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Maturing Dendritic Cells Depend on RAGE for In Vivo Homing to Lymph Nodes

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The mobilization of dendritic cells (DCs) from peripheral tissues is critical for the establishment of T cell-dependent immune responses or tolerance, because the physical interaction of DCs with naive T cells takes place in the T cell areas of lymph nodes. The autocrine/paracrine release of the high mobility group box 1 (HMGB1) nuclear protein by DCs controls the outcome of the DC–T cell interaction, influencing the priming/Th1 polarization of naive T cells. We herein present evidence that the receptor for advanced glycation end products (RAGE), a multiligand member of the Ig superfamily of cell-surface molecules that acts as a receptor for HMGB1, plays a nonredundant role in DC homing to lymph nodes. We used noninvasive imaging by magnetic resonance and immunohistochemistry to track DCs after s.c. injection in the footpad of wild-type+/+ or RAGE−/− mice. Maturing DCs expressing RAGE effectively migrated in both conditions. In contrast, RAGE−/− DCs failed to reach the draining popliteal lymph nodes of +/+ and −/− mice, indicating that the integrity of RAGE is required for DC mobilization. Thus the HMGB1-RAGE pathway is a checkpoint in DC maturation and function and a candidate for targeted therapies. The Journal of Immunology, 2008, 180: 2270–2275.

Dendritic cells (DCs) undergo a complex program in response to microbial components, referred to as maturation. Maturing DCs up-regulate the membrane expression of molecules involved in T cell activation and costimulation, including MHC class I and class II CD40, CD80, and CD86 molecules. This process is necessary for productive activation of naïve T cells, which takes place in the lymph nodes (1).

DCs committed to maturation in peripheral tissues reach the lymph nodes through afferent lymphatic vessels: they release inflammatory chemokines, which induce through an autocrine/paracrine loop the down-regulation of the CCR1 and CCR5 chemokine receptors (2). Later on, DCs up-regulate the expression of CCR7 and CXCR4, thus acquiring responsiveness to lymph node chemokines. DCs that do not express CCR7 cannot reach the lymph nodes. Conversely, DC migration abates in mice lacking lymph node chemokines or the γ-isof orm of phosphoinositide-3 kinase, which is required for response to chemotactic agonists (3, 4). Lymphatic vessels also express chemokines (5), which are in turn involved in the attraction of maturing DCs. Finally, signals initiated within draining lymph nodes of immunized animals cause the expansion of lymphatic vessels, further sustaining and amplifying the effective homing of DCs (6, 7).

High mobility group box 1 (HMGB1) nuclear protein is a constituent of chromatin, which in the extracellular environment is an important mediator of inflammation that regulates the tissue response to infection and injury (8–10). HMGB1, in particular, controls the migratory properties of cells of various origins, including monocytes, smooth muscle cells, vessel-associated stem cells, and endothelial progenitor cells (11–14).

HMGB1 also controls DC function in vitro (15, 16) and exerts potent adjuvant effects on the immunogenicity of soluble Ags and apoptotic lymphoma cells in vivo (15). Potential sources of environmental HMGB1 are dying cells, or bone-marrow derived APCs, which express HMGB1 in the nucleus and secrete it in response to inflammatory stimuli (8–10, 17, 18). HMGB1 is a constituent of the immune complexes that stimulate cytokine production via activation of the TLR9, an event that has been recently demonstrated to involve the receptor for advanced glycation end products (RAGE) (19, 20).

Myeloid DCs committed to maturation translocate HMGB1 from the nucleus into the cytosol and secrete it in the microenvironment while remodeling the actin-based cytoskeleton, up-regulating CCR7 and CXCR4, and acquiring the ability to respond in vitro to chemokine receptor ligands (21). All events abate in the presence of antagonists of HMGB1 or of its best characterized membrane receptor, RAGE (21, 22), suggesting that HMGB1 secretion and DC mobilization could be causally linked. RAGE-mediated in vitro chemotaxis is G-protein dependent, because it is inhibited by pertussis toxin (22). In this study we verified whether RAGE expression is causally required for the migration of maturing DCs to lymph nodes.
FIGURE 1. Effective tracking of DCs in vitro and in vivo by MRI. DCs were labeled with SPIO particles, as detectable by Prussian blue staining/nuclear fast red counterstaining of cytospins (SPIO 640 µg/ml) (A) or by MRI of tubes containing water (no. 1) or pellets of DCs incubated with increasing concentrations of SPIO (in µg/ml: no. 2, 0; no. 3, 80; no. 4, 160; no. 5, 320; no. 6, 640); the amount of internalized iron correlates with the intensity of the reduction of the signal (B). C–F, SPIO+ DC were injected into the footpads. MRI analysis of draining popliteal lymph nodes was conducted immediately after (time 0, D) and 24 and 48 h after injection (E and F, respectively). C, Reduction of signal (signal-to-noise ratio, y-axis) associated with iron particles in the popliteal lymph node at different times after injection (hours, x-axis). G–I, Immunohistochemical staining of the draining popliteal lymph nodes 48 h after injection: at this time point iron particles (G), CD11c+ cells (H), and CD45.2+ cells (I) were clearly detectable in the subcapsular zone. Results are from representative routine experiments.

Materials and Methods

Cells

DCs were derived from bone marrow precursors of wild-type C57BL/6-Ly5.1, C57BL/6-Ly5.2, or of C57BL/6 RAGE−/− mice (23). When indicated, CD45.2+ DCs were derived from C57BL/6-Ly5.2 mice and injected into C57BL/6-Ly5.1 mice, which do not express the CD45.2 membrane molecule. In some experiments, DCs were treated with LPS (1 mg/ml, Sigma-Aldrich) for 48 h and/or with superparamagnetic iron oxide particles (SPIO, Endorem, Guerbet) for 16 h (24) and retrieved by magnetic sorting. DCs exposed to SPIO (0, 80, 160, 320, 640 µg/ml) were studied by flow cytometry after staining with FITC-labeled annexin V (Bender MedSystems and Valter Occhiena) and propidium iodide. DCs exposed to SPIO (0, 80, 160, 320, 640 µg/ml) were studied by flow cytometry and propidium iodide. DCs exposed to SPIO (0, 80, 160, 320, 640 µg/ml) were studied by flow cytometry and propidium iodide. DCs were derived from bone marrow precursors of wild-type C57BL/6-Ly5.2 mice and injected into C57BL/6-Ly5.1 mice, which do not express the CD45.2 membrane molecule.

Immunohistochemistry

Prussian blue staining was used to reveal SPIO-labeled DCs in situ. Spleen and liver were retrieved and analyzed at 2, 24, and 48 h after i.v. DC injection. The draining popliteal lymph nodes were removed 48 h after s.c. injection (hours, x-axis). G–I, Immunohistochemical staining of the draining popliteal lymph nodes 48 h after injection: at this time point iron particles (G), CD11c+ cells (H), and CD45.2+ cells (I) were clearly detectable in the subcapsular zone. Results are from representative routine experiments.

Magnetic resonance imaging

MR imaging (MRI) of the mice was performed in vivo on a 1.5T clinical whole-body MRI system (Gyroscan Intera, Philips Medical System) equipped with a 30 mT/m gradient system (slew rate 150 T/m/s) using a 43-mm circular coil (Microcoil 43, Philips Medical System). Mice were studied with MRI before and 2, 24, and 48 h after i.v. SPIO+ DC injection and before and 48 h after s.c. SPIO+ DC injection. MRI included coronal T1-weighted fast field echo (TE 4.6 ms, TR 180 ms, matrix 512 × 256, 1.5 mm, gap 0 mm), coronal T2-weighted turbo spin echo (TE 90 ms, TR 3700 ms, matrix 512 × 256, Th 1 mm, gap 0 mm), and coronal T2*-weighted, three-dimensional fast field echo sequences (TE 14 ms, TR 32 ms, matrix 512 × 256, Th 1 mm, gap 0 mm). The T2+ images were used to measure the signal-to-noise ratio of the liver, spleen, and draining popliteal lymph nodes by an experienced investigator who was blinded about the status of the mice.

Migration of endogenous DCs

Migration of endogenous DCs was assessed as described (27) with minor modifications as follows: wild-type and RAGE-null mice were painted on the ear with 20 µl of a TRITC solution (final dilution 8 mg/ml, Sigma-Aldrich) prepared in acetone and dibutyl phthalate (1:1, v/v), immediately before the painting. Twenty-four hours later, mice were sacrificed retroauricular lymph nodes and were cut into small fragments and digested in collagenase type II (1 mg/ml, Worthington Biochemical) and DNase (1 µg/ml grade II bovine pancreatic DNase, Boehringer Mannheim). Single-cell suspensions were analyzed for TRITC-positive cells expressing CD11c using an APC-conjugated mAb (BD Pharmingen). Analyses were performed with a FACSCalibur flow cytometer (BD Biosciences).

Results

We followed and quantitatively monitored by MRI the migration of DCs injected i.v. or in the footpads of mice in the presence of CFA. As expected (28), i.v.-injected DCs early and preferentially accumulated in the liver and spleen (not shown). In contrast, s.c.-injected DCs preferentially migrated to draining lymph nodes, where they were easily detectable at 48 h (Fig. 1). The immunohistochemical analyses of tissues (spleen, liver, draining and nondraining lymph nodes) retrieved confirmed the
results obtained by MRI. Lymph node histochemical analysis in particular confirmed that internalized iron particles and cells expressing the CD11c DC marker accumulated in the subcapsular region of draining lymph nodes. We exclude that CD11c/H11001 DC cells were originally derived from the injected animal, because they expressed the CD45.2 membrane molecule. Cells derived from C57BL/6-Ly5.2 mice, which we used as a source of bone marrow progenitors to propagate DCs, indeed selectively expressed CD45.2; in contrast, C57BL/6-Ly5.1 mice, in which DCs were injected, failed to express CD45.2 (Fig. 1).

MRI relies on the detection of DCs labeled with inert iron particles. This treatment per se was not toxic, and iron particle internalization did not interfere with the ability of DCs to mature when challenged with LPS or to activate Ag-specific T cells (Fig. 2).

A maturation signal is necessary to ensure that injected DCs acquire the ability to respond to the chemotactic gradient that originates from the lymphatic vessels and the draining lymph nodes (28). To verify the hypothesis that the autocrine/paracrine production of HMGB1 by maturing DCs could lead to RAGE activation, facilitating maturation and mobilization of DCs, we tested whether RAGE expression by maturing DCs might be required for migration to the draining lymph node. As shown in Figs. 3 and 4, A and B, RAGE−/− DCs injected into the footpad of wild-type or RAGE−/− mice failed to migrate to the draining popliteal lymph node. Conversely, wild-type DCs efficiently migrated to the lymph nodes in both wild-type and RAGE−/− animals, further supporting the notion that expression of RAGE

**FIGURE 2.** Conserved function of DCs that internalized SPIO particles. DCs derived from bone marrow precursors were treated or not with SPIO particles and, when indicated, exposed to LPS for the last 48 h of in vitro culture. Internalization of SPIO particles did not influence the basal CD40, CD80, and CD86 expression or their up-regulation after treatment with LPS. A, Fluorescence associated with each mAb (filled profiles; x-axis) compared with the relevant negative control (dotted profiles). B, Relative fluorescence intensity per each population (RFL, y-axis). The treatment with SPIO did not induce features of programmed cell death (annexin V or propidium iodide binding) at the concentrations we tested (0–0.64 mg/ml, x-axis, C and D) and did not influence the ability of DCs to activate OVA-specific hybridoma T cells, as assessed by evaluating IL-2 secretion (ng/ml, y-axis). Results are from representative routine experiments.

**FIGURE 3.** Selective migration of RAGE+/+ DCs to draining lymph nodes. MRIs of wild-type (bottom panel) or RAGE−/− mice 48 h after injection into the footpads of DCs labeled with SPIO. DCs were either wild-type (right panels) or RAGE−/− (left panels). Histograms indicate the signal-to-noise ratio (y-axis) calculated in the draining lymph node areas relative to each condition (**, significantly different from control, p < 0.001).
by maturing DCs, but not on lymphatic endothelium, is required for DC migration.

Skin painting with fluorescent dyes allows the identification of skin-derived DCs within draining lymph nodes. In this model, migration is facilitated by coadministration of dibutyl phthalate (27). By this procedure we found that the migration of maturing DCs abated in the absence of RAGE, as assessed by comparing the number of fluorescent CD11c⁺ DCs that appeared in draining lymph nodes 24 h after ear painting (Fig. 4C).

Discussion

MRI is an optimal approach for the dynamic tracking of labeled cells in vivo, including DCs (24, 26). With this technique we were able to follow and quantitatively monitor the migration of DCs labeled with inert iron particles injected in vivo. The internalization of iron particles was not toxic and did not interfere with the ability of DCs to mature or with their interaction with Ag-specific T cells (Fig. 2). This is in agreement with previous reports that exploited iron particles for noninvasive tracking of myeloid cells in vivo (24, 26, 29–31). Not surprisingly (3, 32, 33), we found that DCs injected subcutaneously in the presence of CFA preferentially migrated to draining lymph nodes.

A maturation signal (in our case provided by the CFA) is necessary to ensure that injected DCs up-regulate the expression of CCR7 and CXCR4, thus acquiring the ability to respond to the chemotactic gradient that originates from the lymphatic vessels and the draining lymph nodes (28). We previously found that the HMGB1 secretion downstream from the recognition of maturation stimuli was required for CCR7 and CXCR4 up-regulation and for the response to CCL19 and/or CXCL12 in vitro: all events abated if DCs were committed to maturation in the presence of HMGB1-blocking Abs or of the HMGB1 box A recombinant fragment, which behaves as an antagonist (21).

The hindrance of RAGE had similar effects in vitro on the HMGB1-elicited mobilization of DCs (21, 22). RAGE has a well-characterized role in promoting the recruitment and extravasation of inflammatory leukocytes in various experimental models via long-term activation of NF-κB (34–36). The latter event is apparently critical for DCs that mature in response to

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**FIGURE 4.** Selective migration of RAGE⁺/⁺ DCs to draining lymph nodes. A, Histochemical analysis of SPIO⁺ cells in the popliteal lymph nodes retrieved from wild-type (bottom panels) or RAGE⁻/⁻ mice 48 h after their injection into the footpads. DCs were either wild-type (right panels) or RAGE⁻/⁻ (left panels). B, Histograms indicate the number of positive cells/field of view (FOV) (y-axis) relative to each condition (**, significantly different from control, p < 0.001). C, Migration of endogenous skin DCs into the draining lymph node 24 h after TRITC painting in wild type (+/+ ) or RAGE null (−/−) C57BL/6 mice. The total number of CD11c⁺ TRITC⁺ DCs per lymph node was calculated as described in Materials and Methods. Data are from one representative experiment.

**FIGURE 5.** Schematic representation of the RAGE-mediated modulation of the DC migratory ability. RAGE activation occurs in DCs that are committed to maturation in peripheral tissues. It depends on the release of endogenous RAGE ligands, including the nuclear protein HMGB1, and controls the expression and function of receptors for lymph node chemokines, such as CXCL12 and CCL19 (21, 22). As a consequence, DCs acquire the ability to reach secondary lymphoid organs, where they initiate the clonal expansion of Ag-specific T cells (top). The genetic deletion of RAGE interrupts this circuit, possibly limiting the initiation of T cell-dependent immune responses.
microbial or endogenous stimuli (10, 19, 21–23, 37). The autocrine/paracrine production of HMGB1 by maturing DCs leads to RAGE activation, facilitating the maturation and mobilization of DCs. Taken together, the results of this study indicate that RAGE expression by maturing DCs (but not on other cells) is necessary for migration to the draining lymph node (Fig. 5).

Structurally heterogeneous ligands, including advanced glycation end products, members of the S100 calgranulin family, the β2 integrin Mac-1, amyloid-β peptide, and β-sheet fibrils, have been described for RAGE. Several such ligands are preferentially generated or modified in vivo during prolonged oxidative stress, inflammation, aging, and hyperglycemia, and they contribute to persisting inflammation and eventual tissue damage (38). Diabetes and aging in particular are associated with a deregulated and less effective immune response. Interestingly, advanced glycation end products down-regulate in vitro the ability of DCs to express costimulatory signals and to activate T cells (39). Similar results have been described after blockade of the autocrine secretion of HMGB1 and of RAGE activation (9, 23, 37). Our results suggest that advanced glycation end products could interfere with DC maturation by competition with endogenous ligands, causing a defective migration toward secondary lymphoid organs (Figs. 3 and 4) and possibly less effective nascent Ag-specific immune responses. Further studies are warranted to address this issue.

DC migration depends on several signal transduction events, including the phosphorylation of kinases and activation of the NF-κB pathway (28, 40). These events are initiated by the original maturing stimulus; however, the autocrine/paracrine activation of the RAGE receptor by secreted HMGB1 sustains the late activation of kinases, including MAPK p38, and of NF-κB (9, 23).

DCs are highly mobile cells. They acquire Ags in peripheral tissues, possibly in the presence of microbial signals, and present them to T cells in lymphoid organs, after a time-consuming migration process through lymphoid vessels. It is crucial for effective activation of T cells that DCs maintain the memory of the environment in which they acquired the Ag (41, 42). It is tempting to speculate that maturing DCs secrete HMGB1 and activate RAGE to mimic an “alerting” environment even in anatomical districts in which the original proinflammatory stimuli are not present anymore. The autocrine/paracrine activation of RAGE would maintain the DC-activated state during the migration through the lymphatic vessels, that is, long enough to allow their productive encounter with naïve T lymphocytes.

Disclosures
The authors have no financial conflicts of interest.

References


