

2B4 (CD244) IS INVOLVED IN EOSINOPHIL ADHESION AND CHEMOTAXIS, AND ITS SURFACE EXPRESSION IS INCREASED IN ALLERGIC RHINITIS AFTER CHALLENGE

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A role for the subtypes of CD2 Ig superfamily receptors has been recently demonstrated in eosinophilic inflammation in experimental asthma and atopic asthmatics. We investigated the functions of 2B4 (CD244) molecules in eosinophil adhesion and chemotaxis, and correlated the results to the pathophysiology of allergic rhinitis (AR). Herein, we show that agonistic stimulation of 2B4 by C1.7, the anti-human 2B4 functional grade purified antibody, resulted in significant increase of eosinophils and eosinophil cell line (Eol-1 cells) adhesion to collagen type IV, and random migration. These functions were associated with tyrosine kinase phosphorylation of several protein residues of low molecular weight. Flow cytometry (FACS) experiments demonstrated that Eol-1 cells, normal peripheral blood eosinophils and eosinophils from AR patients, express surface 2B4 molecules. *In vitro* AR model demonstrated that the CC-chemokine receptor CCR3 stimulation by eotaxin induced significant increase in the expression of surface 2B4 in eosinophils and Eol-1 cells. Immunofluorescence confocal microscopy images showed that eotaxin induces also redistribution of 2B4 molecules towards the pseudopods in eosinophils and Eol-1 cells, changing their shape. Blocking of 2B4 molecules by the corresponding neutralizing antibody inhibited eotaxin induced Eol-1-adhesion, chemotaxis and the cytoskeleton changes. Pretreatment of Eol-1 cells with 1 μ M genistein blocked eotaxin-induced Eol-1 adhesion, chemotaxis and 2B4 up-regulated expression. *In vivo* correlation demonstrated the expression of 2B4 molecules in eosinophils from AR patients to be significantly increased, after nasal provocation challenge. These results identify a novel role for 2B4 molecules in eosinophil functional migratory response and may point to a novel tyrosine kinase-mediated ligation between CCR3 receptor and 2B4 co-receptor in eosinophil chemotaxis. If so, then 2B4 molecules would be a novel target for therapeutic modalities in diseases characterized by eosinophilia such as AR.

The subtypes of CD2 Ig superfamily, are essential for the cross-talk between immune cells and co-stimulation (1-3). 2B4 (CD244) receptor together with CD48, Ly-

9, NTB-A/Ly-108A and CRACC form the members of SLAM family (4-7). Characteristically these receptors possess single transmembrane immunoglobulin-like

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domain in its extracellular region and several tyrosine-based intracellular motifs. In humans, 2B4 is expressed in several haemopoietic cells including NK cells, T lymphocytes especially CD8⁺ T cells as well as subsets of basophils, monocytes and eosinophils (6, 8-12). Recently, reports have demonstrated a role for CD2 in eosinophilic inflammation in experimental asthma and from atopic asthmatics (13-15). Nonetheless, little is known about the role of these novel molecules in eosinophil recruitment and the pathophysiology of AR.

Hypereosinophilia with tissue infiltration is involved in a wide spectrum of immunological conditions such as allergic inflammation, parasitic infections, hypereosinophil syndrome (HES) and chronic eosinophilic leukemia (CEL). Eotaxin, which binds only to the seven-transmembrane G-protein-coupled receptor CCR3 abundantly expressed on eosinophils, possesses the highest selectivity to eosinophils in controlling cell migration among the eosinophil chemoattractants (16-19). In addition to the well reported roles of eotaxin in allergic airways eosinophilic inflammation, a role for it in environmental interaction to microorganisms in inducing or exaggerating eosinophilic inflammation in chronic rhinosinusitis (20), and in chronic leukemia, has been also suggested (21-22). Although reports on the signal transduction of eotaxin involved in recruiting eosinophils are well characterized, to the best of our knowledge no single study has investigated the role of 2B4 as effector co-stimulatory molecules in eosinophil recruitment against eotaxin.

Eol-1 is an ideal cell line to study cellular proteins and the ability of n-butyrate to induce Eol-1 differentiation to mature eosinophils make the cell line also ideal for the study of allergic inflammation in diseases such as AR and asthma. Accordingly, the current *in-vitro* model was designed to study the expression, regulation and migratory functional roles of 2B4 molecules in eotaxin-induced eosinophil recruitment utilizing eosinophils and Eol-1 cells. An *in-vivo* investigation by allergen nasal provocation was also performed, to correlate the biochemical results to a more clinical application.

MATERIALS AND METHODS

Cell preparation

Eosinophils and NK cells were isolated by Percoll (66%) solution separation. Eosinophils and NK cells were

further purified by negative selection immunomagnetic cell separation (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), using anti-CD16 magnetic beads for eosinophil purification and a cocktail of biotin-conjugated antibodies against lineage-specific antigens and a cocktail of microbeads (NK Cell Isolation Kit-human, Miltenyi Biotec, Bergisch Gladbach, Germany) for NK (CD56⁺ve CD3⁻ve) cells purification.

EOL-1 cell line

Human eosinophilic leukemia (Eol-1) cell line (Riken BioResource Center, Japan) was used in the current biochemical study. Cell differentiation into mature eosinophils was induced by histone deacetylase inhibitors, n-butyrate precisely as described earlier (23). Successful differentiation was confirmed by morphological changes and cell viability by trypan blue that was always >98%.

2B4 antibodies

Two anti-2B4 antibodies were tested on human eosinophils and Eol-1 cells: Antagonistic anti-2B4 antibody (R&D systems) and agonistic C1.7 the anti-human 2B4 functional grade purified (eBioscience). As for the FACS and confocal microscopy experiments, two conjugated antibodies were used that gave similar results: conjugated PE anti-human 2B4 antibody (Biolegend) and conjugated -FITC anti-2B4 antibody (R&D systems).

Adhesion assay

Eol-1 at concentration of 2×10^6 cells/ml (unless stated otherwise) or eosinophils at 1×10^6 /ml were primed for 1 h with 100ng/ml eotaxin or buffer, followed by two washes with buffer saline. Then 30 μ l of Eol-1 suspension were placed in 1 μ -Slide VI coated (collagen IV) cell microscopy chamber (Ibidi integrated BioDiagnostics, Munich-Germany) and left to adhere for 1 h. After two washes, adherent cells were fixed with cold methanol and conserved in ProLong[®] Gold antifade with DAPI (Invitrogen Molecular Probes Eugene Oregon, USA). Cells were then counted under microscopy (magnification x20). As for blocking experiments, the Eol-1 cells were pretreated with: buffer, 10 μ g/ml irrelevant blocking antibody (isotype), 1, 5 & 10 μ g/ml anti-human 2B4 antibody (R&D systems) or 1 μ M Genistein (Sigma) before stimulation for 1 h with 100ng/ml eotaxin.

Chemotaxis assay

Chemotaxis assays were performed in triplicate in a 48-well microchemotaxis Boyden chamber incubated in 5% CO₂ at 37°C for 90 min. Aliquots of 29 μ l of the chemotactic agent eotaxin (R&D Systems; Minneapolis, MN, USA) at 100ng/ml or buffer were placed in the lower wells and 50 μ l of Eol-1 or eosinophils suspension

(10^6 cells/ml) were placed in the upper wells. The two chambers were separated by a 5.0 μ m pore polycarbonate membrane (Nuclepore, Whatman, Middlesex, UK). Migrated cells adherent to the lower surface were counted in 5 selected high power fields/well under a light microscope (5hpf; $\times 400$). For blocking experiments, the Eol-1 cells were pretreated with: buffer, 10 μ g/ml irrelevant blocking antibody (isotype), 1, 5 & 10 μ g/ml anti-human 2B4 antibody (R&D systems) or 1 μ M Genistein (Sigma), for 60 min at 37°C. Cells were then washed twice, their viability were rechecked by trypan blue that was unchanged during the incubation period, and was always >98%. Cells were re-suspended in buffer medium and their chemotaxis was checked as stated above.

Western blot

Western blot analyses for tyrosine kinase protein residues was performed from whole cellular extract from peripheral blood eosinophils and Eol-1 cells utilizing anti-phosphotyrosine 4G10[®] (Upstate-CA/Millipore).

Confocal microscope images

30 μ l of cell suspensions at a concentration of 10^6 cells/ml were placed in 1 μ -Slide VI coated (collagen IV) cell microscopy chamber (Ibidi integrated BioDiagnostics, Munich-Germany) and left to adhere for 1 h. After two washes, stimulation with either buffer or eotaxin 100ng/ml were then performed for 1 and 15 minutes. For blocking experiments, adhered cells were incubated for 30 min with 10 μ g/ml anti-human 2B4 before being stimulated with 100ng/ml eotaxin. After two additional washes with PBS the cells were fixed with 4% formaldehyde for 20 mins and Nomarski-differential interference images were obtained using confocal microscopy (Leica TCS SP2). For immunofluorescence images, fixed cells were stained with conjugated PE anti-human 2B4 antibody (Biolegend) or Alexa fluor 488[®] – Phalloidin diluted 40x (Invitrogen Molecular probes[®]) for 1 h in the dark, on ice, washed twice with PBS and analyzed by confocal microscopy.

Fluorescence-activated cell sorting analysis

2B4 surface expression on NK cells, eosinophils and Eol-1 were analysed by FACS (FACS CANTO II BD Systems) as reported previously (24). Briefly, stimulated or unstimulated cells were fixed with 4% paraformaldehyde for 15 minutes. The cells were then washed and incubated with the corresponding antibody (surface expression) or permeabilised by 0.1% saponin (total expression) and then incubated with Alexa fluor 488[®] – Phalloidin diluted 40x (Invitrogen Molecular probes[®]), for 60 mins in the dark, on ice. After two additional washes, cells were conserved in paraformaldehyde 1% and then analysed for their

fluorescence intensity.

Statistical analysis

Statistical significance was performed by paired *t*-test and ANOVA. Values of $P < 0.05$ were considered significant.

RESULTS

Effects of 2B4 antibodies on eosinophil and Eol-1 adhesion and random migration

Two anti-2B4 antibodies were tested on human eosinophils and Eol-1 cells: anti-2B4 antibody (R&D systems) and C1.7 the anti-human 2B4 functional grade purified (eBioscience). The former activated neither the random migration, spontaneous adhesion nor protein phosphorylation of tyrosine kinase residues in eosinophils or Eol-1 cells. However, the latter increased random migration, spontaneous adhesion and the phosphorylation of several tyrosine kinase residues in eosinophils and Eol-1 (Table I, Fig. 1 A). Similarly, C1.7 but not anti-2B4Ab induced cell shape changes and F-actin reorganization (data not shown). Accordingly, anti-2B4 antibody (R&D systems) was used as neutralizing antibody in the following experiments. Of note, the increased random migration and aggregations of cells in response to C1.7 was blocked by Genistein (Fig. 1B, compares representative photos of migrated Eol-1 cells to 5.0 μ m pore polycarbonate membranes). Genistein alone reduced neither the spontaneous adhesion nor random migration (data not shown). This indicates that the agonistic stimulation of 2B4 induced increase in eosinophils adhesion and random migration is mediated by tyrosine kinase activity.

Surface expression of 2B4 receptor

NK cells abundantly express 2B4 receptor and therefore serve as our positive control cells. Fig. 2 demonstrates that the surface expression of 2B4 by NK cells was much higher and abundant than eosinophils from healthy subjects and Eol-1 cells. However, stimulation of eosinophils from healthy subjects and Eol-1 cells, by the optimal dose of 100ng/ml eotaxin, resulted in significant CCR3 mediated up-regulation of surface 2B4 molecules. The confocal microscopy images in Fig. 3A and B demonstrate that the novel up-regulated surface expression of 2B4 (in red) in eosinophils and Eol-1

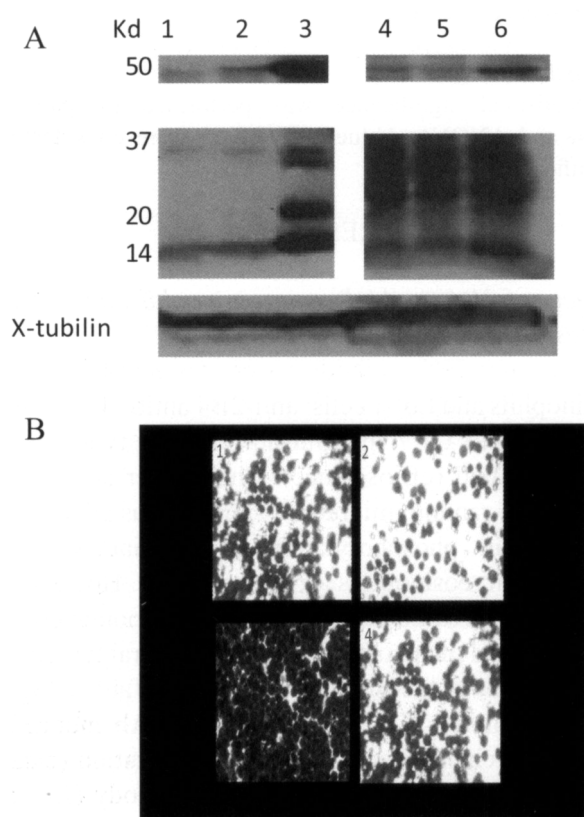


Fig. 1. **A)** Western blot of tyrosine kinase phosphorylation in eosinophils from AR patients (1-3) and Eo1 cells (lanes 4-6). Lanes 1 and 4 represent stimulation with buffer. Lanes 2 and 5 represent stimulation with anti-2B4 Ab (R&D systems). Lanes 3 and 6 represent stimulation with C1.7 Ab. Note the increase in the tyrosine protein phosphorylation of several protein residues in both eosinophils and Eo1 in similar fashion by C1.7 Ab but not anti-2B4. **B)** The effect of the agonistic C1.7 Ab and the neutralizing anti-2B4 Ab, on Eo1 cells random migration. Photos represent Eo1 cell random migration against buffer medium (RPMI) as follows: (1) Eo1 treated with buffer medium alone, (2) Eo1 treated with anti-2B4 Ab, (3) Eo1 treated with C1.7 Ab and (4) Eo1 treated with C1.7 Ab in presence of genistein.

cells was accompanied by 2B4 reorganization within pseudopods. These results pointed to a close relation between cytoskeletal elements, shape changes and 2B4.

Involvement of 2B4 in F-actin reorganization and Eo1 cells shape changes

We earlier reported that eotaxin induces a

rapid increase in the F-actin contents and its intracellular organization that is linked to eosinophil shape changes (25). In Fig. 4 (I) we demonstrate that 100ng/ml stimulation of Eo1 resulted in similar F-actin reorganization and shape changes. Interestingly, blockage of 2B4 by 10 μ g/ml of the corresponding neutralizing antibody resulted in blockage of both the F-actin increase contents, reorganization and the cell shape changes. 2B4 Ab treatment did not induce either apoptosis or necrosis in Eo1 cells as judged by FACS analysis utilizing PE Annexin V and 7AAD; BD Bioscience (data not shown). Of note, 2B4 irrelevant antibody (isotype) did not modify eotaxin-induced cell shape changes and F-actin contents and reorganization. The mean fluorescence intensity (MFI) for F-actin was 13 ± 2 for the buffer stimulation, 13.8 ± 5 for eotaxin stimulation and 13.05 ± 3 for eotaxin stimulation in presence of 2B4 Ab.

The signal transduction is a fast event that takes place after the ligand stimulates its specific receptor on the cell surface. Therefore, we next double stained Eo1 cells with Phalloidin and anti-2B4 Ab to investigate the co-localization of these molecules after 1 min stimulation by 100ng/ml eotaxin. As can be seen in Fig. 4 (II), there was a fast reorganization and co-localization of these molecules at as early as 1 min stimulation of CCR3.

Taken collectively, the results presented in Figs. 3 and 4 strongly indicate a co-stimulatory role for 2B4 with CCR3-induced cytoskeleton changes. As cytoskeletal elements and shape changes are prerequisites for cell migration, we hypothesize that 2B4 are important co-stimulatory molecules for eotaxin-induced eosinophil chemotaxis. Accordingly, the following migratory functional assays were performed.

Involvement of 2B4 in Eo1 adhesion and chemotaxis is tyrosine kinase-dependent

Fig. 5 demonstrates that blockage of 2B4 by neutralizing anti-2B4 antibody blocked completely eotaxin-induced Eo1 adhesion (Fig. 5A) and chemotaxis (Fig. 5B) in dose dependency. 2B4 possesses intracellular tyrosine motifs, and eotaxin induced eosinophil chemotaxis involves tyrosine kinase pathway (25-26). Therefore we next, tested the ability of genistein, a tyrosine kinase inhibitor,

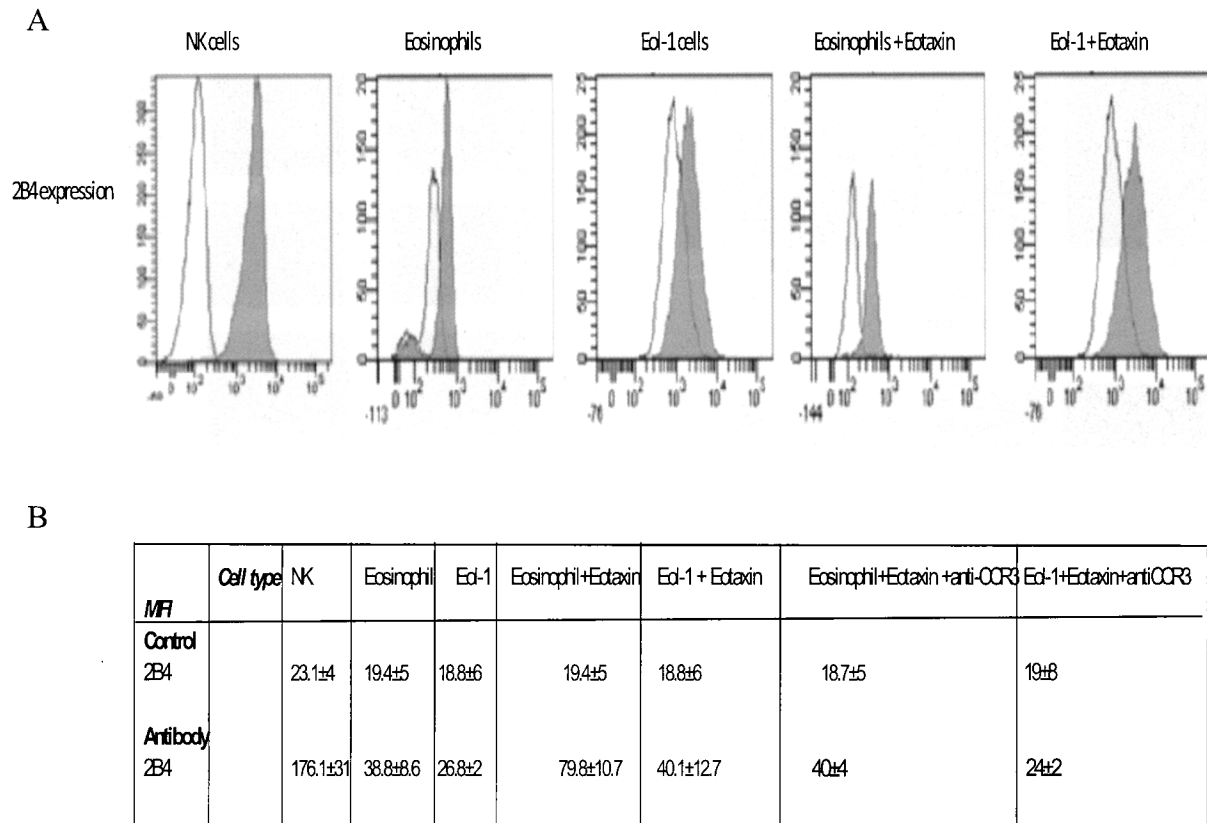


Fig. 2. **A)** The levels of 2B4 expressed on cell surfaces were analyzed by flow cytometry. White represents isotype staining (control) while grey represents 2B4 antibody staining. Results are one histogram representative of 5 independent experiments all showing similar results. **B)** The mean fluorescence intensity (MFI) of the five independent experiments.

in modulating eotaxin-induced EoL-1 adhesion and chemotaxis. Equal concentration of EoL-1 cells (1×10^6 cells/ml) was pretreated with genistein for 1 h before their stimulation with eotaxin. As seen in Fig. 6A and B, $1 \mu\text{M}$ concentration of genistein blocked both eotaxin-induced EoL-1 adhesion and chemotaxis. These results indicate the involvement of 2B4 and tyrosine kinase, in eotaxin-induced EoL-1 adhesion and chemotaxis signal transduction.

Eotaxin-induced 2B4 up-regulated expression by EoL-1 is tyrosine kinase-dependent

Experiments were performed to investigate whether the eotaxin-induced 2B4 up-regulated expression is a downstream event to tyrosine kinase activity or through independent pathway. EoL-1 cells were incubated with $1 \mu\text{M}$ genistein before being stimulated with 100 ng/ml eotaxin. In Fig. 6C, FACS

analysis shows that the up-regulated expression of 2B4 in EoL-1 by eotaxin was blocked by genistein. These results may indicate that phosphorylation of multiple intracellular tyrosine protein residues by CCR3 stimulation is an important upstream event for the co-stimulatory effects of 2B4 in EoL-1 migratory functional response by eotaxin.

Up-regulation of 2B4 in eosinophils from allergic rhinitis patients

Finally, to have an idea of the effect of 2B4 molecules in the pathophysiology of AR and to validate our cellular and molecular results, we performed nasal provocation challenge with HDM allergen on 8 patients sensitized against HDM. These patients reported nasal allergic symptoms consistent with HDM allergen and their skin prick tests confirmed positive results to HDM allergen

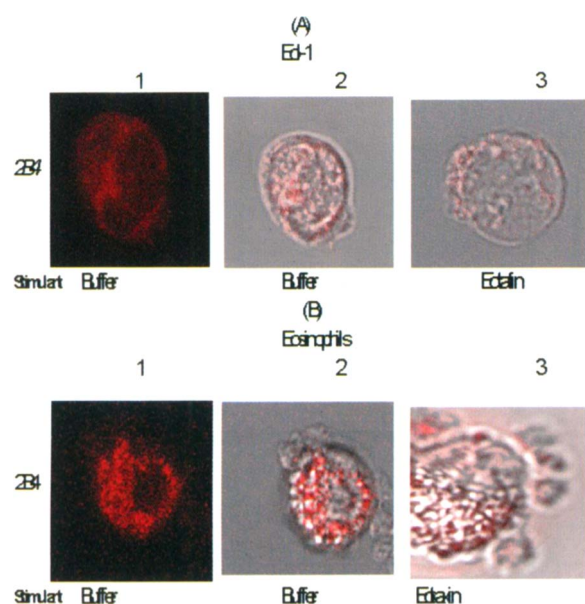


Fig. 3. Confocal images of (A) Eo1 cells and (B) peripheral blood eosinophils, showing the relationship of cell shape changes and the up-regulated expression of 2B4 (red) in cells treated with 100ng/ml eotaxin. Images are of one experiment representative of 3. Note the peripheral and membrane bound distribution of 2B4.

as well as the allergosorbant test (RAST). None of them had previously taken antihistamines or nasal/systemic cortisone. After obtaining their consent, the allergen provocation was induced by blotting paper discs soaked with either the diluent only or the allergen dilution (Stallergenes 100IR/ml) in contact with the mucosa of the inferior turbinate. Non-specific responsiveness was always eliminated before each challenge by introducing the paper disc in contact with the inferior turbinate mucosa. The patients' vital signs were monitored for at least 30 min after challenge. The patients' peripheral blood eosinophils were purified and analyzed by FACS for their expression of 2B4 before challenge and at 6 and 24 h post challenge. Two patients served as control and were challenged with diluent only. They did not show changes in 2B4 expression after challenge.

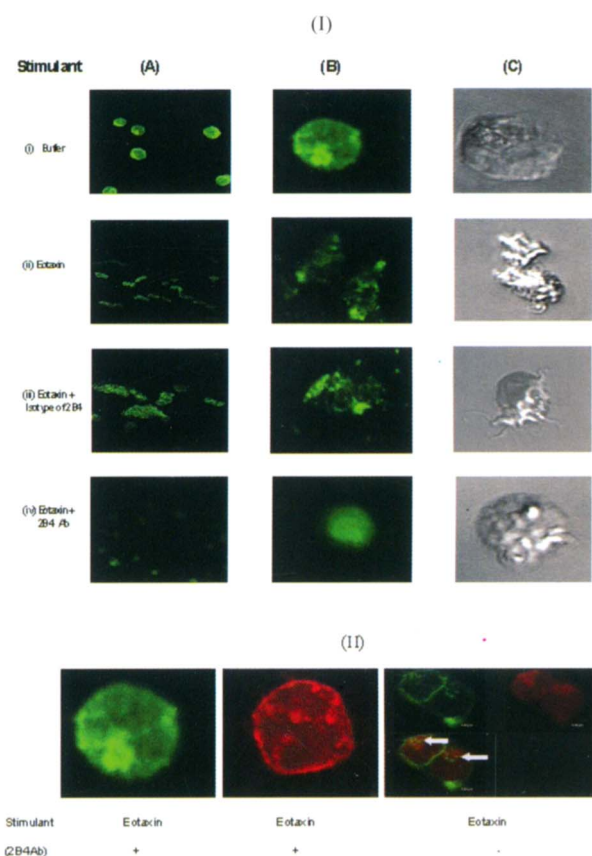


Fig. 4. Effect of 2B4 blockade on 100ng/ml eotaxin-induced Eo1 shape changes and F-actin contents reorganisation. (I) Stimulations are for 15 min with (i) buffer (PBS), (ii) eotaxin 100ng/ml, (iii) eotaxin 100ng/ml after pretreatment of cells with 10µg/ml 2B4 irrelevant Ab (isotype) for 30 min. or (iv) eotaxin 100ng/ml after pretreatment of cells with 10µg/ml anti-2B4 Ab for 30 min. A) Green represents F-actin of images of group of cells from the same slide (IPF, magnification x20). B) One cell representative of the group of cells in panel (A) (magnification x40). C) Nomarski-differential interference images. Results are of one experiment representative of 6 independent experiments all showing similar results. (II) Stimulation is with 100ng/ml eotaxin for 1 min. Grey arrows point to co-localisation of F-actin (in green) and 2B4 (in red).

On the other hand, as demonstrated in Fig. 7 A, C and D, in patients who were challenged with HDM allergen their pre-challenge expression of surface 2B4 was expressed on $28 \pm 5\%$ of cells with MFI of 44.4 ± 7.1 . Interestingly, 6 h after challenge, 2B4 surface expression increase to $50 \pm 7\%$ with MFI

Table 1. Results are the mean $\pm$ SEM of 6 independent experiments performed in triplicate. Freshly isolated peripheral blood eosinophils were purified from 6 healthy volunteers.

Stimulant	Eosinophil		Eol-1	
	Adhesion	Migration	Adhesion	Migration
Buffer	200 $\pm$ 5	98 $\pm$ 3	250 $\pm$ 4	100 $\pm$ 7
Irrelevant Ab (Isotype)	198 $\pm$ 4	100 $\pm$ 7	230 $\pm$ 6	105 $\pm$ 5
Anti-2B4 Ab	202 $\pm$ 5	90 $\pm$ 3	245 $\pm$ 9	98 $\pm$ 12
C1.7 Ab	380 $\pm$ 8*	320 $\pm$ 10*	500 $\pm$ 14*	400 $\pm$ 9*

of 75 $\pm$ 6.5. These effects lasted up to 24 h post-challenge. At 24 h post-challenge, 2B4 expression was in 38% $\pm$ 13 of cells with MFI of 73.3 $\pm$ 12. These results indicate the involvement of these molecules in the pathophysiology of AR and further support and validate the results of our biochemical migratory assays. Furthermore, the involvement of 2B4 in the pathophysiology of AR seems to be specific to eosinophils, since comparative study on NK cells from the same patients neither modulated the number of cells expressing 2B4 nor 2B4 MFI, before challenge and 6 h post-challenge (Fig. 7A). Furthermore, confocal images taken directly from eosinophils adherent to collagen IV coated slides (Fig. 7B) also demonstrated increased fluorescence intensity of 2B4 after challenge with increase in cytoskeleton changes. This further supports a possible role for 2B4 in the pathophysiology of AR.

DISCUSSION

The migratory function of eosinophil 2B4 molecules is not yet fully elucidated. We observed that the stimulation of eosinophils from AR patients and Eol-1 cells by C1.7 the agonistic monoclonal Ab leads to tyrosine phosphorylation and an increase in spontaneous adhesion and random migration of eosinophils. This is attributed to the functional ability of C1.7 Ab to share with CD48 the involvement to the same residue in the V domain of human 2B4.

This provides a novel role for CD48-2B4 molecules in eosinophil adhesion and chemotaxis through tyrosine kinase cascade.

To further elaborate on the possible role of 2B4 in chemokine-directed eosinophil migration, we next investigated the possible ligation of CCR3 receptors to 2B4 molecules utilizing the potent and relatively specific eosinophil cc-chemokine eotaxin and Eol-1 cells. This cell line was ideal for the *in-vitro* migratory assays, especially FACS analysis and confocal images which showed that eosinophils and Eol-1 express surface 2B4 in a similar fashion. Cell migration is preceded by several complex biochemical events including adhesion, protein kinase activity and actin cytoskeletal re-organization and cell shape changes (28). These events have been speculated to be linked to co-stimulatory transmembrane molecules, such as integrins, that have the ability to bind through their intracytoplasmic tail to filamins, the large, actin-cross-linking proteins that connect transmembrane signaling molecules to the cytoskeleton (29). Herein, our results support this hypothesis since the transmembrane molecules 2B4 were molecularly linked to F-actin reorganization and cytoskeleton changes. Interestingly, 2B4 expression and cytoskeleton changes were blocked by genistein, a tyrosine kinase inhibitor. This confirms our previous observation that HCK and c-Fgr, the Src family kinases, are involved in eotaxin-induced eosinophil chemotaxis (25). To this end, signal transduction

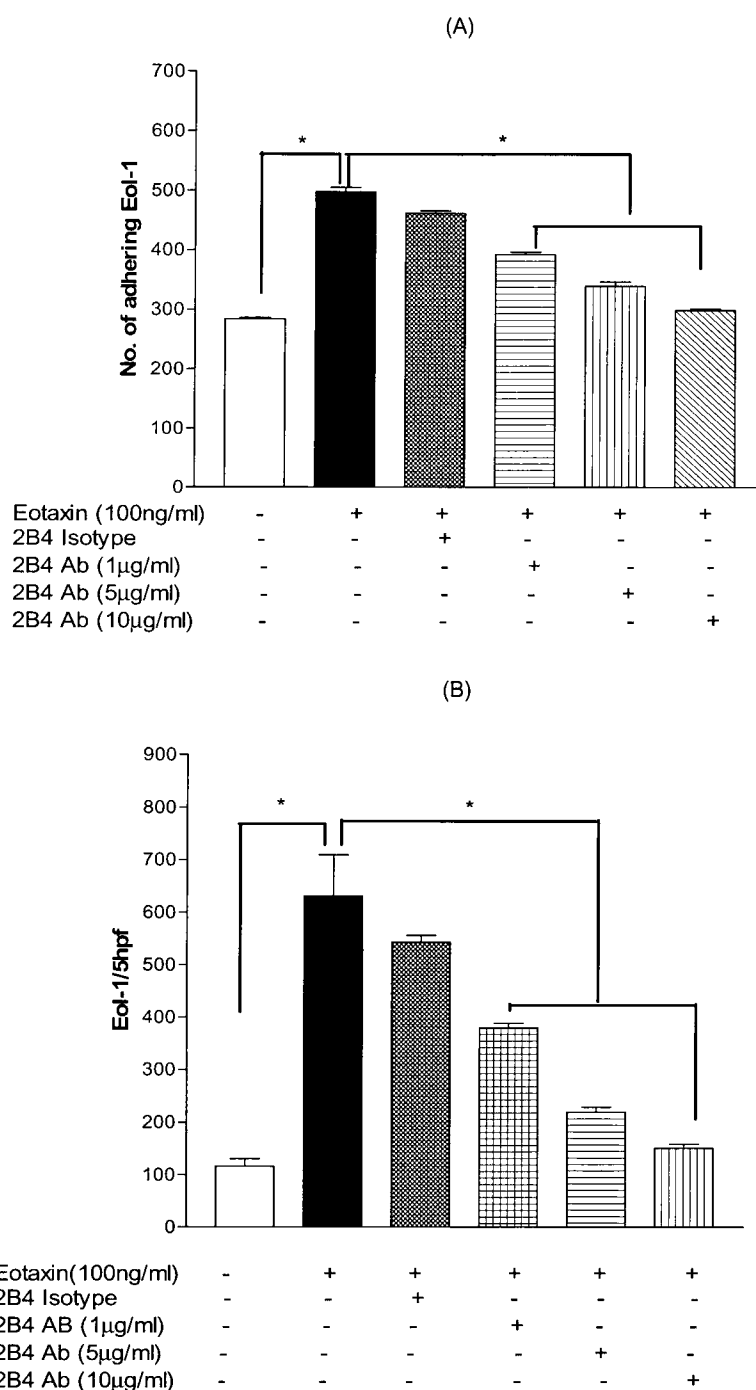


Fig. 5. Comparison of the inhibitory effect of anti-2B4 Ab on 100ng/ml eotaxin-induced Eol-1 adhesion and chemotaxis. Data are the mean $\pm$ SEM of 6 independent experiments performed in triplicate. Asterisks indicate P value < 0.05 . **A)** Adhesion of Eol-1 to 1 μ -Slide VI coated (collagen IV) cell microscopy chambers. Data are the number of adhering cells during 1 h assay (magnification $\times 20$). Of note, neither the isotypes nor 2B4 Ab modulated the spontaneous adherence of Eol-1 to the buffer control (data not shown). **B)** Eotaxin induced Eol-1 chemotaxis. Data are the number of migrating Eol-1 in selected 5hpf (magnification $\times 40$). Similar results were obtained from differentiated Eol-1 cells to mature eosinophils. Concentration of the 2B4 isotype is 10 μ g/ml. No statistical differences is seen between isotype and eotaxin treatment, while $P < 0.05$ between isotype and 2B4 antibodies.

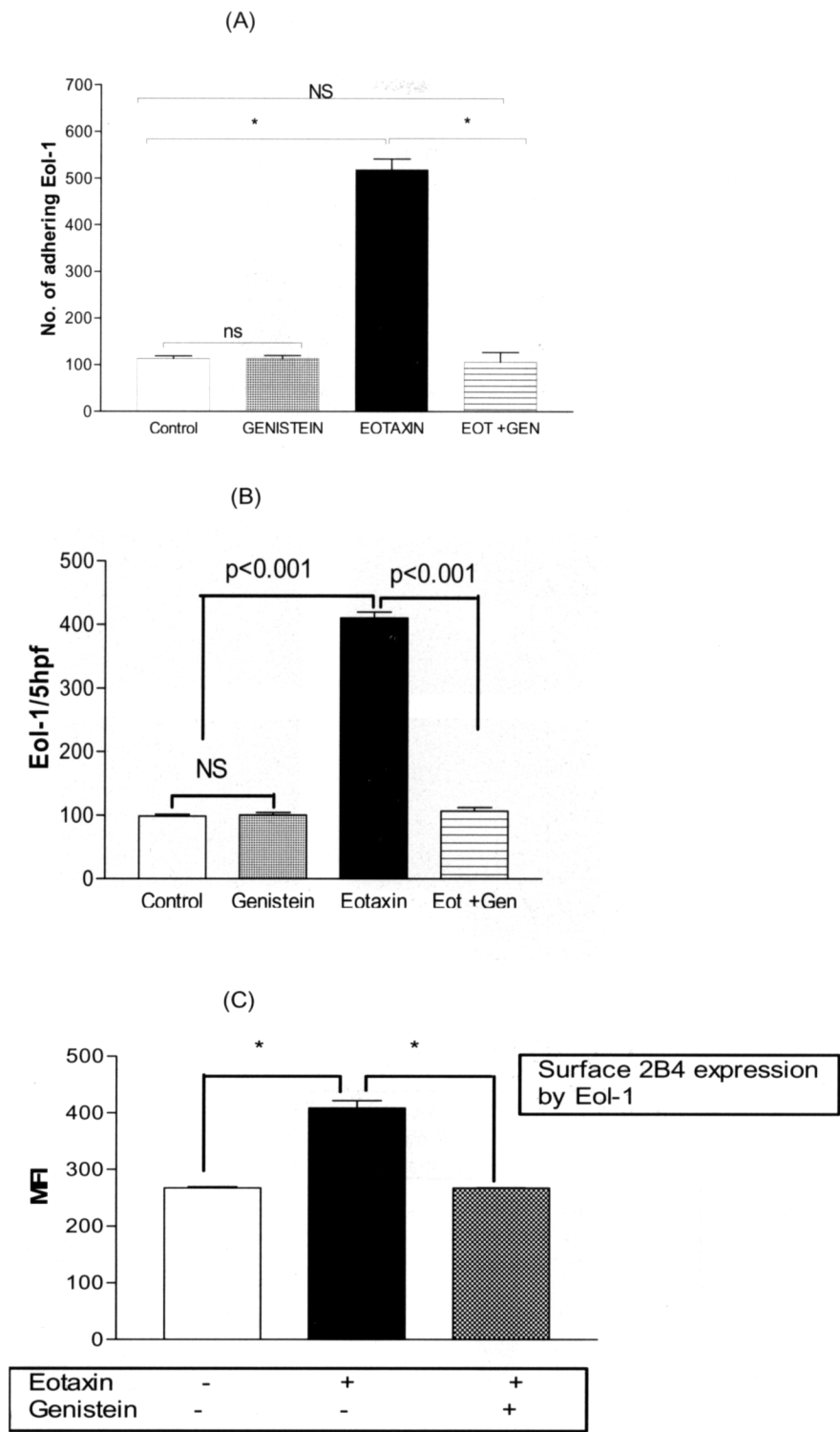


Fig. 6. Effect of genistien on eotaxin-induced upregulated expression of 2B4 and the migratory assays. *A and B*) Blocking effect of 1 μ M genistein on 100ng/ml eotaxin-induced Eol-1 adhesion and chemotaxis. Data are the mean $\pm$ SEM of 6 independent experiments performed in triplicate. Asterisks indicate *P* value <0.05. *C*) Data are the mean $\pm$ SEM of 3 independent experiments performed in triplicate. Results represent the surface expression of 2B4. Asterisks indicate *P* value <0.05.

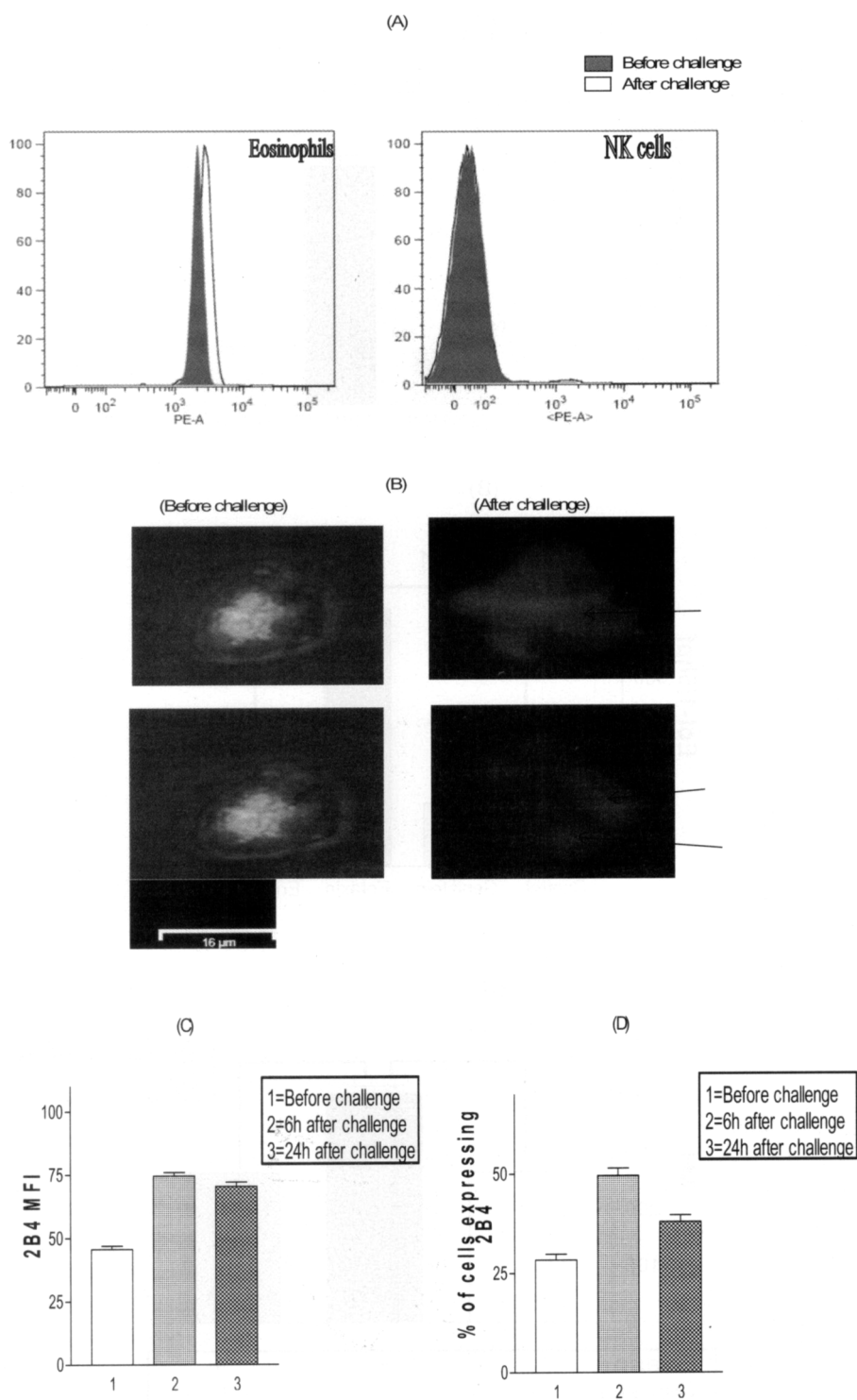


Fig. 7. *A)* The levels of 2B4 expressed on peripheral blood eosinophils and NK cells of AR patients sensitised to HDM, before and 6 h after HDM allergen nasal challenge. Histograms are of one patient representative of 6 all showing similar results. *B)* Confocal images of 2B4 fluorescence (arrow) on eosinophils from the same allergic patient. Images are taken before (left panel) and at 6 h after HDM allergen nasal challenge (right panel). *C-D)* MFI and percentage of eosinophils, from AR patients, expressing 2B4, before, 6 and 24 h after allergen nasal challenge. Results are the mean $\pm$ SEM of 6 independent experiments. $P < 0.001$ by Friedman one way ANOVA test.

through 2B4 in activated NK cells has been shown to be linked to phosphorylation of tyrosine motifs in its cytoplasmic tail and to the recruitment of SAP and the Src family kinase Fyn (30-31). Furthermore, we demonstrate that stimulation of eosinophils from AR patients and Eo1-1 cells by C1.7 leads to tyrosine phosphorylation of several low molecular weight proteins in eosinophils and Eo1-1.

It is then possible that following CCR3 stimulation on the surface of eosinophils by eotaxin, a co-receptor stimulation via 2B4 helped by the physical association of Hck and c-Fgr and possibly others to CCR3 in a compartment fashion triggers signal transduction through tyrosine phosphorylation. Taken collectively, our biochemical assays demonstrate that 2B4 co-stimulatory receptor is functional and involves adhesion, cytoskeletal changes and chemotaxis against eotaxin.

Single nasal antigen challenge provokes immediate anaphylactic reaction in nasal mucosa within 5-15 minutes, and the duration may last for less than 1 hour. The symptoms, mainly nasal obstruction, reappear 6-8 hours after antigen challenge. This phenomenon is called late phase reaction of allergic rhinitis and is characterized by eosinophil recruitment, which can last up to 24-48 hours (very late reaction). In this study, we show that HDM nasal challenge in patients with AR to HDM resulted in up-regulation of the expression of 2B4 in the peripheral blood eosinophils of these patients. This up-regulation lasted up to 24 hours post-challenge. This may indicate the involvement of 2B4 molecules in the pathophysiology of AR and support their novel role(s) in eosinophil activation and recruitment. However, due to the involvement of many *in-vivo* mediators in the pathophysiology of AR, it is difficult to say which mediator(s) is specifically involved in this up-regulation. Nonetheless eotaxin remain one of these important allergic mediators.

In conclusion, therapeutic modalities aiming at targeting either the CCR3/2B4 receptors and/or the tyrosine kinase intracellular activity would be a suitable target for allergic airway eosinophilic inflammation.

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