ABSTRACT
Chemotaxis and integrin activation are essential processes for neutrophil transmigration in response to injury. CalDAG-GEFI plays a key role in the activation of β1, β2, and β3 integrins in platelets and neutrophils by exchanging a GDP for a GTP on Rap1. Here, we explored the role of CalDAG-GEFI and Rap1b in integrin-independent neutrophil chemotaxis. In a transwell assay, CalDAG-GEFI−/− neutrophils had a 46% reduction in transmigration compared with WT in response to a low concentration of LTB4. Visualization of migrating neutrophils in the presence of 10 mM EDTA revealed that CalDAG-GEFI−/− neutrophils had abnormal chemotactic behavior compared with WT neutrophils, including reduced speed and directionality. Interestingly, Rap1b−/− neutrophils had a similar phenotype in this assay, suggesting that CalDAG-GEFI may be acting through Rap1b. We investigated whether the deficit in integrin-independent chemotaxis in CalDAG-GEFI−/− neutrophils could be explained by defective cytoskeleton rearrangement. Indeed, we found that CalDAG-GEFI−/− neutrophils had reduced formation of F-actin pseudopodia after LTB4 stimulation, suggesting that they have a defect in polarization. Overall, our studies show that CalDAG-GEFI helps regulate neutrophil chemotaxis, independent of its established role in integrin activation, through a mechanism that involves actin cytoskeleton and cellular polarization. J. Leukoc. Biol. 88: 313–319; 2010.

Introduction
Neutrophil chemotaxis and transmigration toward a source of inflammation are two crucial processes for host defense against infection. Circulating neutrophils respond to a variety of inflammatory stimuli such as cytokines, chemokines, and LTs [1–3]. Through a multi-step process, neutrophils first roll on the activated endothelium via a mechanism that depends on P-selectin glycoprotein ligand 1 on the neutrophil and selectins on the endothelium [4]. Rolling leukocytes on the endothelium become activated [5, 6]. In this process, integrins on their surface transition from a low-affinity state that does not allow ligand binding to a high-affinity state that does allow ligand binding [7]. Integrins in the high-affinity conformation allow leukocyte firm adhesion to the endothelium. Finally, the neutrophil transmigrates into the tissue, where it migrates to the site of tissue injury or infection.

Although neutrophil adhesion to the inflamed endothelium is dependent on integrin function [4, 8], several authors have shown that cell migration is also possible in the absence of functional integrins. Neutrophils have been shown to chemotactically migrate in the presence of EDTA and blocking antibodies against β1 and αvβ3 [9]. In addition, neutrophils from a patient with leukocyte adhesion deficiency type-I (lacking β2 integrins) were also able to polarize and chemotax [10]. More recently, Lammermann and colleagues [11] showed that dendritic cells lacking all integrins on their surface were able to migrate to the lymphatic nodes when injected into the dermis, revealing that integrin-independent migration is not restricted to in vitro experimental settings but can also occur in vivo.

CalDAG-GEFI is a signaling molecule that in response to calcium and diacylglycerol, activates the small GTPase Rap1 by exchanging a GDP for a GTP [12]. Its expression is restricted to the basal ganglia and blood cells [12]. In platelets and neutrophils, CalDAG-GEFI is required for integrin activation [13, 14], and in human T cells, it is needed for LFA-1-mediated adhesion [15]. As a result, mice lacking CalDAG-GEFI present impaired inflammatory responses as a result of reduced leukocyte infiltration into the inflamed tissues and deficient platelet aggregation manifested as bleeding [13, 14]. Rap1 proteins are
GTpases of the Ras family broadly expressed in tissues. There are two isoforms of Rap1: Rap1a and Rap1b. Rap1b is the major isoform in platelets [16], B cells [17], and neutrophils [18], and Rap1b−/− mice show a bleeding phenotype and impaired integrin activation similar to that of CalDAG-GEFI-deficient mice [19].

We were interested in examining whether CalDAG-GEFI may have biological activities other than integrin activation. Here, we report that CalDAG-GEFI plays a role in neutrophil chemotaxis, independent of integrin function, by a mechanism that involves F-actin distribution and cell polarization.

MATERIALS AND METHODS

Mice

Six- to eight-week-old C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). CalDAG-GEFI−/− [13] and CD18−/− [20] mice were bred and housed in our animal facility. Rap1b−/− mice [19] and their WT controls were on a mixed background and obtained from the Blood Research Institute, Blood Center of Wisconsin (Milwaukee, WI, USA). Experimental procedures were approved by the Animal Care and Use Committees of the Immune Disease Institute and Harvard Medical School (Boston, MA, USA).

Neutrophil isolation

Mouse neutrophils were isolated from bone marrow by negative immunoselection as described [14]. Bone marrow cells were incubated with a cocktail of rat anti-mouse mAb against non-neutrophil cell markers, followed by a second incubation with anti-rat IgG coupled to magnetic beads (Miltenyi Biotec, Auburn, CA, USA). The cells were run through a magnetic column (Miltenyi Biotec) and the eluted neutrophils counted by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). Only samples with >90% of neutrophils were used.

Transwell migration assay

Neutrophils (10⁵) in RPMI 1640 with 10 mM HEPES and 0.5% BSA were placed in the upper well of a 12-transwell plate (Corning Inc., Acton, MA, USA). Different concentrations of LTB₄ (Sigma Chemical Co., St. Louis, MO, USA) were placed in the medium (unstimulated) only 1% of WT and CalDAG-GEFI−/− neutrophils transmigrated toward LTB₄ in a dose-dependent manner (Fig. 1A). When no LTB₄ was added to the medium (unstimulated) only 1% of WT and CalDAG-GEFI−/− neutrophils transmigrated toward LTB₄, in a dose-dependent manner (Fig. 1A). When no LTB₄ was added to the suspension 10 min before starting the experiment.

Adhesion assay

Neutrophils (10⁵) in RPMI 1640 with 10 mM HEPES and 0.5% BSA were loaded into a 24-well plate in the presence or absence of 10 mM EDTA and were activated or not with 10 ng/mL LTB₄. Cells were let to adhere 1 h at 37°C and 5% CO₂. After washing twice with PBS, adherent neutrophils were counted using light microscopy.

Chemotaxis assay

Neutrophils were subjected to horizontal chemotaxis using the EZ-TAXIScan apparatus [21, 22] (Effector Cell Institute, Tokyo, Japan). Between 70 and 100 neutrophils in RPMI 1640 + 10 mM HEPES + 0.1% BSA + 10 mM EDTA were aligned on one edge of the chemotaxis channel. At the other end, 1 pg LTB₄ was injected, creating a gradient along the channel. Pictures were taken every 30 s for 20 min, and recorded movies were analyzed using DIAS (Soll Technologies Inc., Iowa City, IA, USA).

F-actin quantification by flow cytometry

Neutrophils in suspension (10⁶) were incubated for 10 min with 10 mM EDTA and stimulated with 0.1 ng/mL LTB₄ for 30, 60, 180, or 300 s. Cells were fixed with 4% paraformaldehyde. Unstimulated cells (Time 0) received the same volume of PBS and were fixed immediately. Cells were permeabilized and stained with 0.1% Triton X-100 and Alexa 488 phalloidin (6.6 nM; Molecular Probes). Mean fluorescent intensity of phalloidin-stained neutrophils was measured by flow cytometry.

F-actin staining and visualization

Isolated neutrophils (10⁶) were incubated with 10 mM EDTA for 10 min and stimulated in suspension with 0.1 ng/mL LTB₄ for 30 s. Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with Alexa 488 phalloidin (6.6 nM) and Hoechst (1 μg/mL). Cells were observed in wide-field fluorescence microscopy (100×). Neutrophils with pseudopods with a width and length that exceeded 10% of the cell’s diameter were counted by two individuals blinded to their source.

Statistical analysis

Results were expressed as mean ± sem. Student’s t-test was performed, and results were considered to be statistically significant when P < 0.05.

RESULTS AND DISCUSSION

CalDAG-GEFI−/− neutrophils have an integrin-independent defect in chemotactic transmigration

To reach a site of inflammation or infection, neutrophils have to adhere to the inflamed endothelium, transmigrate through endothelial cells, and chemotax through the extravascular tissue matrix toward the focus of inflammation [8]. Although integrins are critical for the adhesion of neutrophils to activated endothelium, their role in chemotaxis through the extravascular space is less understood. CalDAG-GEFI is a small signaling molecule that has been shown to play a key role in integrin activation by activating the small GTPase Rap1 [12]. Here, we show that CalDAG-GEFI−/− neutrophils, whose only known phenotype is defective integrin activation, present impaired chemotaxis in integrin-independent assays as compared with WT neutrophils. In a transwell assay, WT and CalDAG-GEFI−/− neutrophils transmigrated toward LTB₄ in a dose-dependent manner (Fig. 1A). When no LTB₄ was added to the suspension 10 min before starting the experiment with a blocking antibody (GAME-46) or 10 mM EDTA. WT neutrophils stimulated with 0.1 or 10 ng/mL LTB₄ were able to transmigrate into the
lower well when 10 mM EDTA was used. Addition of a β2-blocking antibody did not affect the ability of WT neutrophils to transmigrate toward 10 ng/mL LTB₄. These results suggest that integrin function was not required for transmigration (Fig. 1B). In contrast, CytoD, an actin polymerization inhibitor, abolished neutrophil transmigration completely (Fig. 1B). We confirmed that 10 mM EDTA inhibits neutrophil integrins by performing adhesion assays on plastic in the presence or absence of 10 mM EDTA (data not shown). The results revealed that WT and CalDAG-GEFI−/− neutrophils (lacking β2 integrins) transmigrated in the absence of integrin function [9–11, 23].

Our results are consistent with other studies showing that neutrophil chemotaxis and migration on nonendothelial surfaces can occur independently of integrin function [9–11, 23]. In vitro studies showed that CD4⁺ T cells were able to migrate in a collagen matrix even when β1, β2, β3, and αv integrins were blocked [23]. In vivo studies showed that dendritic cells, in which all of their integrins were genetically ablated from their surface, were able to migrate to lymph nodes when injected into the dermis of a mouse. However, they could not migrate if they were injected into the circulation, a process that would require adhesion of the cells to the endothelium via integrins [11]. This study also showed that cell migration within a three-dimensional matrix is mediated through actin flow at the front of the cell and actomyosin contraction at the back, squeezing the cell within the scaffold of the matrix [11].

**Impaired horizontal chemotaxis in CalDAG-GEFI−/− neutrophils (integrin-independent)**

To study further the defect of CalDAG-GEFI−/− neutrophils in chemotaxis, we used the EZ-TAXIscan apparatus that allows real-time visualization of neutrophil movement toward a source of chemoattractant on a horizontal glass surface. In these assays, neutrophils were aligned to one edge of the glass surface, and the chemoattractant was deposited on the other edge of the channel, creating a gradient of chemoattractant in the channel. To focus on neutrophil chemotaxis, independent of integrins, we used 10 mM EDTA in all buffers and cell suspensions. Because in the transwell assay, we saw the defect in transmigration at a low concentration of LTB₄, we tested different concentrations of LTB₄ and used 1 pg LTB₄ (1 μL from a 1 ng/mL LTB₄ solution), as it was the lowest concentration that induced chemotaxis (data not shown). The results revealed that WT and CalDAG-GEFI−/− neutrophils were able to migrate toward the gradient LTB₄, showing again that neutrophil chemotaxis is possible in the absence of integrin function.

Using DIAS, we analyzed the migration characteristics of WT and CalDAG-GEFI−/− neutrophils. Single cells were tracked, and their paths were analyzed (Fig. 2, A and B). We saw that the chemotactic defect observed in CalDAG-GEFI−/− neutrophils could be explained by significant abnormalities in their chemotactic behavior. First, CalDAG-GEFI−/− neutrophils had slower migration speed compared with WT neutrophils...
Neutrophils. CalDAG-GEFI present an overall defective chemotaxis compared with WT chemottractant. In summary, CalDAG-GEFI straight the cell goes from its starting point to the source of chemotactant and the real distance moved. It indicates how ratio between the distance moved in the direction of chemotactant and the number and frequency of turns a cell makes. A higher number indicates more turns and thus, less efficient chemotaxis. Third, CalDAG-GEFI neutrophils had a reduced directionality index (Table 1).

The directionality index indicates how straight a cell moves from point A to point B. It represents the minimum distance between A to B divided by the actual distance the cell migrated. Therefore, the straighter the cell migrates, the close its directionality index will be to 1 and more efficient chemotaxis. Lastly, CalDAG-GEFI neutrophils showed reduced upward directionality (Table 1). This parameter is the ratio between the distance moved in the direction of the chemotactant and the real distance moved. It indicates how straight the cell goes from its starting point to the source of chemotactant. In summary, CalDAG-GEFI neutrophils present an overall defective chemotaxis compared with WT neutrophils. CalDAG-GEFI neutrophils are slower, make unnecessary turns, are less apt to migrate in a straight line,

and do not move in the direction of the source of chemottractant as efficiently as WT neutrophils. Thus, CalDAG-GEFI facilitates neutrophil migration toward LTB₄ and this, in the absence of integrin-mediated adhesion.

**Impaired pseudopod formation and F-actin distribution in stimulated CalDAG-GEFI⁻/⁻ neutrophils**

Our group has shown previously that CalDAG-GEFI⁻/⁻ neutrophils sense LTB₄ normally, as measured by calcium flux after stimulation [14], suggesting that intracellular signaling immediately downstream of the LTB₄ receptor is normal in CalDAG-GEFI⁻/⁻ neutrophils. Cytoskeleton rearrangement and in particular, F-actin polymerization and distribution are crucial processes for correct neutrophil chemotaxis. Indeed, blocking F-actin polymerization with CytoD resulted in abolished neutrophil transmigration in the transwell assay (Fig. 1B). We therefore wondered whether loss of CalDAG-GEFI could be affecting the actin cytoskeleton in response to LTB₄. To address this question, we stimulated WT and CalDAG-GEFI⁻/⁻ neutrophils and measured total F-actin polymerization by flow cytometry. As in the previous experiments, we used a low concentration of LTB₄ (0.1 ng/mL), and to assure an integrin-independent environment, we stimulated neutrophils in suspension in the presence of 10 mM EDTA. WT and CalDAG-GEFI⁻/⁻-unstimulated neutrophils (Time 0) did not show any change in their F-actin levels, but addition of 0.1 ng/mL LTB₄ produced a rapid increase in F-actin in both genotypes (Fig. 3A). WT and CalDAG-GEFI⁻/⁻ neutrophils showed similar kinetics of F-actin polymerization, reaching a peak after 30 s and decreasing rapidly after 1 min (Fig. 3A).

### Table 1. Impaired Directionality in CalDAG-GEFI⁻/⁻ Neutrophils

<table>
<thead>
<tr>
<th></th>
<th>WT (mean±SEM)</th>
<th>CalDAG-GEFI⁻/⁻ (mean±SEM)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Directionality index</td>
<td>0.92±0.01</td>
<td>0.84±0.02</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Upward directionality</td>
<td>0.90±0.01</td>
<td>0.83±0.02</td>
<td>&lt;0.05</td>
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(15±0.9 µm/s CalDAG-GEFI⁻/⁻ vs. 22±0.8 µm/s WT; P<0.05; Fig. 2C). Second, CalDAG-GEFI⁻/⁻ neutrophils exhibited increased direction change (13±0.7° WT vs. 25±2° CalDAG-GEFI⁻/⁻; P<0.05; Fig. 2D). The direction change is a parameter that measures, in degrees, the number and frequency of turns a cell makes. A higher number indicates more turns and thus, less efficient chemotaxis. Third, CalDAG-GEFI⁻/⁻ neutrophils had a reduced directionality index (Table 1). The directionality index indicates how straight a cell moves from point A to point B. It represents the minimum distance between A to B divided by the actual distance the cell migrated. Therefore, the straighter the cell migrates, the closer its directionality index will be to 1 and more efficient chemotaxis. Lastly, CalDAG-GEFI⁻/⁻ neutrophils showed reduced upward directionality (Table 1). This parameter is the ratio between the distance moved in the direction of the chemotactant and the real distance moved. It indicates how straight the cell goes from its starting point to the source of chemotactant. In summary, CalDAG-GEFI⁻/⁻ neutrophils present an overall defective chemotaxis compared with WT neutrophils. CalDAG-GEFI⁻/⁻ neutrophils are slower, make unnecessary turns, are less apt to migrate in a straight line,
These results indicate that CalDAG-GEFI is not required for total F-actin polymerization.

Because of the chemotactic defects in CalDAG-GEFI−/− neutrophils, we next wished to assess if there were any differences in the cellular distribution of F-actin upon stimulation. Neutrophils stimulated as described above were stained with phalloidin green, and F-actin distribution in individual cells was analyzed by fluorescence microscopy. Unstimulated WT neutrophils were round with uniform F-actin staining throughout the cytoplasm. Addition of LTB4 induced cell polarization with pseudopod formation and redistribution of the F-actin to the pseudopods in 28 ± 6% of the WT cells. In contrast, only 14 ± 2% of CalDAG-GEFI−/− neutrophils formed pseudopods with F-actin after stimulation (P<0.05; Fig. 3B). Those neutrophils that did not respond to LTB4 showed a round morphology and uniform F-actin distribution in the cell cytoplasm (Fig. 3C).

These results suggest that the defective chemotaxis seen in CalDAG-GEFI−/− neutrophils (a lower speed and defective chemotactic directionality compared with WT neutrophils) is likely a result of impaired cell polarization and defective F-actin localization at the pseudopods upon LTB4 stimulation, although their total F-actin polymerization is normal.

**Impaired chemotaxis in Rap1b−/− neutrophils**

Small GTPases such as Rac, RhoA, and Cdc42 have emerged as key regulators of cell polarization by controlling formation of lamellipodia at the front of the cell (Rac) [24], organizing the actomyosin meshwork (RhoA) [25] or establishing cell polarity directly (Cdc42) [26]. In a recent study, depletion of Cdc42 in dendritic cells resulted in impaired chemotaxis and abnormal cytoskeleton organization with normal actin polymerization [27]. This phenotype highly resembles the chemotactic phenotype of CalDAG-GEFI−/− neutrophils, suggesting that there could be a possible link between CalDAG-GEFI and Cdc42. In neurons, Rap1b was shown to activate Cdc42 regulating axon specification and neuronal polarity [28]. Interestingly, CalDAG-GEFI is highly expressed in neurons of the basal ganglia [12, 29], and it is known to activate Rap1 in platelets and neutrophils [12, 14]. In platelets, Rap1b rapidly incorporates into the cytoskeleton upon activation [30]. Therefore, we wondered whether the signaling pathway CalDAG-GEFI uses to regulate chemotaxis in neutrophils is through Rap1b. Rap1b−/− neutrophils and their respective WT control neutrophils were subjected to horizontal chemotaxis using the EZTAXIscan apparatus in the presence of 10 mM EDTA. WT and Rap1b−/− neutrophils sensed and migrated toward the gradient of LTB4 (Fig. 4, A and B). Rap1b−/− neutrophils presented reduced speed when compared with WT neutrophils (15±0.8 μm/s WT vs. 11±0.9 μm/s Rap1b−/−; P<0.05; Fig. 4C), and their direction change was higher than WT neutrophils (15±1.3° WT vs. 19±1.5° Rap1b−/−; P<0.05; Fig. 4D). On the other hand, the directionality index and upward directionality were similar in Rap1b−/− and WT neutrophils (data not shown).

These results show that Rap1b−/− neutrophils present some degree of impaired chemotaxis. Although these results cannot be compared directly with the results obtained with CalDAG-GEFI−/− neutrophils, as CalDAG-GEFI−/− and Rap1b−/− mice were on a different genetic background, the defect observed in Rap1b−/− neutrophils was reminiscent of that seen in CalDAG-GEFI−/− neutrophils. Considering these similarities, we propose that CalDAG-GEFI is controlling neutrophil integrin-independent chemotaxis, at least in part, by a mechanism involving Rap1b. The fact that CalDAG-GEFI is expressed in neurons and that Rap1b is known to have a role in establishing neuron polarity suggests that CalDAG-GEFI could also be involved in the regulation of neuron polarity, but to the best of our knowledge, this has not been investigated.

Increasing concentrations of LTB4 rescued the defect of CalDAG-GEFI−/− neutrophils in the transwell assay (Fig. 1A).
and also in the horizontal chemotaxis (not shown), suggesting that alternative pathways may exist in the regulation of neutrophil chemotaxis. Interestingly, increasing concentrations of the agonist overcame the defect in Rap1 activation in CalDAG-GEF\textsuperscript{I/-} neutrophils [14], and protein kinase C signaling has been proposed as an alternative signaling pathway for integrin activation in CalDAG-GEF\textsuperscript{I/-} platelets [31]. Throughout our studies, we used concentrations of LTB\textsubscript{4} of 1 or 0.1 ng/mL, as these were the concentrations where a major defect in neutrophil chemotaxis was seen in CalDAG-GEF\textsuperscript{I/-} neutrophils. We believe these concentrations are biologically relevant, as they are in the concentration range described in inflammatory diseases, for example, in the synovial fluid of rheumatoid arthritis [32] or in the sputum of patients with chronic obstructive pulmonary disease [33].

Although our results strongly support the existence of integrin-independent migration of neutrophils [9, 10], the exact mechanism these cells use to migrate is not known. In the EZ-TAXIScan machine, the depth of the channel through which the neutrophils migrate is 4 \( \mu \)m, meaning that the neutrophils, averaging 10–12 \( \mu \)m in diameter, have to squeeze in between the upper and lower surfaces of the channel. A similar setting was used by Malawista and de Boisfleury Chevance [9], where human neutrophils were located between a glass coverslip and a glass slide, separated by 5.7 \( \mu \)m. Here, they showed that human neutrophil chemotaxis is possible, even in the presence of 10 mM EDTA or blocking antibodies to \( \beta \)1 and a\( \alpha \)V\( \beta \)3 integrins [9]. In addition, neutrophils from a patient with leukocyte adhesion deficiency I (lacking \( \beta \)2 integrins), similar to the mouse \( \beta \)2-integrin-deficient neutrophils, underwent chemotaxis [9, 10]. The authors called this type of movement “chimney” and suggest that like a rock climber in a chimney, the cells use the contact with the surfaces as a force to locomote, minimizing the need for adhesion molecules. This is probably the same strategy WT and CalDAG-GEF\textsuperscript{I/-} neutrophils used to migrate in the channel of the EZ-TAXIScan chamber. However, the exact mechanism the cells use to migrate in these confined environments is still unknown. Hawkins et al. [34] proposed a mathematical model in which the cell migration in a channel relies on the coupling of actin polymerization to geometric confinement without the need for specific adhesion points. This model would be in accordance with our studies that suggest that the actin cytoskeleton directs cell movement when no integrin function is present. Indeed, Renkawitz and coworkers [35] documented recently that integrin-independent migration of ameboid dendritic cells is the result of retrograde flow of the actin filaments and accelerated actin polymerization at the leading edge. This mechanism maintains constant membrane protrusion and migration velocity of the cells [35]. It remains to be defined exactly how CalDAG-GEF and Rap1 regulate this type of integrin-independent ameboid movement of the neutrophils.

**AUTHORSHIP**

C.C., T.G., H.R.L., and D.D.W. designed research; C.C., D.D., T.G., H.H., J.S., and S.M.C. performed research; C.C., D.D., H.R.L., and D.D.W. analyzed data; G.C.W. and M.C-W. bred and provided the rare Rap1b\textsuperscript{-/-} animals; and C.C. and D.D.W. wrote the paper.
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