005A</td>
</tr>
<tr>
<td>1.5 mM Ca2+</td>
<td>278 ± 39</td>
<td>0.0007A</td>
</tr>
<tr>
<td>2.5 mM Ca2+</td>
<td>296 ± 35</td>
<td>0.0007A</td>
</tr>
<tr>
<td>4.5 mM Ca2+</td>
<td>307 ± 35</td>
<td>0.0003A</td>
</tr>
<tr>
<td>1.5 mM Ca2+ and agonist R</td>
<td>460 ± 52</td>
<td>0.0135A</td>
</tr>
<tr>
<td>1.5 mM Ca2+ and agonist S</td>
<td>417 ± 48</td>
<td>0.0205A</td>
</tr>
</tbody>
</table>

Monocytes were exposed to varying concentrations of Ca2+ and to the selective CaR activator NPS R-467 (1 μM) or NPS S-467 (1 μM) in the presence of 1.5 mM Ca2+. The median fluorescence intensity for CCR2 expression was measured for each Ca2+ concentration and in the presence of NPS R-467 or NPS S-467. Mean values ± SEM from three independent experiments are shown. *Student’s t test vs. 0 mM Ca2+; 1Student’s t test vs. 1.5 mM Ca2+. |

In **vivo monocyte migration toward calcium.** To determine whether Ca2+ was active as a chemoattractant for monocytes in vivo, Ca2+ alone (5.0 mM), MCP-1 alone, or both Ca2+ and MCP-1 were injected subcutaneously into C57BL/6 mice, and the tissue was excised 18 hours later. Accumulation of mononuclear cells staining positive for the monocytic marker Mac-1 was noted in mice injected with either Ca2+, MCP-1 (Figure 3), or 10 μM NPS R-467. No subcutaneous infiltrates of cells were detected in mice injected with PBS alone. The greatest degree of monocyte infiltration into subcutaneous tissues was seen with 5 mM Ca2+ plus MCP-1, supporting in vivo the synergistic relationship observed in vitro (Figure 3). No significant infiltrate of neutrophils into the site of subcutaneous injection was seen under any of the conditions used, as determined by hematoxylin and eosin staining of skin sections.

**Discussion**

Ionic calcium is highly regulated in vivo, yet can be elevated in specific tissue microenvironments such as the bone marrow, and is particularly altered in the context of ongoing cell death and inflammation. Hypothesizing that this phenomenon may serve as a primitive regulator of immune cells, we examined a number of hematopoietic cell types for expression of the CaR. Whereas this receptor is expressed on primitive hematopoietic cells (I. Olszak, manuscript in preparation), it is strikingly abundant on mature monocyctic cells and is therefore a candidate mediator of immunologic function of this member of the innate immune system.

Localization of monocytes/macrophages at sites of injury or inflammation is crucial for initiation of their role in host defense, and has been demonstrated to be regulated by a number of members of the chemokine family. The CaR is a seven-transmembrane-spanning and G protein–coupled receptor that has been shown to signaling through the CaR in a Gαi-coupled pathway mediated by tyrosine kinase, but not PI-3 kinase. The activation of this receptor by either polycationic cognate ligands (e.g., high Ca2+ or spermine) or NPS R-4E7, an allosteric activator of the CaR, resulted in the transmigration of monocyctic cells, providing a mechanism to explain the prior observation of others that calcium may enhance the migration of monocytes to oxidized LDL (30). The absence of chemotaxis of CaR−/− monocytes toward Ca2+ even though they responded to a control chemokine shows the specificity of the phenomenon for the CaR. Conjecturing that the Ca2+ signal would probably...
interact with other mediators of cell response, we assessed whether the migratory response to MCP-1 was similarly modulated by CaR activation. The impact of CaR signaling was manifested in upregulation of MCP-1 receptor, mediated by a mechanism of altered intracellular trafficking rather than new protein synthesis. A reciprocal increase in CaR expression was noted with stimulation of CCR2 with MCP-1, documenting a clear interrelationship of these two unrelated members of the 7-TMR family. This interaction was evident in vivo as well as in vitro when ligands were used in isolation or in combination in animals, and generated marked monocytic infiltrates at the site of injection. Thus ionic calcium serves to directly and indirectly influence monocyte migratory response. It can play a role in the tissue localization of primary monocytes, and may potentiate protein-mediated induction of chemotaxis. The contribution of this signal to inflammation is dependent upon local calcium concentrations, and therefore may be relevant for settings of either extensive injury or in the context of chronic inflammation.

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**Figure 3**

Ca²⁺ is a chemoattractant for monocytes in vivo. Frozen sections of skin were obtained after subcutaneous injection of mice with PBS alone (a and b), 5.0 mM CaCl₂ alone (c and d), or 5.0 mM CaCl₂ plus 10 ng/mL MCP-1 (e and f). Sections were stained with hematoxylin and eosin (a, c, e) or alkaline phosphatase-conjugated, macrophage-specific Mac-1 antibody (b, d, f). Occasional cells in skin from mice injected subcutaneously with PBS alone stained positively for Mac-1. However, CaCl₂ alone and the combination of CaCl₂ plus MCP-1 caused a dense infiltrate of mononuclear cells that stained positively for Mac-1 (indicated by arrows in c, d, and f). Data are representative of one of two independent experiments.