

Priming of Eosinophil Migration Across Lung Epithelial Cell Monolayers and Upregulation of CD11b/CD18 Are Elicited by Extracellular Ca²⁺

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In patients with asthma, eosinophils are primed and massively infiltrate lung tissues and migrate across epithelia into airways. Using blocking monoclonal antibodies, we found that eosinophil transmigration across a lung epithelial cell monolayer depended on the functions of α M β 2 integrin CD11b/CD18. To study the role of Ca²⁺ in eosinophil priming and transepithelial migration, we treated eosinophils with eotaxin or thapsigargin (TG), reagents that increase cytoplasmic free Ca²⁺ concentrations by receptor- or nonreceptor-mediated mechanisms, respectively. Pretreatment of eosinophils with TG enhanced CD11b/CD18-dependent transmigration across lung epithelium. Within minutes, TG time- and dose-dependently upregulated the expression of CD11b/CD18 but did not upregulate the expression of α L (CD11a) or β 1 (CD29) integrin. The upregulation of CD11b/CD18 expression by eotaxin or TG was prevented when Ca²⁺ entry was blocked. The priming of eosinophil transmigration by TG was also abrogated by the blockade of Ca²⁺ entry. Our results indicate that induction of Ca²⁺ entry by the depletion of Ca²⁺ from intracellular stores upregulates CD11b/CD18 expression on eosinophils and primes eosinophil transmigration across lung epithelium. Both responses are therefore elicited by extracellular Ca²⁺. We suggest that, as an important priming signal for human eosinophil functional responses, store-operated Ca²⁺ entry may be one of the underlying mechanisms of eosinophilic inflammation in asthma.

An important feature of allergic asthma is the infiltration and accumulation of eosinophils in the lungs (1, 2). In patients with asthma, eosinophils in blood are found primed or activated *in vivo* and show enhanced responses to different stimuli *in vitro*. The activated eosinophils can be obtained also from the bronchoalveolar lavage fluid (BALF), indicating that eosinophil extravasation and transmigration across airway epithelia have taken place. The extravasated eosinophils exert enhanced functional responses in comparison with eosinophils from blood, indicating that eosinophils are

further primed or activated during transmigration across endothelia and epithelia (2–4). Increasing evidence suggests that these activated eosinophils contribute to the pathogenesis and severity of allergic asthma.

Recent studies demonstrate that eosinophil transmigration is influenced by cytokines, chemokines, and other locally produced soluble mediators and is controlled by interactions between adhesion molecules on the surface of eosinophils and their counter-structures on the opposing cells or matrix proteins (2, 5, 6). It is known that eosinophil adherence, migration, and interactions with chemokines and many other soluble mediators induce intracellular Ca²⁺ increases (7–11), and these intracellular Ca²⁺ changes presumably have a large impact on cell functions. An increase in intracellular Ca²⁺ concentrations has been shown to prime cellular responses (12). Priming is necessary for eosinophils to migrate across endothelia or lung epithelia (13–15). We have recently established an *in vitro* model to study the regulation of human eosinophil transmigration across a monolayer of lung epithelial cells (15, 16). We found that priming eosinophils with cytokines such as interleukin (IL)-5 or with platelet-activating factor (PAF) enhances their transmigration across lung epithelium toward chemoattractant factors (15). Nonetheless, the mechanisms through which the Ca²⁺ increases prime eosinophil functional responses remain to be elucidated.

In this work, we have studied the role of Ca²⁺ in the priming of eosinophil transmigration across lung epithelial monolayers by using eosinophil chemoattractants (e.g., eotaxin) and thapsigargin (TG), reagents that increase intracellular Ca²⁺ concentrations in eosinophils through receptor- and nonreceptor-mediated mechanisms, respectively. We have investigated the role of α M β 2 integrin (CD11b/CD18) in eosinophil transmigration across lung epithelium and its relationship with the role of intracellular Ca²⁺ changes and the priming of eosinophil functions.

Materials and Methods

Reagents

L- α -phosphatidylcholine, β -acetyl- γ -O-hexadecyl (PAF), TG, recombinant human (rh) complement fragment 5a (C5a), and synthetic LTB₄ were purchased from Sigma (St. Louis, MO). Fura-2/acetoxymethyl ester (fura-2/AM) was purchased from Calbiochem (La Jolla, CA). Rh eotaxin (CCL11) was from Pepro Tech EC (London, UK). Rh MCP-3 (CCL7), RANTES (CCL5), and IL-8 (CXCL8) were from R&D Systems (Abingdon, UK). Lanthanum chloride (LaCl₃) was from BDH Chemicals (Poole, UK). C5a and PAF were dissolved in phosphate-buffered saline (PBS) supplemented with 0.5% or 2% (wt/vol) HSA (Central Laboratory of the Netherlands Blood Transfusion Service (CLB), Amsterdam,

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Abbreviations: bronchoalveolar lavage fluid, BALF; bovine serum albumin, BSA; Central Laboratory of the Netherlands Blood Transfusion Service, CLB; complement fragment 5a, C5a; cytoplasmic free Ca²⁺ concentration, [Ca²⁺]_i; eosinophil cationic protein, ECP; fluorescein isothiocyanate, FITC; fura-2/acetoxymethyl ester, fura-2/AM; interleukin, IL; monoclonal antibodies, mAbs; mean fluorescence intensity, MFI; platelet-activating factor, PAF; phosphate-buffered saline, PBS; recombinant human, rh; thapsigargin, TG.

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The Netherlands), respectively. TG and fura-2/AM were dissolved in dimethyl sulfoxide, and LaCl_3 was dissolved in distilled water. All reagents were stored as stock solutions at -20°C . The concentrations of the chemoattractants used in transepithelial migration assay were PAF and LTB_4 , $1\ \mu\text{M}$; C5a, MCP-3, RANTES, and IL-8, $10\ \text{nM}$. Cell culture medium and supplements were purchased from Life Technologies (Paisley, UK). The HEPES medium used in this study contained $132\ \text{mM NaCl}$, $6\ \text{mM KCl}$, $1\ \text{mM MgSO}_4$, $1.2\ \text{mM KH}_2\text{PO}_4$, $20\ \text{mM HEPES}$, $5.5\ \text{mM glucose}$, and 0.5% (wt/vol) HSA (pH 7.40). HEPES+ Ca^{2+} medium and HEPES- Ca^{2+} medium were HEPES medium supplemented with $1\ \text{mM CaCl}_2$, and with $0.5\ \text{mM EGTA}$, respectively. In experiments applying La^{3+} , modified HEPES+ Ca^{2+} medium was used in which phosphates and sulphates were replaced by chlorides.

Monoclonal Antibodies

The following mouse monoclonal antibodies (mAbs) were used to block eosinophil transepithelial migration. Purified mAb clone CLB-LFA-1/1 (IgG1) is directed against the integrin $\beta 2$ chain (CD18) and was purchased from CLB. Purified blocking mAbs clones 38 (IgG2a) and 3.9 (IgG1) against αL (CD11a) and αX (CD11c) of $\beta 2$ integrins, respectively, were from R&D Systems. Mouse isotype control IgG1 (clone 203) and IgG2a (clone 20,102.1) were purchased from CLB and R&D Systems, respectively. Mouse ascites fluids containing mAbs clone 44 (IgG2a) and clone 94 (IgM) against integrin αM chain (CD11b) were kindly provided by Dr. R. F. Todd, III (University of Michigan, Ann Arbor, MI) (17). Negative control NS-1 mouse ascites fluid was purchased from ICN Biomedicals (Aurora, OH). PBS supplemented with 0.001% Merthiolate was used as the buffer for these mAbs and their controls.

The following fluorescein isothiocyanate (FITC)-conjugated mouse mAbs were used to determine eosinophil surface antigen expression. Anti- αL (CD11a, clone MHM 24, IgG1); anti- $\beta 2$ (CD18, clone MHM 23, IgG1); anti- $\beta 1$ (CD29, clone K20, IgG2a) mAbs; and mouse control IgG1, IgG2a, and IgG2b were purchased from DAKO A/S (Glostrup, Denmark). Anti- $\alpha 6$ (CD49f, clone 450-30A, IgG1) mAb was from Serotec (Oxford, UK). Anti- αM (CD11b, clone Bear1, IgG1), anti-PECAM-1 (CD31, clone 5.6E, IgG1), anti-CD44 (clone J.173, IgG1), and anti- $\alpha 4$ (CD49 d, clone HP2/1, IgG1) mAbs were from Immunotech (Marseille, France).

Eosinophil Purification

Granulocytes were isolated using freshly processed buffy coats from $450\ \text{ml}$ peripheral blood obtained from healthy blood donors by density gradient centrifugation at room temperature over 67% isotonic Percoll (Pharmacia, Uppsala, Sweden) as described (18). After centrifugation, the cells were processed on ice or at 4°C and with ice-cold buffer unless otherwise stated. The erythrocytes were removed from the pellet by hypotonic lysis. The granulocytes obtained were washed twice with PBS and resuspended in PBS containing 2% heat-inactivated newborn calf serum (Life Technologies).

Eosinophils were purified via removal of neutrophils (CD16-positive) using a negative immunomagnetic selection technique as described elsewhere (16). The purified eosinophils were collected, washed, and suspended in HEPES medium. The purity and the viability of the eosinophils were more than 96% and 99% , respectively.

Epithelial Cell Culture

Human lung mucoepidermoid carcinoma H292 cell line cells (American Type Culture Collection CRL-1848, Rockville, MD)

(19, 20) were maintained in 25-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) in cell culture medium containing RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin ($100\ \text{U/ml}$), streptomycin ($100\ \mu\text{g/ml}$), and $2\ \text{mM}$ glutamine. After reaching confluence, cells from the third to thirtieth passages were routinely subcultured in an inverted position on polycarbonate membranes ($3.0\text{-}\mu\text{m}$ pore size, $6.5\ \text{mm}$ diameter) of Transwell cell culture inserts (C-93415; Corning Costar, Cambridge, MA) according to a protocol described in detail elsewhere (16). The culture medium in the Transwell system was replaced with fresh medium 24 h before the transmigration assay. The confluence of epithelial cell monolayers, as determined by May-Grünwald/Giemsa staining and light microscopy, was reached within 3 d before being used in transmigration experiments.

Eosinophil Transepithelial Migration

A detailed protocol of eosinophil transmigration assay has been described elsewhere (16). Briefly, the upper and lower compartments of the cell culture chamber inserts with confluent epithelial cell monolayers were washed twice with 37°C -prewarmed HEPES+ Ca^{2+} medium. Purified eosinophils were washed and re-suspended in HEPES+ Ca^{2+} medium and kept at 0°C . They were adjusted to 10^6 cells/ml, treated if necessary (*see below*), and prewarmed at 37°C for 3 to 4 min before being added into the upper compartments. Chemotactic solutions were added to lower compartments and were prewarmed to 37°C . The 24-well culture plates containing these inserts were then incubated at 37°C with 5% CO_2 and maximal humidity for 2 h.

TG was added at different concentrations to eosinophils in HEPES+ Ca^{2+} medium, and the cells were incubated for 5 min, or for time indicated, in a 37°C -water bath with gentle agitation. The concentration of dimethyl sulfoxide was $< 0.1\%$ in all cases in this study. After TG treatment, eosinophils were immediately chilled in ice-cold water, and ice-cold HEPES+ Ca^{2+} medium (> 5 vols) was quickly added. The cells were then washed at 4°C for 5 min and re-suspended at 10^6 cells/ml in HEPES+ Ca^{2+} medium.

To block $\beta 2$ integrins, purified eosinophils (at 10^6 cells/ml) in HEPES+ Ca^{2+} medium were incubated with different purified mouse mAbs, mouse ascites fluid, or their controls at room temperature for 15 min and then in a shaking water bath at 37°C for 3 to 4 min just before the start of the transmigration assay.

After the 2-h transmigration assay, the cell suspensions from upper and lower compartments were collected separately as described previously (16). Samples were stored at -20°C for later quantification of eosinophil cationic protein (ECP). The epithelial cell monolayers remained intact after transmigration assays, as determined by fixation, staining, and examination of the filter membranes under light microscope (15, 16).

ECP Quantification

The ECP content in samples was measured as described (16). The percentage of eosinophil transmigration was calculated from the ECP content of the lower compartment relative to the total ECP contained in the cells added to the insert at the beginning of the assay. The recovery of ECP was always $> 90\%$.

Measurement of Cytoplasmic-Free Ca^{2+} Concentration

Purified eosinophils suspended in HEPES+ Ca^{2+} medium (at 10^7 cells/ml) were loaded with $2\ \mu\text{M}$ fura-2/AM for 40 min at 37°C in the dark under gentle agitation. The cells were washed once, kept

in the dark for 15 min at room temperature, and washed again, resuspended in HEPES medium to the original concentration, and kept on ice. Fura-2-loaded cells were diluted 10 times in HEPES medium and pre-warmed for 5 min at 37°C before being transferred to a cuvette containing 10^6 cells/ml. Unless otherwise stated, the concentration of Ca^{2+} in cell suspension was adjusted to 1 mM. The cells were magnetically stirred and kept at 37°C, and fluorescence changes were monitored with a dual-excitation wavelength spectrofluorometer (Model F-2000; Hitachi, Tokyo, Japan), with 340 and 380 nm as excitation and 510 nm as emission wavelengths. To calibrate the fura-2 fluorescence as a function of cytoplasmic-free Ca^{2+} concentration ($[Ca^{2+}]_i$), maximal fluorescence was achieved by adding Triton X-100 (0.05%) and minimal fluorescence by 10 mM EGTA/150 mM Tris (pH > 8.3). Absolute $[Ca^{2+}]_i$ was calculated according to the formula described by Grynkiewicz and co-workers (21) with 224 nM as the dissociation constant for Ca^{2+} -fura-2 complex at 37°C.

Flow Cytometry

Purified human eosinophils were spun down and resuspended to 10^6 cells/ml in PBS with 0.5% (wt/vol) bovine serum albumin (BSA). Aliquots of cells (50 μ l) were incubated at 4°C in the dark for 30 min with FITC-conjugated mouse mAbs or negative isotope control Ig. The cells were then washed twice at 4°C and re-suspended in a volume of 200 μ l in PBS with 0.5% BSA and analyzed with a flow cytometer (Coulter EPICS-XL; Coulter Corporation, Miami, FL). The negative isotope control Ig was used to set the background fluorescence level. The expression of specific antigens on eosinophils was quantitated as mean fluorescence intensity (MFI) of each specific FITC-conjugated mAb bound to eosinophils after subtracting the background fluorescence.

Statistics

Data are presented as means \pm SEM. Two value sets were compared using Student's *t* test. The statistical differences among the multiple groups were analyzed by ANOVA and post hoc comparisons by the Tukey-Kramer Multiple Comparison Test (InStat2; GraphPad Software, San Diego, CA). A *P* value < 0.05 was considered significant.

Results

Priming Effect of Eotaxin and TG on Eosinophil Trans epithelial Migration

Pretreatment of eosinophils with chemoattractants primes eosinophil transmigration across lung epithelial cell monolayers. Our preliminary data indicated that chemoattractant-treated eosinophils did not transmigrate toward medium alone. Pretreatment of eosinophils with PAF or RANTES enhanced these cells to transmigrate across lung epithelium toward C5a but not toward PAF or RANTES, respectively (desensitization). Pretreatment with C5a enhanced trans epithelial migration toward PAF and RANTES but not toward C5a (our unpublished observations). Figure 1A shows the priming effect of eotaxin at different concentrations on eosinophil trans epithelial migration toward C5a. A common feature of these chemoattractants on eosinophils is to increase $[Ca^{2+}]_i$ and upregulate CD11b expression. This $[Ca^{2+}]_i$ increase consists of Ca^{2+} release from intracellular stores and Ca^{2+} influx across plasma membrane; the latter has been shown, at least in neutro-

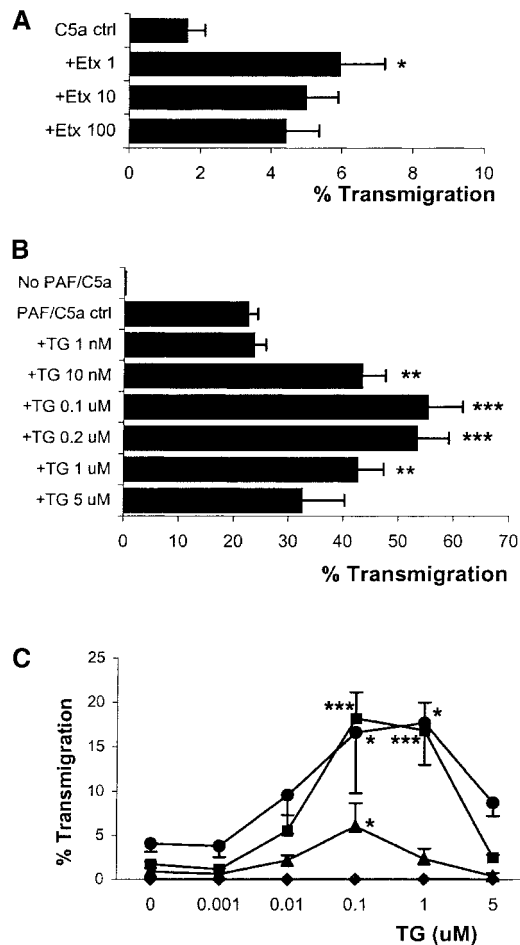


Figure 1. Effect of eotaxin and TG on eosinophil trans epithelial migration. Purified eosinophils were treated with different concentrations of eotaxin or TG at 37°C for 5 min and washed, and the trans epithelial migration was determined. (A) Eotaxin (Etx) primes eosinophil trans epithelial migration toward C5a ($n = 3$). TG enhances eosinophil trans epithelial migration toward (B) the combined chemotaxins PAF and C5a, and (C) different single chemoattractants. Data are means \pm SEM. In B, four to five independent experiments were performed with each single measurement done with monolayers in duplicate. In C, the number of independent experiments is indicated in parentheses. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, compared with each corresponding non-eotaxin or non-TG controls (ANOVA). Diamonds, Medium (4); triangles, RANTES (3); circles, C5a (3); squares, PAF (4).

phils, to be due mostly to the depletion of Ca^{2+} from intracellular stores (22). To understand the impact of depletion of Ca^{2+} from stores on the functions of eosinophils, we treated eosinophils with TG, a specific inhibitor of sarcoplasmic reticulum Ca^{2+} -ATPase, which bypasses membrane receptors and increases $[Ca^{2+}]_i$ in cells via depletion of Ca^{2+} in intracellular stores and subsequent induction of Ca^{2+} influxes across plasma membrane (23–25), and tested the trans epithelial migration of these eosinophils.

We found that short-term treatment of eosinophils with TG substantially increased their transmigration across lung epithelial cell monolayers toward the combined chemotactic

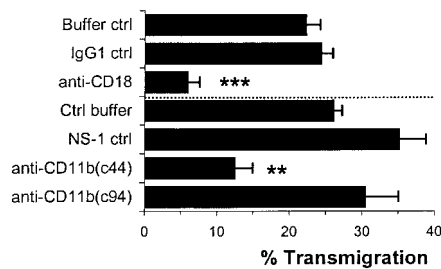


Figure 2. Effect of mAbs against $\beta 2$ integrins on eosinophil trans-epithelial migration. Freshly purified eosinophils were incubated with purified anti-CD18 mAbs (10 $\mu\text{g}/\text{ml}$), control IgG1, or 1/200 dilution of mouse ascites fluid containing anti-CD11b mAbs clone 44 (c44), clone 94 (c94), or negative NS-1 mouse ascites fluid as described in MATERIALS AND METHODS. Eosinophil transmigration was induced by combined chemotaxins PAF and C5a. Data are means \pm SEM of four experiments for the effect of anti-CD18 or three experiments for the effect of mAbs clones 44 and 94. *** $P < 0.001$, and ** $P < 0.01$ when compared with buffer, IgG1, or with NS-1 controls, respectively (ANOVA).

solutions of PAF and C5a or the single chemoattractants PAF, C5a, and (to a lesser extent) RANTES but not toward medium alone (Figures 1B and 1C). This priming effect of TG was concentration dependent, the optimal concentration being around 100 nM. Eosinophils pre-treated with cyclopiarazonic acid, another microsomal Ca^{2+} -ATPase inhibitor, or with Ca^{2+} ionophore ionomycin also exhibited an enhanced transmigration across lung epithelial monolayers toward chemoattractants (data not shown).

Role of $\alpha\text{M}\beta 2$ Integrin (CD11b/CD18) in Eosinophil Transepithelial Migration

We incubated eosinophils with blocking mAbs against different subunits of $\beta 2$ integrins and tested their capacity for transepithelial migration. mAb against the $\beta 2$ chain (CD18, CLB-LFA-1/1) potently inhibited eosinophil transmigration toward the combined chemotaxins PAF and C5a (Figure 2). When the concentration of this mAb was increased from 10 to 40 $\mu\text{g}/\text{ml}$, inhibition of transmigration was complete. Eosinophil transepithelial migration was potently inhibited by anti- αM (CD11b) mAb clone 44, whereas clone 94 had no effect (Figure 2). The anti- αM mAb clone 44 has been well characterized and is known to inhibit adhesion and lectin site-dependent functions of CD11b/CD18, whereas clone 94 has been shown to inhibit CD11b/CD18-dependent resotting but not adhesion (17, 26). Two purified commercial mAbs against two different α chains, CD11a and CD11c, clones 38 and 3.9, respectively, were found ineffective in our model (not shown). These data indicate that the transmigration of human eosinophils across a lung epithelial cell monolayer is CD11b/CD18 dependent.

Because eosinophil transepithelial migration was potently enhanced by the short-term pretreatment of eosinophils with TG (Figure 1), we examined whether eosinophil transepithelial migration is also CD11b/CD18 dependent after TG treatment. Using the same mAbs, we tested the

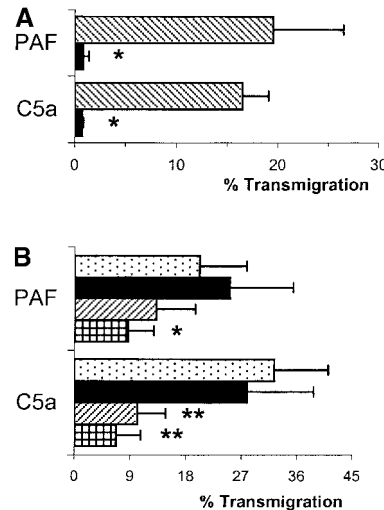


Figure 3. Effect of mAbs against CD18 (A, 40 $\mu\text{g}/\text{ml}$) or against CD11b (B, clone 44) on the transepithelial migration of eosinophils pre-treated with TG. After TG treatment (100 nM, 5 min) and washing, eosinophils were incubated with mAbs or the controls and used in the transmigration assay. The NS-1 supernatant diluted 1/200 was used as negative control. PAF or C5a was used as chemoattractant in these experiments. Data are means \pm SEM ($n = 3$). In A, * $P < 0.05$ when compared with

the IgG1 control (by paired t test). *Striped bars*, IgG1 control; *solid bars*, anti-CD18. In B, * $P < 0.05$ or ** $P < 0.01$ when compared with the buffer control (by ANOVA). *Dotted bars*, buffer; *solid bars*, NS-1 control; *striped bars*, c44, 1/200; *hatched bars*, c44, 1/50.

transmigration of eosinophils, pre-treated with TG and then incubated with mAbs, toward a single chemoattractant PAF or C5a. After TG treatment, eosinophils still depended on CD11b/CD18 for transepithelial migration (Figure 3). We concluded that transmigration of TG-treated and untreated eosinophils across lung epithelium is mediated by CD11b/CD18.

TG-Induced Intracellular Ca^{2+} Changes in Eosinophils

We tested the effect of TG on human eosinophil $[\text{Ca}^{2+}]_i$ using fura-2. We found that the basal $[\text{Ca}^{2+}]_i$ of eosinophils suspended in a 1 mM Ca^{2+} -containing medium was 53 ± 6 nM (mean \pm SD, $n = 18$). The addition of TG in the presence of 1 mM Ca^{2+} induced a gradual increase of $[\text{Ca}^{2+}]_i$ after an initial delay of ~ 10 s (Figure 4A). This increase in $[\text{Ca}^{2+}]_i$ was dose dependent. After the lag phase, the elevation of $[\text{Ca}^{2+}]_i$ was prolonged and sustained for at least 10 min.

To investigate the relative contribution of intracellular and extracellular Ca^{2+} to the TG-induced $[\text{Ca}^{2+}]_i$ changes, we measured $[\text{Ca}^{2+}]_i$ in the presence of EGTA. After 5 min of TG, the addition of EGTA to a final concentration of 0.5 mM rapidly decreased $[\text{Ca}^{2+}]_i$ to the basal level (Figure 4B, *solid curve*). On the other hand, when TG was added to eosinophils suspended in Ca^{2+} -free medium containing 0.5 mM EGTA, basal $[\text{Ca}^{2+}]_i$ and the $[\text{Ca}^{2+}]_i$ increase were much lower than in medium containing 1 mM Ca^{2+} . The $[\text{Ca}^{2+}]_i$ increase was not sustained in the absence of Ca^{2+} in medium, decreasing to its original level within 3 min (Figure 4B). The addition of Ca^{2+} to the cell suspension after the Ca^{2+} response had ceased produced an immediate increase in $[\text{Ca}^{2+}]_i$, indicating that the cells had retained their capacity to increase $[\text{Ca}^{2+}]_i$ (Figure 4B, *dotted line*). We observed that in 1 mM Ca^{2+} -containing medium, after $[\text{Ca}^{2+}]_i$ had increased for at least 6 min, the addition of La^{3+} , a

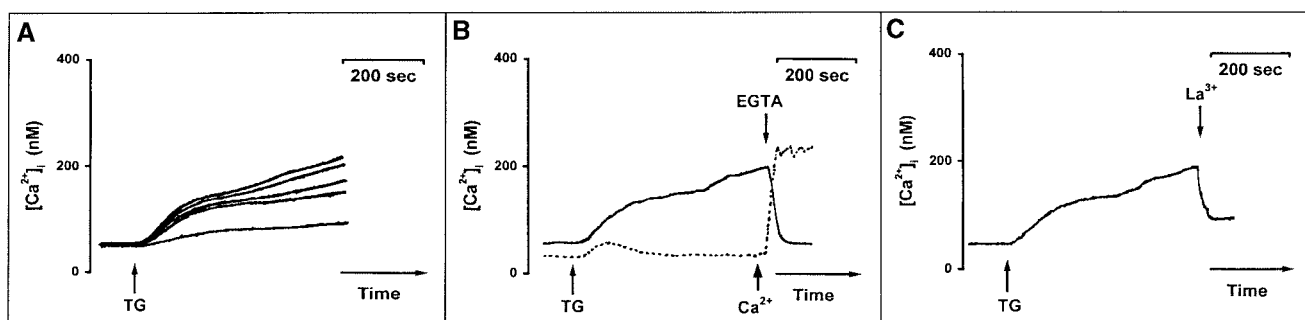


Figure 4. TG-induced $[Ca^{2+}]_i$ changes in human eosinophils and the role of extracellular Ca^{2+} . (A) Eosinophil $[Ca^{2+}]_i$ was measured upon addition of different concentrations of TG. From bottom to top, the traces show $[Ca^{2+}]_i$ after addition of 10 nM, 0.1 μ M, 0.2 μ M, 1 μ M, and 2 μ M of TG, respectively. The curves shown are from one out of three independent experiments performed in HEPES+ Ca^{2+} medium (1 mM Ca^{2+} present). (B) Eosinophil $[Ca^{2+}]_i$ was monitored upon TG addition (0.2 μ M) in HEPES+ Ca^{2+} medium (1 mM Ca^{2+} present, *solid curve*) or in HEPES- Ca^{2+} medium (0.5 mM EGTA present, *dotted curve*), respectively. Six to 8 min after TG, two additions were made: (i) EGTA (to 0.5 mM final concentration) to the eosinophils in medium with Ca^{2+} (*solid curve*) or (ii) Ca^{2+} (to 1 mM final concentration) to the eosinophils in medium without Ca^{2+} (*dotted curve*). In the former, after EGTA addition, external Ca^{2+} was replenished to 1 mM final concentration according to a protocol previously described (12) before calibration of fura-2 fluorescence. Similar curves were observed in two independent experiments. (C) The effect of La^{3+} on TG-induced $[Ca^{2+}]_i$ changes was determined in modified HEPES+ Ca^{2+} medium. The addition of TG (0.2 μ M) increased $[Ca^{2+}]_i$ in eosinophils similar to the profiles shown in A and B. Subsequent addition of 0.1 mM La^{3+} rapidly decreased $[Ca^{2+}]_i$. A similar decrease in $[Ca^{2+}]_i$ was observed in three different experiments.

nonspecific Ca^{2+} channel blocker, resulted in a decrease of $[Ca^{2+}]_i$ (Figure 4C). Our findings suggest that TG-induced $[Ca^{2+}]_i$ increase in eosinophils is initially caused by Ca^{2+} release from intracellular stores, but subsequent extracellular Ca^{2+} influxes contribute to the sustained $[Ca^{2+}]_i$ increase. In control experiments, we found that the addition of La^{3+} or 3 mM Ca^{2+} to unstimulated eosinophils did not change the basal $[Ca^{2+}]_i$ (not shown).

TG-Induced CD11b/CD18 Upregulation in Eosinophils

The fact that the transmigration of TG-primed eosinophils across lung epithelium depends on CD11b/CD18 led us to assume that the priming effect of TG may originate from an upregulation of CD11b/CD18 expression. TG upregulated CD11b/CD18 expression on eosinophils in a dose-dependent fashion (Figure 5A). Similar to the dose-response curve for enhanced transepithelial migration (Figures 1B and 1C), TG-induced CD11b/CD18 upregulation was optimal at 100 nM or above. The upregulation of CD11b/CD18 was accompanied by exocytosis of these eosinophils, as evidenced by the release of ECP to extracellular medium (Figure 5A, *inset*). The highest release amounted to < 2% of the total content of cellular ECP. The dose-response curves of ECP release and CD11b/CD18 upregulation after TG treatment were similar. However, after eosinophils had been incubated with TG and washed, they did not release ECP during the 2-h transepithelial migration assay (data not shown). In a time-course study, we found that a 5- to 10-min incubation with 100 nM TG resulted in a maximal upregulation of CD11b/CD18 expression by eosinophils (Figure 5B). Because high concentrations of TG were found to have some toxic effects on eosinophils, such as cell death and homotypic aggregation, we considered the treatment of eosinophils with 100 nM TG for 5 min as optimal for priming.

Under the optimal condition of eosinophil treatment

with TG, we examined the expression of a number of surface markers that have been found to be involved in eosinophil adhesion and migration in different models. TG treatment upregulated eosinophil CD11b/CD18 but not integrin α L (CD11a), α 4 (CD49d), α 6 (CD49f), or β 1 (CD29) chains or CD31 (PECAM-1) or CD44 (Figure 5).

Role of Extracellular Ca^{2+} in Eotaxin- or TG-Induced CD11b/CD18 Upregulation

Eotaxin has been shown to upregulate CD11b expression on eosinophils (10, 11). We confirmed this finding and found that the upregulation of CD11b/CD18 by short-term eotaxin treatment was mostly dependent on the presence of Ca^{2+} in medium (Figure 6A). Because TG depletes Ca^{2+} in the same intracellular stores and induces Ca^{2+} influxes as chemoattractants do, to understand the importance of Ca^{2+} influxes for CD11b/CD18 upregulation in eosinophils, we examined the influence of EGTA and La^{3+} on the TG-induced upregulation of CD11b/CD18 expression. We observed that the expression of CD11a was unaffected by TG, EGTA, or both and that, although 0.5 mM EGTA did not change the basal CD11b/CD18 expression on eosinophils, it completely inhibited the upregulation of CD11b/CD18 induced by TG (Figure 6B). Pre-treatment of eosinophils with La^{3+} (from 1 to 100 μ M) dose-dependently inhibited the upregulation of CD11b/CD18 by TG (Figure 6C). At concentrations from 1 μ M to 0.2 mM, La^{3+} alone did not change the CD11b/CD18 expression on unstimulated eosinophils (data not shown). We also tested L-type Ca^{2+} channel blockers and observed that nifedipine at concentrations from 10 nM to 10 μ M had no effect on TG-induced upregulation of CD11b/CD18 on eosinophils and that verapamil at 10 μ M only weakly inhibited (< 25% inhibition) the upregulation of CD11b. This suggests that Ca^{2+} influx via L-type Ca^{2+} channels is unlikely to be involved in TG-

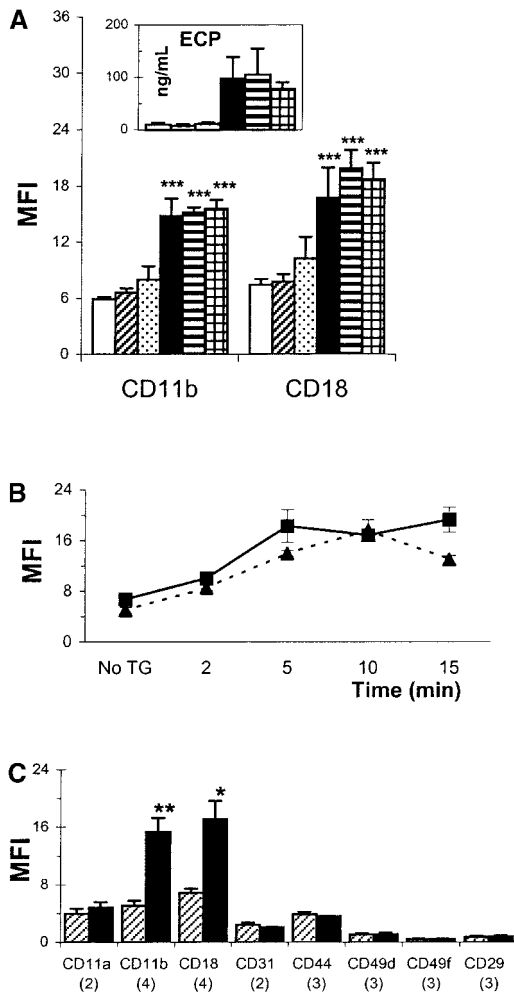


Figure 5. Effect of TG on the expression of eosinophil surface antigens. (A) Dose-response of TG-induced upregulation of CD11b/CD18 expression on eosinophils. Eosinophils (10^6 cells/ml) were incubated with different concentrations of TG for 5 min, washed, labeled with indicated FITC-conjugated mAbs, and analyzed by flow cytometry. At 5 min of TG treatment, aliquots of the cell suspension were collected and immediately centrifuged at $600 \times g$. The ECP concentrations in supernatants of TG-treated eosinophils (10^6 cells/ml) are shown (inset). Data are means \pm SEM of four (A) or three (inset in A) independent experiments. $***P < 0.001$ when compared with non-TG controls (ANOVA). Open bars, Eos control; diagonally striped bars, +TG 1 nM; dotted bars, +TG 10 nM; solid bars, +TG 0.1 μ M; horizontally striped bars, +TG 1 μ M; hatched bars, +TG 5 μ M. (B) Kinetics of TG-induced CD11b/CD18 upregulation. Eosinophils were incubated at 37°C with 100 nM TG for the time indicated, chilled in ice-cold water, washed, and labeled with FITC-conjugated mAbs for flow cytometric analysis. Data are means \pm range of two independent experiments. Triangles, CD11b; squares, CD18. (C) Expression of eosinophil surface antigens after TG treatment. Cells treated with 100 nM TG for 5 min were washed, labeled with FITC-conjugated mAbs against different eosinophil surface antigens, and analyzed by flow cytometry. The number of independent experiments is indicated in parentheses. $**P < 0.01$, and $*P < 0.05$, compared with non-TG controls (by paired *t* test). Striped bars, Eos control; solid bars, Eos + TG.

induced CD11b/CD18 upregulation in eosinophils. Our results demonstrate that TG upregulates CD11b/CD18 expression on eosinophils, and this upregulation is completely dependent on the influx of extracellular Ca^{2+} into eosinophils.

Effect of La^{3+} on TG Priming of Eosinophil Transepithelial Migration

Based on the results presented above, we reasoned that the priming of eosinophil transepithelial migration by intracellular Ca^{2+} depletion is dependent on the upregulation of CD11b/CD18 expression or functions mediated by an influx of extracellular Ca^{2+} .

We previously found that the addition of EGTA or La^{3+} to epithelial cells potentially inhibited eosinophil transepithelial migration (16). Because La^{3+} and EGTA show similar inhibitory effects on TG-induced extracellular Ca^{2+} influx (Figure 4) and the upregulation of CD11b/CD18 expression on eosinophils (Figure 6), we studied the effect of La^{3+} on the priming of eosinophil transepithelial migration by TG. Eosinophils treated with 1 or 10 μM La^{3+} did not differ from control eosinophils in their ability to migrate (Figure 7). Instead, La^{3+} at 100 μM increased eosinophil transepithelial migration toward PAF ($P < 0.05$), although not the transmigration toward C5a ($P > 0.05$). Nonetheless, La^{3+} at concentrations between 10 to 100 μM significantly inhibited ($P < 0.01$) the priming effect of TG on eosinophil transepithelial migration toward C5a, and this inhibition was concentration dependent.

To examine the possibility of La^{3+} having other actions on eosinophils, we also tested the effect of La^{3+} on eosinophil transepithelial migration after eosinophils had been pretreated with TG. We found that La^{3+} at 100 μM exerted only a weak inhibitory effect on transmigration (18% and 16% inhibition of the transmigration of TG-treated eosinophils toward PAF and C5a, respectively), indicating that the presence of La^{3+} does not substantially affect the transmigration of primed eosinophils. Because eosinophil transepithelial migration is CD11b/CD18-dependent, this suggests that La^{3+} does not affect the CD11b/CD18 functions. Moreover, the addition of L-type Ca^{2+} channel blockers verapamil or nifedipine to TG-primed eosinophils did not inhibit transepithelial migration.

Discussion

Using human primary bronchial epithelial cells and lung mucoepidermoid carcinoma H292 cells, we have recently demonstrated *in vitro* that priming is required for eosinophils to migrate efficiently across lung epithelial cell monolayers (15). In the present study, we have investigated the role of Ca^{2+} in the priming of eosinophil transepithelial migration by using the chemoattractant eotaxin and the pharmacological tool TG, both of which increase eosinophil $[\text{Ca}^{2+}]_i$. Our main findings are (i) human eosinophil transmigration toward chemoattractants across a lung epithelial cell monolayer is CD11b/CD18 dependent; (ii) TG induces gradual and sustained increases of $[\text{Ca}^{2+}]_i$ in eosinophils, initially due to the depletion of Ca^{2+} from intracellular stores and subsequently to Ca^{2+} entry into cells; (iii) eotaxin and TG upregulate CD11b/CD18 surface expression in an

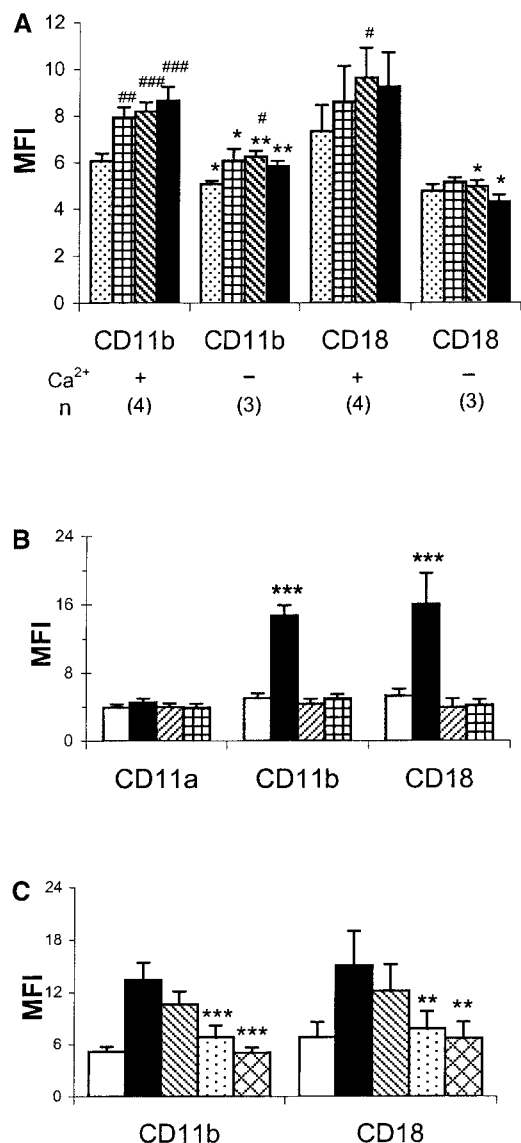


Figure 6. Role of extracellular Ca^{2+} in the upregulation of eosinophil CD11b/CD18 expression induced by (A) eotaxin and (B) TG. (A and B) Eosinophils were suspended in HEPES+ Ca^{2+} medium and HEPES- Ca^{2+} medium, respectively; incubated with buffer, eotaxin (Etx), or 100 nM TG for 5 min; and washed with the same medium. The expression of CD11b, CD18 (and CD11a in B) was analyzed by flow cytometry. Data are means \pm SEM of three to four independent experiments. (A) $^*P < 0.05$, $^{##}P < 0.01$, and $^{###}P < 0.001$, compared with each buffer control, respectively, by ANOVA. $^*P < 0.05$, and $^{**}P < 0.01$ when compared with the corresponding groups in the presence of 1 mM Ca^{2+} , by unpaired *t* test. Dotted bars, Eos control; hatched bars, +Etx 1 nM; striped bars, +Etx 10 nM; solid bars, +Etx 100 nM. (B) $^{***}P < 0.001$ compared with the groups of the + Ca^{2+} buffer control or EGTA buffer control, or with the EGTA+TG group, by ANOVA. Open bars, Eos + Ca^{2+} ; solid bars, + Ca^{2+} + TG; striped bars, +EGTA; hatched bars, +EGTA + TG. (C) La^{3+} at different concentrations (La, μM) was added to eosinophils in modified HEPES+ Ca^{2+} medium (1 mM Ca^{2+} present) at room temperature for 3 to 4 min before TG treatment. TG was added to all cell suspensions except control eosinophils and was incubated at 37°C for 5 min. The cells were then washed with modified HEPES+ Ca^{2+} medium con-

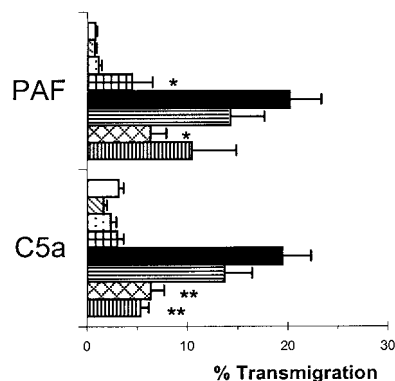


Figure 7. Effect of La^{3+} on the priming of eosinophil transepithelial migration toward PAF or C5a. Epithelial cell monolayers were washed twice with 37°C-pre-warmed medium before the transmigration assay. Purified eosinophils were incubated with different concentrations of La^{3+} (La, μM) and then with 100 nM TG or control buffer, and washed. Thereafter, the

cells were pre-warmed at 37°C in the presence of the same concentrations of La^{3+} for 3 to 4 min before the start of the 2-h transmigration assay. Modified HEPES+ Ca^{2+} medium was used for washing and incubation. Data are means \pm SEM of four to six independent experiments. $^*P < 0.05$, and $^{**}P < 0.01$ compared with the corresponding non- La^{3+} controls, respectively (ANOVA). Open bars, Eos control; diagonally striped bars, +La 1; dotted bars, +La 10; square-hatched bars, +La 100; solid bars, Eos + TG control; horizontally striped bars, +La 1 + TG; diagonally hatched bars, +La 10 + TG; vertically striped bars, +La 100 + TG.

extracellular Ca^{2+} -dependent manner and prime eosinophil transepithelial migration; and (iv) Ca^{2+} influxes prime eosinophil functions and upregulate eosinophil CD11b/CD18 expression.

Integrin CD11b/CD18 is an important molecule involved in various leukocyte functions (26, 27). In eosinophils, it mediates or modulates responses such as degranulation, superoxide production (28–30), transendothelial migration (31), and adhesion to epithelial cells (32). It has been reported to be important in mediating eosinophil infiltration *in vivo* (33). Using fluorescence-conjugated *Escherichia coli* and flow cytometry, we have found that the complement-dependent phagocytosis of *E. coli* by eosinophils was dramatically enhanced after TG treatment (unpublished observations). The transmigration of human eosinophils and neutrophils across monolayers of intestinal epithelial cells depends on the function of CD11b/CD18 (34, 35). We showed recently that human neutrophil transmigration across a monolayer of human lung epithelial cells is dependent on CD11b/CD18 (36). We now demonstrate the dependence on CD11b/CD18 of eosinophil transmigration across lung epithelium. The discrepancy between the effects of the two anti- αM mAbs used in this study is probably due to their different epitope specificities. Clone 94 is known only to inhibit CD11b/CD18-dependent rosetting, whereas the well-characterized clone 44 exerts a broad inhibitory effect on adhesion and lectin site-related functions of CD11b/

taining the same concentrations of La^{3+} as those present in the incubations before they were labeled with FITC-conjugated mAbs and analyzed by flow cytometry. $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with TG controls without La^{3+} ($n = 3$, ANOVA). Open bars, control Eos; solid bars, Eos + TG; striped bars, +La 1; dotted bars, +La 10; hatched bars, +La 100.

CD18 (17, 26). Moreover, we show that the enhancement of eosinophil transmigration evoked by Ca^{2+} influxes is paralleled by CD11b/CD18 upregulation.

Integrins newly recruited from leukocyte intracellular storage sites are not functionally competent (37–39). However, when physiologic ligands or other activators such as chemoattractants are present, as in our case, integrins are immediately activated, presumably by increasing their affinities or avidity to the ligands (39–41). The fact that EGTA or La^{3+} blocks the upregulation of eosinophil CD11b/CD18 expression and TG-induced priming of eosinophil transepithelial migration, which is CD11b/CD18 dependent (Figures 6 and 7), suggests that the translocation of CD11b/CD18 from intracellular pools is one significant cause of the enhancement of eosinophil transepithelial migration. Changes of the affinity of CD11b/CD18 could also contribute to the regulation of transepithelial migration. This is supported by the fact that at low TG concentrations (10 nM), migration was stimulated (Figure 1B), whereas expression was unchanged (Figure 5A). Conversely, at high TG concentrations (5 μM), expression was increased, whereas migration was unchanged. Previous investigations of integrin expression and function suggest that qualitative changes are apparent at concentrations of a given stimulus lower than those resulting in quantitative changes (27, 39).

TG is a naturally occurring sesquiterpene lactone often used in cellular calcium signaling studies. It specifically and universally inhibits the intracellular sarco-endoplasmic reticulum Ca^{2+} -ATPase, thus preventing the uptake of Ca^{2+} from the cytoplasm into intracellular Ca^{2+} stores (23, 24, 42). Depletion of Ca^{2+} from intracellular stores activates Ca^{2+} influx across plasma membrane, a process termed store-operated Ca^{2+} entry or capacitative Ca^{2+} entry (42). Together, these events evoke a sustained $[\text{Ca}^{2+}]_i$ increase in different cell types (16, 23–25, 42, 43). In this way, TG, unlike chemoattractants, bypasses the receptor-mediated signaling mechanisms that lead to inositol (1,4,5)-trisphosphate production and increases in $[\text{Ca}^{2+}]_i$ (23, 24, 43). Nevertheless, the downstream events that follow (i.e., the depletion of intracellular Ca^{2+} stores and initiation of store-operated Ca^{2+} entry) are common to TG- and chemoattractant-driven processes (22).

In the present study, we demonstrated that the Ca^{2+} responses induced by TG in human eosinophils consist of intracellular Ca^{2+} depletion and subsequent Ca^{2+} entry and that the intracellular Ca^{2+} release is not prevented by blocking extracellular Ca^{2+} entry (Figure 4). Because the upregulation of CD11b/CD18 expression in eotaxin- and TG-treated eosinophils is abolished in the presence of EGTA, our results indicate that $[\text{Ca}^{2+}]_i$ increases, and particularly Ca^{2+} entry, are essential for the upregulation of CD11b/CD18 expression. However, it is possible that the physiologic ligand eotaxin triggers additional signaling pathways besides those affected by TG.

In neutrophils, a small number of CD11b/CD18 receptors are localized on the plasma membrane after gentle purification. The bulk of them are stored in cytoplasmic granules of three different types: secondary and tertiary granules and secretory vesicles (44). Upon activation, they can be rapidly mobilized mainly from secretory vesicles. In eosinophils, CD11b/CD18 has also been found in secretory

vesicles (45). However, a detailed knowledge of CD11b/CD18-containing granules and vesicles and of the molecular machinery of exocytosis in eosinophils is largely lacking. In fast-excitabile cells such as neurons and endocrine cells, Ca^{2+} -regulated exocytosis (via Ca^{2+} influx) involves protein-protein interactions between so-called v-SNARE and t-SNARE complexes (46, 47). Some protein members of the SNARE complexes have been found in human neutrophils (44) and eosinophils (48, 49). These proteins, involved in the Ca^{2+} -regulated exocytosis of inflammatory cells, are likely to participate in the upregulation of CD11b/CD18 expression evoked by Ca^{2+} influxes in human eosinophils.

The Ca^{2+} entry-driven upregulation of CD11b/CD18 expression on eosinophils may be an important priming mechanism. Eosinophils are frequently primed *in vivo* in patients with asthma, probably because these cells have encountered cytokines or chemoattractants *in vivo* (14, 50, 51). Besides, chemoattractants are known to evoke Ca^{2+} increases in cells with the contribution of store-operated Ca^{2+} entry (22), which is accompanied by CD11b/CD18 upregulation (10, 11, 40). Eosinophils isolated from sputum or from BALF from patients with asthma (i.e., cells that had migrated or extravasated *in vivo*) show upregulated surface CD11b/CD18 phenotype and enhanced functionality *in vitro* (4, 52). In keeping with these observations, *in vitro* experiments have shown that the transmigration of neutrophils or eosinophils across a monolayer of endothelial cells results in the upregulation of CD11b/CD18 expression and primed functional responses (53–55). We suggest that the recruitment of eosinophils from the bloodstream into extravascular spaces, which involves eosinophil interaction with chemoattractants, adhesion to and migration across endothelium, and migration through interstitial matrix proteins, could further prime them (e.g., through upregulation of CD11b/CD18) by a mechanism involving store-operated Ca^{2+} entry. In this manner, eosinophils become fully competent for their transmigration across epithelium. These fully primed and migrated eosinophils exert enhanced, destructive functions in the airways and contribute to the pathogenesis of allergic asthma.

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