

TLR3 in Human Eosinophils: Functional Effects and Decreased Expression during Allergic Rhinitis

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Key Words

Allergic rhinitis • Eosinophil • Adhesion molecule • Chemokine • Signal transduction • p38 MAP kinase • Nuclear factor- κ B

Abstract

Background/Aim: Viral respiratory infections are increasingly implicated in allergic exacerbations. Virus-induced activation of eosinophils through Toll-like receptors (TLRs) could be involved. The present study was designed to examine TLR3 expression in eosinophils from bone marrow (BM) and peripheral blood (PB) during symptomatic allergic rhinitis, and to evaluate the functional responsiveness of TLR3 in purified eosinophils. **Methods:** BM and PB samples were obtained from healthy volunteers and patients with seasonal allergic rhinitis outside and during the pollen season. Eosinophils were analyzed for TLR3 expression by flow cytometry. Polyinosinic:polycytidylic acid [poly(I:C)], an agonist for TLR3, was used to assess its functional role in purified eosinophils and the intracellular signaling pathways involved. **Results:** TLR3 expression was demonstrated in BM and PB eosinophils. It was higher in BM-derived than in circulating

cells and it was downregulated in both compartments during symptomatic allergic rhinitis. TLR3 expression was also downregulated in the presence of interleukin (IL)-4 and IL-5. Stimulation with poly(I:C) increased the percentage of CD11b+ cells and enhanced the secretion of IL-8, effects mediated via the p38 mitogen-activated protein kinases and nuclear factor- κ B signaling pathways. Moreover, pretreatment with IL-5 augmented the poly(I:C)-induced IL-8 release. **Conclusions:** Eosinophils activated via TLR3 might be more able to home and recruit leukocytes to sites of inflammation. The decreased TLR3 expression during symptomatic allergic rhinitis and in the presence of Th2 cytokines indicates a role in allergic airway inflammation. Thus, eosinophils might function as a link between viral infections and exacerbations of allergic disease.

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Introduction

The accumulation of eosinophils, releasing an array of tissue-damaging mediators, is a hallmark of asthma and allergy-associated inflammation [1, 2]. Viral respiratory infections are believed to contribute to allergic sensitization and development of asthma, and in subjects with already established asthma, viral infections are known to exacerbate the disease [3]. The mechanisms behind this virus-induced airway inflammation are unclear. One possible explanation might be a direct activation of viral components through the Toll-like receptors (TLRs). The TLR family comprises 10 different members in humans (TLR1–TLR10), each recognizing a conserved microbial structure and thereby initiating a host defense response [4, 5]. The virus-recognizing TLRs are TLR3, TLR7/8 and TLR9, which respond to viral double-stranded RNA, single-stranded RNA and CpG-DNA, respectively [5–8].

Eosinophils have been reported to express messenger RNA (mRNA) for several TLRs [9–11], and protein analyses have revealed TLR1, -2, -4, -5, -6, -7 and -9 expression using Western blot and flow cytometry [12]. The predominant TLR in eosinophils is indisputably TLR7. Previous studies using the synthetic ligands R-848 (both TLR7 and TLR8 agonists) and imiquimod/R-837 (selective TLR7 agonist) have shown activation of eosinophils manifested by a regulated adhesion molecule expression, induction of cytokine secretion, generation of superoxide and prolongation of eosinophil survival [9, 12, 13]. A recent study performed at our lab has also demonstrated that purified eosinophils respond to R-837 and CpG by inducing survival, cytokine release, regulation of adhesion molecule expression, migration and degranulation, and that the atopic status of the donor and the presence of a Th2-like cytokine milieu affect the response [14]. However, knowledge about the other virus-recognizing TLRs is limited.

Screening different leukocyte subsets in bone marrow (BM) and peripheral blood (PB) acquired from patients with seasonal allergic rhinitis outside and during the pollen season as well as from healthy controls, we have investigated alterations in TLR expression. Results have shown TLR9 expression in several different cell types; the expression was generally higher in BM, and the onset of allergic rhinitis did not affect TLR9 expression [8]. Activated expression of TLR2 and TLR4 was also found in leukocytes from BM and PB during symptomatic allergic rhinitis [unpubl. obs.]. To our surprise, we also found signs of TLR3 expression in eosinophils. With the excep-

tion of a recent study on murine splenic eosinophils [15], there are no existing data showing TLR3 proteins in eosinophils. Thus, the present study was designed to explore the expression of TLR3 in eosinophils from BM and PB obtained from healthy individuals and patients with allergic rhinitis both outside and during the pollen season in detail. We also aimed at evaluating the functional importance of this receptor in purified eosinophils and the intracellular signaling pathways involved.

Materials and Methods

Subjects

Nine healthy volunteers with no history of allergic disorders and 21 patients with birch- and/or grass-pollen-induced seasonal allergic rhinitis (11 patients with symptomatic allergic rhinitis and 10 patients outside the pollen season) were included in the study. The study was approved by the Ethics Committee of the Lund University, and informed consent was obtained from all participants. A detailed description of the subjects, including age, sex, diagnosis and symptoms, is presented in table 1. None of the subjects were taking any medication at the time of the study. The diagnosis was based on a positive history of seasonal allergic rhinitis for at least 2 years and a positive skin prick test to birch and/or timothy pollen (wheal reaction diameter >3 mm, roughly corresponding to a 3+ or 4+ reaction compared to histamine). Control subjects were symptom-free, had no history of allergic rhinitis and had a negative skin prick test to the standard panel of allergens. Exclusion criteria included a history of perennial symptoms, upper airway infection within 2 weeks before the visit and treatment with local or systemic corticosteroids within 2 months before the visit. Subjects with chronic rhinosinusitis, hypertrophy of the turbinates, severe septum deviation or nasal polypsis were excluded as well.

BM Aspiration

One sample containing 1–2 ml of BM was aspirated from the posterior iliac crest following local anesthesia with lidocaine hydrochloride (10 mg/ml). The sample was immediately placed in a culture medium containing buffered trisodium citrate solution (0.129 M), RPMI-1640 with 2 mM HEPES and N-acetyl-L-alanyl-L-glutamine (FG1233 Biochrom AG, Berlin, Germany). BM aspiration was obtained from 7 patients with symptomatic allergic rhinitis, from 9 allergic patients outside the pollen season and from 8 healthy controls.

Blood Sample Collection

One sample containing 4 ml of PB was collected in a test tube containing EDTA (Vacuette® 454209, Greiner Bio-One, Frickenhausen, Germany) and analyzed for total leukocyte differential count on a cell counter (Beckman Coulter LH750, Marseille, France). An additional sample containing 4 ml of blood was collected in a test tube containing buffered trisodium citrate solution (BD Vacutainer™ 367704, Beckton Dickinson, Franklin Lakes, N.J., USA) and analyzed by flow cytometry. Blood samples were obtained from all subjects.

Table 1. Clinical data of the subjects participating in the study

	AR patients during the pollen season	AR patients outside the pollen season	Healthy volunteers
Number of subjects	11	10	9
Sex	7 males, 4 females	6 males, 4 females	6 males, 3 females
Age, years	20–48 (median 27)	22–49 (median 28)	22–51 (median 40)
History of AR	≥2 years	≥2 years	no
SPT (birch and/or timothy)	>3 mm wheal reaction diameter	>3 mm wheal reaction diameter	negative
Symptoms	moderate to severe rhinoconjunctivitis	none	none

AR = Allergic rhinitis; SPT = skin prick test.

Flow Cytometry of Eosinophils in BM and PB

Samples of BM and PB were acquired as described above and used for cell analysis by flow cytometry. BM samples were filtrated prior to preparation. Fluorescence measurements were performed on a Coulter Epics XL flow cytometer (Beckman Coulter). A total of 30,000 events were collected. Data were analyzed using Expo32 ADC analysis software (Beckman Coulter). Analysis was performed for both extracellular and intracellular occurrence of TLR3. All samples were labeled with CD16-phycoerythrin-cyanine 5 (CD16-Pcy5, clone 3G8) and CD45-energy-coupled dye (CD45-ECD, J.33) from Immunotech (Beckman Coulter) for 15 min at room temperature. The antibody against human TLR3 (40C1285) from ImmunoKontakt (Abington, UK) was labeled with maleimide-activated R-phycoerythrin (RPE) by conjugation to free sulfhydryl groups on the antibody using Phycolink® (Prozyme, San Leandro, Calif., USA). Any remaining free sulfhydryl groups were covalently blocked by treatment with N-ethylmaleimide. In extracellular staining, cells were labeled with TLR3-phycoerythrin (TLR3-PE) for 15 min at room temperature. Erythrocytes in a 50- μ l sample were lysed by mixing with 0.6 ml 0.1% (vol/vol) formic acid for 3–4 s. The ionic strength was rendered iso-osmotic by addition of 0.28 ml 51 mM Na₂CO₃, 0.20 M Na₂SO₄ and 0.22 M NaCl, and cells were washed in PBS and fixed in PBS containing 1% formaldehyde prior to analysis. Intracellular staining was performed using IntraPrep™ Permeabilization Reagent kit (Immunotech) according to the manufacturer's specification. Thus, the cells were fixed and permeabilized prior to incubation with TLR3-PE for 15 min at room temperature. Cells were washed in PBS and resuspended in PBS containing 1% formaldehyde prior to analysis. In control experiments, cells were also incubated with isotype control antibodies, mIgG1-PE (Immunotech). By gating intact leukocytes on forward scatter (FSc) and side scatter (SSc) properties as well as by their CD16 and CD45 signals, eosinophil granulocytes were defined based on their high FSc, high SSc, low CD16 and intermediate CD45.

Cell Separation and Cell Culture

PB was collected into tubes containing heparin (BD Vacutainer™ 367675). PB mononuclear cells and plasma were removed by density gradient centrifugation using Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden), leaving a cell pellet containing granulocytes and erythrocytes. The granulocyte-rich pellet was collected and treated with ammonium chloride

erythrocyte lysis solution (0.8% NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA), centrifuged and washed in complete medium consisting of RPMI-1640 supplemented with 0.3 g/l L-glutamine (PAA, Pasching, Austria), 10% FCS (PAN, Aidenbach, Germany), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, Calif., USA). Eosinophils were negatively separated from other granulocytes using MACS separation (Eosinophil Isolation Kit; Miltenyi Biotec, Cologne, Germany) according to the manufacturer's instructions. Isolated cells were routinely checked by flow cytometry, and eosinophils were defined on the basis of their FSc, SSc and expression of CD16, as FSc^{high}, SSc^{high} and CD16⁺. The purity of the eosinophils was more than 98%; contaminating cells were neutrophils (<1%) and mononuclear cells (<1%). Because of the short lifespan of eosinophils after isolation, all studies were performed with freshly isolated cells that had not been further maintained in culture. Purified cells were cultured (1 $\times$ 10⁶ cells/ml) for 24 h at 37°C and 5% CO₂ in complete medium alone or with various stimuli and inhibitors as indicated.

RNA Extraction, Reverse-Transcription and Real-Time Polymerase Chain Reaction

Freshly isolated eosinophils were placed in RNA-later (Qiagen, Hilden, Germany) for 24 h and were then stored at –80°C until use. RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the supplier's protocol. All samples were treated with DNase (Qiagen). The quality and quantity of the RNA were assessed by spectrophotometry based on the absorbance A₂₆₀/A₂₈₀ ratio (between 1.8 and 2.0 in all preparations). Reverse transcription to cDNA was carried out using the Omniscript Reverse Transcriptase kit (Qiagen) with oligo-dT primer (Novagen, Nottingham, UK) in a final volume of 20 μ l using the Mastercycler Personal PCR machine (Eppendorf AG, Hamburg, Germany), at 37°C for 1 h. Thereafter, real-time polymerase chain reaction (PCR) assays were performed using the SmartCycler system (Cepheid, Sunnyvale, Calif., USA). For the detection of TLR3 and β -actin, intron overspanning oligonucleotide primers were designed (table 2) using Primer Express 2.0 software (Applied Biosystems, Foster City, Calif., USA) and synthesized by DNA Technology A/S (Aarhus, Denmark). PCR was performed using QuantiTect™SYBR® Green PCR kit (Qiagen) in a final volume of 25 μ l. Reactions were incubated at 95°C for 15 min, then incubated for 46 cycles at 94°C for 30 s followed by 55°C for 60 s (initially 65°C,

Table 2. Sequences of primers used for PCR amplification of TLR3 and β -actin

Human TLR3	forward reverse	5'-AACTCCATCTCATGTCCAACCTCAA-3' 5'-GATGACAAGCCATTATGAGACAGATC-3'
Human β -actin	forward reverse	5'-GCCAACC GCGAGAAGATG-3' 5'-ACGGCCAGAGGCGTACAG-3'

followed by 2°C decrease the first 6 cycles). Product specificities were controlled by melting curve analysis. The amplification products were run on a 3% agarose gel containing ethidium bromide. Purified neutrophils served as negative controls [16].

Flow Cytometry Analysis of Purified Eosinophils

Live cells were gated based on FSc and SSc properties, and 10,000–15,000 events were collected. The following antihuman monoclonal antibodies (mAbs) were used: CD11b-fluorescein isothiocyanate (FITC) (Bear1) and CD16-Pcy5 (3G8) from Immunotech, CD16-PE (DJ130c) from DakoCytomation (Copenhagen, Denmark) and TLR3-PE (40C1285) from ImmunoKon-tact. Relevant isotype controls were used for background staining. For the detection of TLR3, intracellular staining of the cells was performed using IntraPrep™ Permeabilization Reagent kit (Immunotech). Cells were incubated with antibodies for 20 min at room temperature, and thereafter washed and resuspended in PBS.

ELISA

The cell culture supernatants were recovered and assayed for IL-8 (sensitivity 3.5 pg/ml) using ELISA plates from R&D Systems (Minneapolis, Minn., USA).

Luminex Multiplex Immunoassay

Measurements of total and phosphorylated Akt, c-Jun NH2-terminal kinase 1/2 (JNK1/2) and p38 mitogen-activated protein kinases (p38 MAPK) were performed using the 3-Plex Assay (Biosource International, Camarillo, Calif., USA). The cell pellet was collected after incubation with or without polyinosinic:poly-cytidylic acid [poly(I:C)], washed twice in ice-cold PBS and resuspended in NP40 lysis buffer (Biosource) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and Protease Inhibitor Cocktail (both Sigma-Aldrich, St. Louis, Mo., USA). After 40 min of incubation on ice with vortexing every 10 min, the suspension was centrifuged. The clear lysate was collected, analyzed for total protein content, and stored at –80°C until use. The simultaneous measurements of total and phosphorylated Akt, JNK1/2 and p38 MAPK were carried out according to the manufacturer's instructions (Biosource). Briefly, the samples were diluted 2-fold with sample treatment buffer and incubated on ice for 20 min. Thereafter, the samples were diluted 4-fold in assay diluent. Beads coupled to total or phosphorylated Akt, JNK1/2 and p38 MAPK were pipetted into 96-well filter plates together with samples or standards, and incubated for 2 h. Next, the beads were washed and mixed with specific biotinylated detector antibodies for 1 h. After an additional washing and subsequent incubation with streptavidin-RPE for 30 min, the beads were washed and resuspended in assay buffer. All incubation steps were carried out on an orbital shaker, at room temperature and in the

dark. The amount of total and phosphorylated Akt, JNK1/2 and p38 MAPK was measured by the Bioplex™ system (Bio-Rad Laboratories, Hercules, Calif., USA). In total, 100 events per specific bead type were read, and the data output was exported to Excel for further analysis.

Detection of Interferon Regulatory Factor 3 Activation

Eosinophils (2×10^6 cells/ml) were cultured for 1, 2 and 20 h in 12-well culture plates with or without 12.5 μ g/ml poly(I:C). Nuclear extracts were prepared using the Nuclear Extract Kit from Active Motif (Carlsbad, Calif., USA). Briefly, cells were collected, washed in ice-cold PBS supplemented with phosphatase inhibitors, and centrifuged. The cell pellet was resuspended in hypotonic buffer, incubated for 15 min on ice, followed by addition of detergent and centrifugation. The pellet was then resuspended in complete lysis buffer, incubated for 30 min on ice, vortexed and centrifuged for 10 min. The nuclear-containing supernatant was then collected and stored at –80°C until use. Thereafter, interferon regulatory factor 3 (IRF-3) activation was analyzed using the TransAM™ IRF-3 ELISA kit from Active Motif.

Reagents

Lyophilized poly(I:C) was purchased from Invivogen (San Diego, Calif., USA), reconstituted in sterile physiological salt solution, heated to 50°C and allowed to cool to room temperature to ensure proper annealing. The purity of the poly(I:C) preparation was tested, and it did not cross-react with other TLRs. Recombinant human interleukin (IL)-4 and IL-5 were obtained from R&D Systems, and R-837 from Invivogen. The inhibitors PD98059 and SB203580 were purchased from Tocris-Cookson (Bristol, UK), SP600125 and LY294002 from Calbiochem (Bad Soden, Germany) and MG-132 from Alexis Biochemicals (Lausen, Switzerland). PD98059, SB203580, SP600125 and LY294002 were all used at a concentration of 10 μ M, whereas MG-132 was used at 1 μ M [17, 18]. All inhibitors were reconstituted in DMSO.

Statistics

Statistical analysis was performed using the software Graph-Pad Prism 5 (GraphPad Software, San Diego, Calif., USA). All data are expressed as mean $\pm$ SEM; n equals the number of subjects. Nonparametric data were analyzed for statistical differences using the Kruskal-Wallis test in combination with Dunn's multiple comparison test for unpaired comparisons and the Wilcoxon signed-rank test for paired comparisons. Parametric data were analyzed using the paired t test (for 2 sets of data) or one-way repeated-measures ANOVA with Dunnett's post-test (≥ 2 sets of paired data). $p < 0.05$ was considered statistically significant.

Table 3. Distribution of eosinophils in PB and BM

	Control	Outside the pollen season	During the pollen season
PB, %	3.2 ± 0.6	3.5 ± 0.4	5.0 ± 0.8
BM, %	2.7 ± 0.5	3.9 ± 0.2	3.7 ± 0.4

Results

Distribution of Eosinophils in PB and BM

The percentage of eosinophils in BM and PB in the 3 groups is presented in table 3. An increased proportion of eosinophils among the allergic patients can be seen, particularly in PB from allergic patients during the pollen season, although the increase is not statistically significant ($p = 0.09$).

Expression of TLR3 in BM and PB

By gating intact leukocytes from BM and PB, eosinophils were defined based on their high FSc, high SSc, low CD16 and intermediate CD45 (fig. 1a) [19, 20]. Intracellular and extracellular expression of TLR3 was investigated by simultaneous staining with TLR3-PE and isotype-matched control antibody. The results show an intracellular expression of TLR3 in eosinophils in both BM and PB (fig. 1b). Extracellular expression, however, could not be demonstrated (data not shown).

Expression of TLR3 in Eosinophils in Different Compartments and during the Pollen Season

First, the expression of TLR3 in eosinophils in the two different compartments was calculated, irrespective of the atopic status of the individuals from whom the cells were obtained, as mean fluorescence intensity ratio (MFIR = MFI for the TLR3 antibody/MFI for the isotype control antibody) [21, 22]. The expression of TLR3 was found to be higher in BM compared to PB (fig. 2a). Next, the influence of allergic inflammation on the expression of TLR3 in eosinophils was examined. Thus, the expression levels of TLR3, as determined by MFIR, were compared between healthy controls and allergic patients outside and during the pollen season. The expression of TLR3 in BM eosinophils was lower in patients with symptomatic allergic rhinitis compared to patients outside the pollen season (fig. 2b). In analogy, the expression of TLR3 in PB eosinophils was lower in patients during the pollen season than in patients outside the season (fig. 2c).

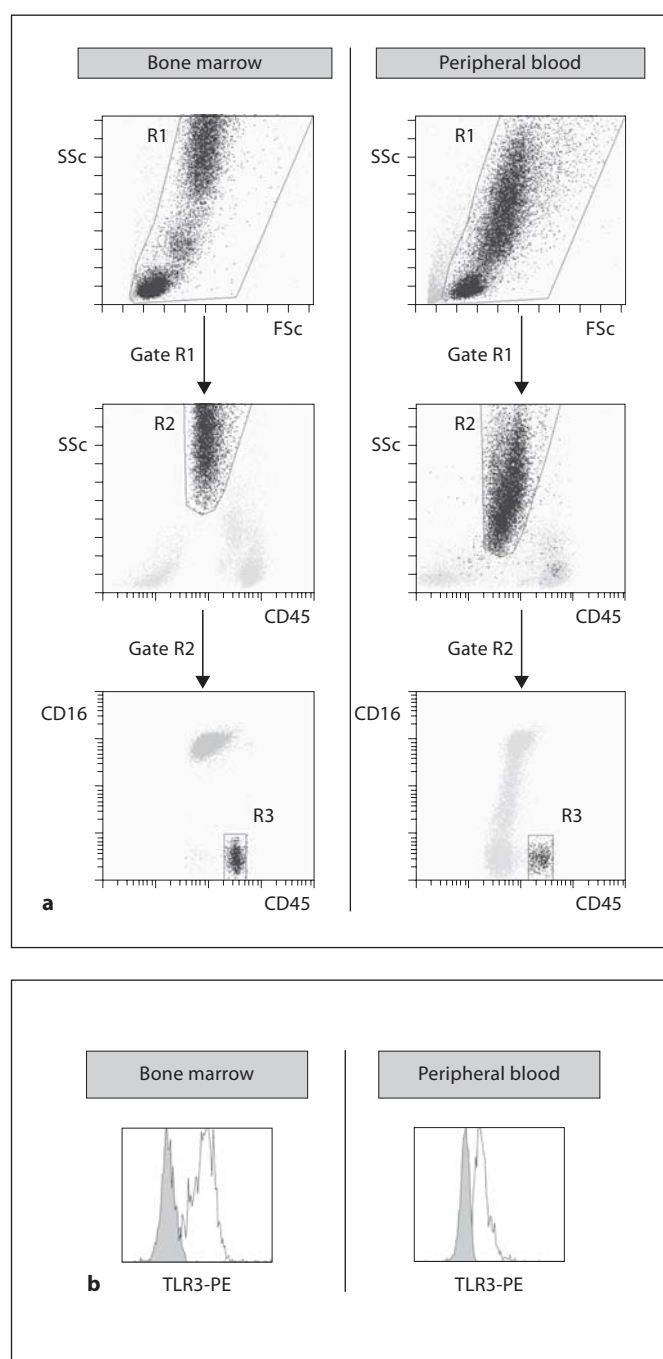


Fig. 1. Expression of TLR3 in eosinophils from BM and PB. **a** Flow cytometry data with dot plots showing gates for eosinophils in BM and PB. FSc versus SSc, with gate R1 representing nucleated leukocytes. CD45 versus SSc of cells gated from R1, representing neutrophils and eosinophils, R2. CD45 versus CD16 of cells gated from R2, representing eosinophils, R3. **b** Eosinophils were analyzed for intracellular expression of TLR3 by flow cytometry using mAbs against TLR3 (open histograms) and appropriate isotype control (shaded histograms). Cells were fixed and permeabilized prior to incubation with mAbs. Data from 1 representative out of 6 independent experiments are shown.

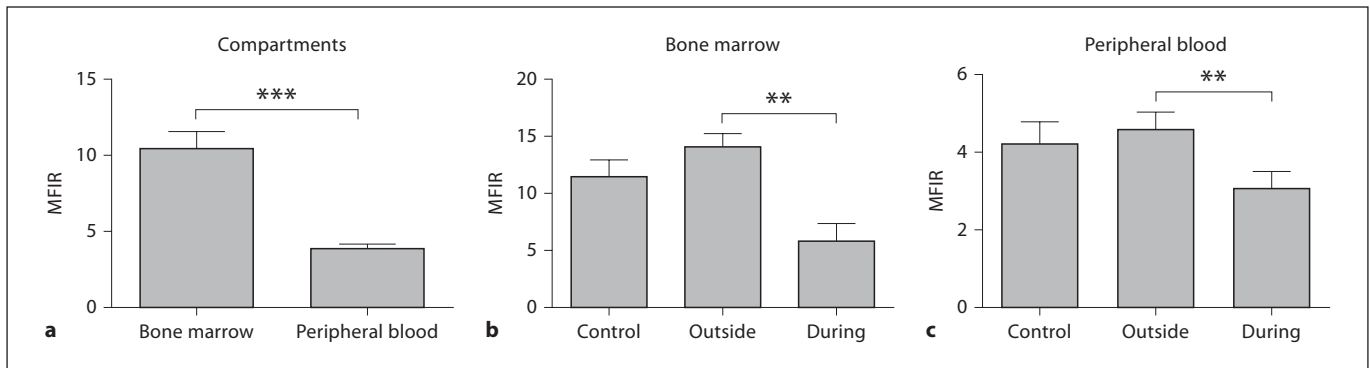


Fig. 2. Expression of TLR3 in eosinophils in different compartments and during the pollen season. Intracellular expression of TLR3 in eosinophils presented as MFIR. **a** Expression of TLR3 in BM (n = 21) and PB (n = 28). **b** Expression of TLR3 in BM in healthy subjects (n = 7), allergic patients outside (n = 7) and dur-

ing the pollen season (n = 7). **c** Expression of TLR3 in PB in healthy subjects (n = 9), allergic patients outside (n = 8) and during the pollen season (n = 11). Data are presented as mean $\pm$ SEM (* p < 0.05, ** p < 0.01, *** p < 0.001).

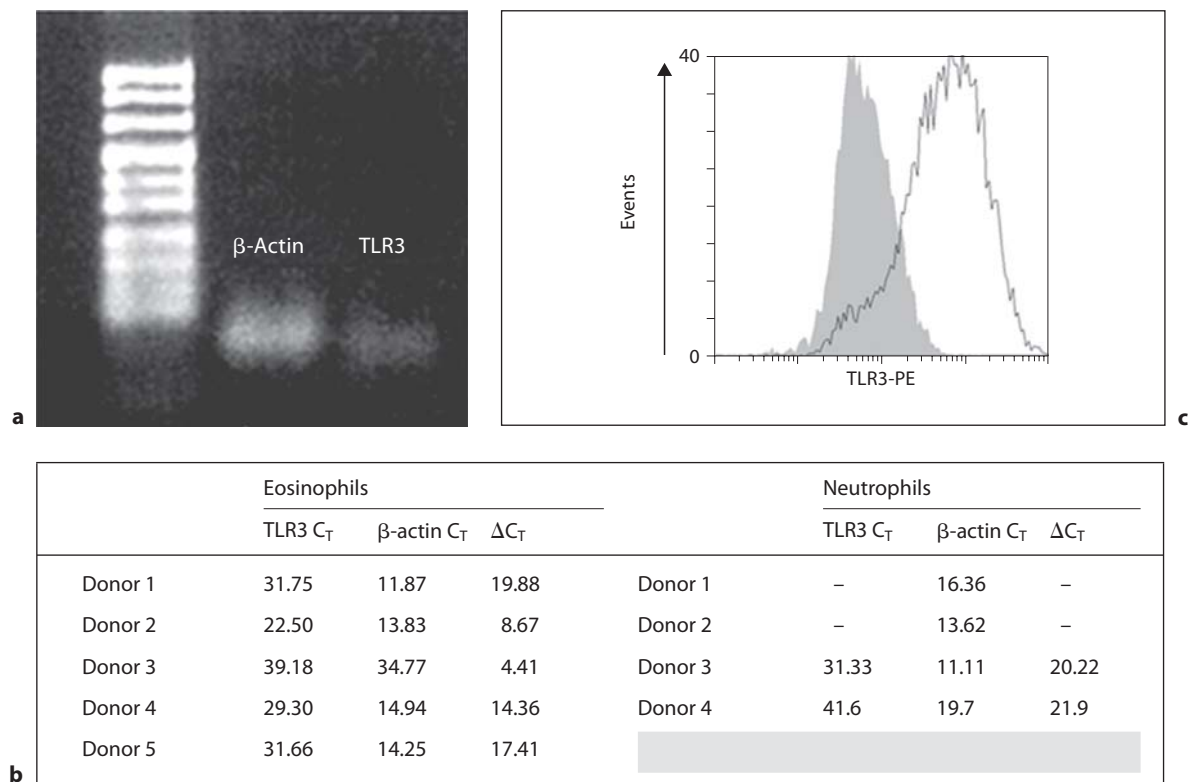


Fig. 3. Expression of TLR3 in purified eosinophils from PB. Freshly isolated eosinophils were analyzed for mRNA and protein expression of TLR3. **a** Total RNA was extracted and analyzed for expression of TLR3 and β -actin using real-time RT-PCR. **b** Table showing C_T values and Δ C_T values for the TLR3 and β -actin primers. Real-time RT-PCR analysis was performed on RNA ex-

tracted from purified eosinophils and neutrophils. **c** Intracellular expression of TLR3. Cells were stained intracellularly with CD16-Pcy5 in combination with TLR3-PE (open histogram) or appropriate isotype control (shaded histogram) and analyzed by flow cytometry. Data from 1 representative out of 3 independent experiments are shown.

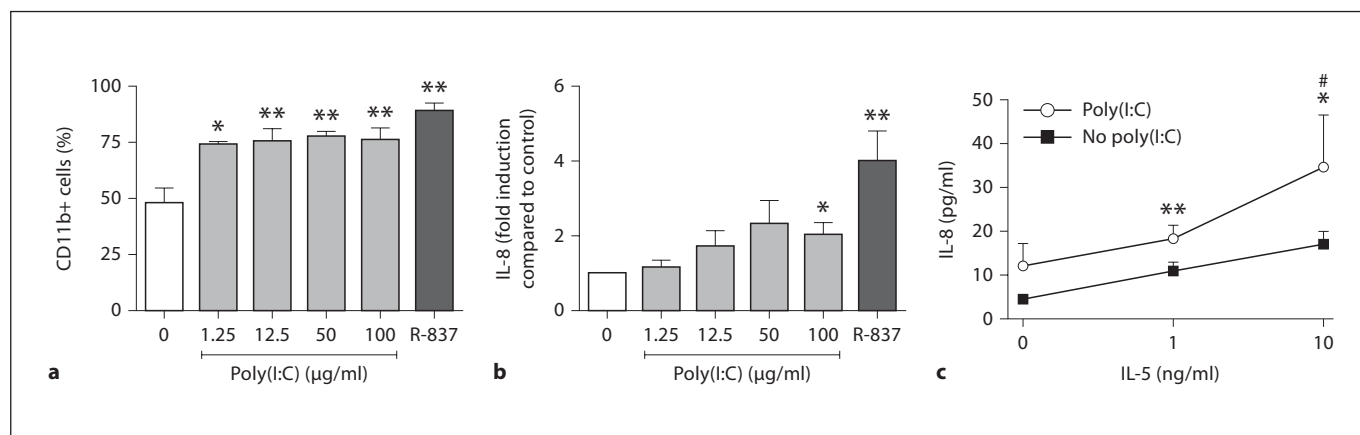


Fig. 4. Upregulation of CD11b and increase in IL-8 secretion by eosinophils isolated from PB in response to poly(I:C) stimulation. Purified cells (1×10^6 cells/ml) were incubated 24 h with medium alone or various concentrations of poly(I:C), IL-5 or R-837 and analyzed for CD11b expression using flow cytometry or IL-8 secretion using ELISA. **a** Percentage of CD11b+ cells, and **(b)** IL-8 production in relation to unstimulated control after poly(I:C)

stimulation. R-837 (10 μ g/ml) was used as a positive control ($n = 4$). **c** Eosinophils were pretreated with IL-5 for 1 h before the addition of 12.5 μ g/ml poly(I:C). Levels of IL-8 were measured in the culture supernatants ($n = 5$). Data are expressed as means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$ (compared to untreated control); # $p < 0.05$ (white circle compared to black square).

Expression of TLR3 in Purified Eosinophils

To confirm the presence of TLR3 seen in eosinophils from PB and BM, real-time PCR and flow cytometry analysis were performed on purified cells. Analysis of RNA extracted from freshly isolated eosinophils showed a weak band on the gel for TLR3 (fig. 3a), and melting curve analysis of TLR3 and β -actin revealed a single peak in each sample. Gene expression was assayed as well using the comparative cycle threshold (C_T) method [21], where the relative amount of TLR3 mRNA was determined by subtracting the C_T value for the gene with the C_T value for β -actin (ΔC_T). C_T and ΔC_T values for TLR3 and β -actin showed a consistent expression of TLR3 in eosinophils, whereas a low expression in neutrophils was found in 2 out of 4 donors (fig. 3b). Further, results from the flow cytometry analysis clearly showed protein expression of TLR3 in purified eosinophils (fig. 3b).

Activation of Eosinophils by Poly(I:C)

Next, we wanted to investigate whether TLR3 exhibited functional activity in eosinophils. Since the intracellular expression of TLR3 was higher in eosinophils from BM than PB, it would have been interesting to investigate whether this difference resulted in a more robust response to poly(I:C) by BM-derived eosinophils. Despite this, we used PB eosinophils in these experiments since samples containing a sufficient amount of cells were eas-

ier to obtain and involved less discomfort for the subjects. In the first set of experiments, responsiveness to poly(I:C) was examined using expression of CD11b and secretion of IL-8 as representative indicators of activation [12, 23, 24]. R-837 (ligand for TLR7) was used as a positive control. It was found that poly(I:C) increased the percentage of CD11b+ eosinophils and enhanced the production of IL-8 after 24 h compared to untreated controls (fig. 4a, b). Also, IL-5, known to prevent apoptosis in vitro, was added to our eosinophil cultures 1 h before the addition of poly(I:C). Stimulation with IL-5 alone gave rise to a small chemokine release, and in combination with poly(I:C) a concentration-dependent increase in IL-8 secretion was seen (fig. 4c). Collectively, these results demonstrate that poly(I:C) has the ability to activate purified eosinophils and that pretreatment with IL-5 augments the response.

Th2 Cytokines Downregulate TLR3 Expression

To investigate possible mechanisms behind the lower expression of TLR3 during the pollen season, purified eosinophils were cultured for 24 h in the absence or presence of the Th2 cytokines IL-4 and IL-5. Thereafter, intracellular expression of TLR3 was determined by use of flow cytometry. Both cytokines were found to downregulate TLR3 expression (fig. 5a, b).

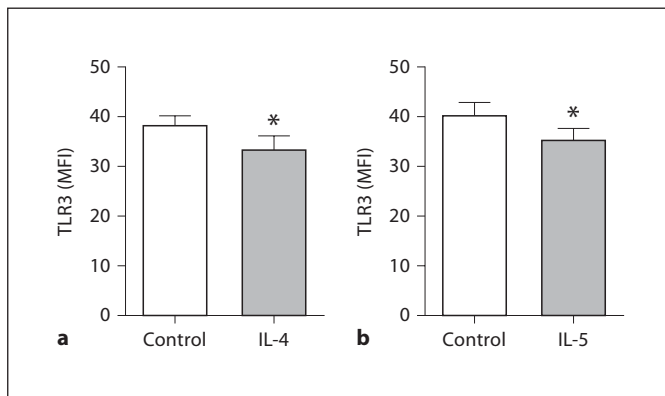


Fig. 5. Th2 cytokines downregulate the expression of TLR3. Purified eosinophils (1×10^6 cells/ml) from PB were cultured for 24 h in the absence or presence of (a) 1 ng/ml IL-4 ($n = 5$) or (b) 10 ng/ml IL-5 ($n = 3$). Thereafter, intracellular expression of TLR3 was determined using flow cytometry. Data are presented as mean fluorescence intensity (MFI), and expressed as means $\pm$ SEM (* $p < 0.05$).

Specific Inhibitors of p38 MAPK and Nuclear Factor- κ B Abolish the Poly(I:C)-Induced Effects in Eosinophils

In order to determine the signal transduction pathways induced by poly(I:C) stimulation, specific inhibitors of different pathways were used. Purified eosinophils were pretreated for 45 min with vehicle (DMSO) or the various inhibitors, further incubated with poly(I:C) for 24 h, and thereafter analyzed for CD11b expression and IL-8 secretion. SB203580, a specific inhibitor of p38 MAPK, and MG-132, a proteasome inhibitor capable of suppressing I κ B α degradation and subsequent nuclear factor- κ B (NF- κ B) nuclear translocation [25, 26], completely abolished the increase in CD11b expression induced by poly(I:C) (fig. 6a). The secretion of IL-8 was affected too, although not statistically significantly (fig. 6b). No effects were seen following SP600125, PD98059 and LY294002, specific inhibitors of JNK1/2, ERK1/2 and phosphoinositide-3 kinase (PI3K), respectively (data not shown). To ensure that the inhibitory effects of SB203580 and MG-132 were not due to unspecific mechanisms, eosinophils were incubated with the various inhibitors in the absence of poly(I:C). Neither the p38 MAPK nor the NF- κ B inhibitor exhibited any unspecific effects on CD11b expression or IL-8 secretion. Therefore, we concluded that the effects exerted by poly(I:C) are most likely mediated via the p38 MAPK and NF- κ B signaling pathways.

Increased Phosphorylation of p38 MAPK in Eosinophils Stimulated with Poly(I:C)

To further verify that intracellular signaling pathways of eosinophils were actually activated by poly(I:C), phosphorylated (activated) and total forms of Akt (comprising PI3K, Akt and praline-rich Akt substrate 40 (PRAS40)), JNK1/2 and p38 MAPK were compared using a Luminex Multiplex Immunoassay. The experiments showed that the expression of total Akt was very low, and phosphorylated Akt was completely absent (data not shown). The amount of total JNK1/2 remained almost constant in poly(I:C)-treated and -untreated eosinophils. No phosphorylation of JNK1/2 was seen in untreated cells, and very low levels of the activated form were detected in cells from 3 of the 8 donors (data not shown). This confirmed the results above that PI3K (Akt) and JNK1/2 are not involved in poly(I:C) signaling. In contrast, total p38 MAPK was abundantly expressed by both untreated and treated cells (fig. 7a). Phosphorylated forms of p38 MAPK were seen both in untreated and treated cells. However, a clear increase in the activated form was seen upon poly(I:C) stimulation (fig. 7b). These results corroborate the preceding data (fig. 6) that stimulation of eosinophils via TLR3 leads to activation of the p38 MAPK signaling pathway. In addition, activation of the transcription factor IRF-3 was measured in the nuclear extracts of poly(I:C)-stimulated cells using ELISA. IRF-3 is constitutively expressed in the cytoplasm of unstimulated cells, but upon activation, it is phosphorylated and translocated into the nucleus [27]. However, poly(I:C) stimulation of eosinophils did not induce activation of IRF-3 ($n = 6$, data not shown).

Discussion

Intracellular expression of TLR3 was demonstrated in eosinophils derived from BM and PB. TLR3 expression was higher in BM than in PB, and it was downregulated in both compartments during periods of symptomatic AR and in the presence of Th2 cytokines. Poly(I:C) stimulation of purified eosinophils increased the expression of CD11b and the release of IL-8, which indicates a functional role for TLR3 in these cells. Priming with IL-5 also amplified the poly(I:C)-induced activation. The stimulation-induced phosphorylation of p38 MAPK, and the poly(I:C)-induced effects were inhibited by SB203580 and MG-132, suggesting that TLR3 activates the p38 MAPK signaling pathway and the transcription factor NF- κ B.

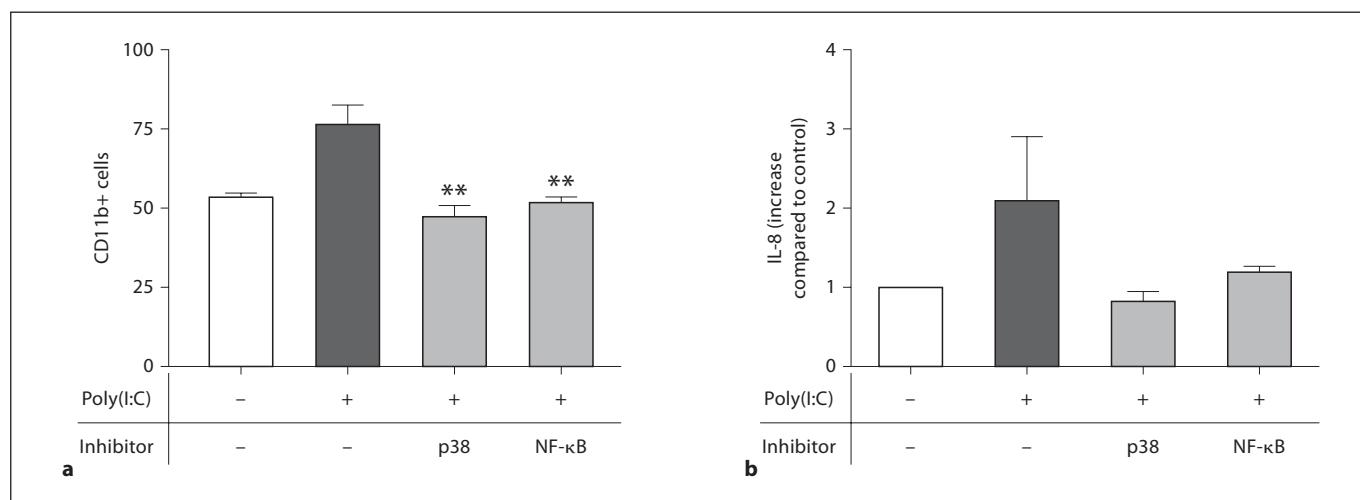


Fig. 6. Activation of eosinophils by poly(I:C) is attenuated by specific inhibitors to p38 MAPK and NF-κB. Eosinophils isolated from PB were pretreated (1×10^6 cells/ml) with vehicle (DMSO) or inhibitors to p38 MAPK (SB203580, 10 μ M) and NF-κB (MG-132, 1 μ M) for 45 min, and further incubated for 24 h with or

without poly(I:C) (12.5 μ g/ml). **a** Percentage of CD11b+ cells determined by flow cytometry. **b** IL-8 secretion in relation to unstimulated control measured by ELISA. Poly(I:C) stimulated cells (dark gray bar) and controls without poly(I:C) stimulation (white bar). Data are presented as means $\pm$ SEM ($n = 4$; ** $p < 0.01$).

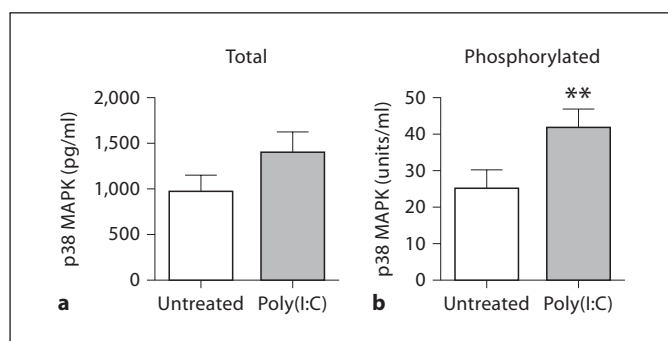


Fig. 7. Poly(I:C) stimulation of eosinophils activates the p38 MAPK signaling pathway. Eosinophils isolated from PB were cultured (1×10^6 cells/ml) for 24 h with or without 12.5 μ g/ml poly(I:C). The cell lysates were analyzed for total and phosphorylated p38 MAPK using a Luminex Multiplex Immunoassay. Data are presented as means $\pm$ SEM ($n = 8$; ** $p < 0.01$). **a** Total p38 MAPK (pg/ml). **b** Phosphorylated p38 MAPK (units/ml).

In the present study, we found mRNA and protein expression of TLR3 in eosinophils using real-time PCR and flow cytometry, respectively. Earlier Western blot and flow cytometry analyses found no or low levels of TLR3 proteins in human eosinophils [11, 12]. However, a recently published study has demonstrated intracellular expression of TLR3 in eosinophils isolated from the

spleens of naïve hypereosinophilic mice by flow cytometry [15]. A proportionally high expression of TLR3 was found in BM eosinophils, which suggests a role for TLR3 in the development and differentiation of these cells. It is also possible that the BM functions as a reservoir of eosinophils with a high expression of TLR3 ready to be discharged in case of a viral infection.

Stimulation of purified eosinophils with the TLR3 agonist poly(I:C) resulted in an increase in the cell surface expression of CD11b and a similar increase in IL-8 secretion. The former is a well-known adhesion molecule and the latter a strong stimulator of leukocyte migration [28, 29]. However, a previous study by Nagase et al. [9] failed to show poly(I:C)-induced expression of adhesion molecules and prolongation of eosinophil survival. For the analysis of adhesion molecule expression, they used a relatively short incubation period (90 min), whereas this period in the present study was 24 h. Further, Wong et al. [12] and Cheung et al. [13] reported that poly(I:C) was unable to induce survival, cytokine secretion and upregulation of adhesion molecules by eosinophils [12, 13]. These discrepancies might be explained by the methodological differences in the eosinophil isolation process. Firstly, in both studies buffy coats obtained from blood transfusion services were used, whereas we used PB acquired directly from the volunteers. It cannot be excluded that the differences in the way the cells were obtained,

resulting in a slightly different cellular milieu, might have affected the basal TLR3 expression and subsequently the effects induced by poly(I:C). Also, the shape and surface morphology of granulocytes have been reported to be substantially affected by the temperature changes and storage time that occur during the separation of buffy coats from whole blood [30]. Possibly, these alterations also influence the expression of TLR3. Secondly, the eosinophil purification processes differed between our study and the two previous ones. The present study has comprehensively investigated and a functional role of TLR3 in purified eosinophils was shown using several activation parameters and several concentrations of poly(I:C). Although the effects exerted by poly(I:C) are not as evident as those by R-837/R-848 and CpG, these findings altogether make the picture complete and show that virus-recognizing TLRs in eosinophils play an important role in allergic airway disease.

Poly(I:C)-induced phosphorylation (activation) of p38 MAPK, and in accordance, inhibition of p38 MAPK and NF- κ B resulted in an attenuation of poly(I:C)-induced effects on CD11b expression and IL-8 secretion in eosinophils. However, no activation of IRF-3 was seen. Stimulation of TLR3 has previously been described to result in the induction of JNK and p38 MAPK pathways leading to the subsequent activation of the transcription factors IRF-3 and NF- κ B that regulate the production of IFN-inducible genes and pro-inflammatory cytokines, respectively [31]. As poly(I:C) failed to activate IRF-3, our findings suggest the sole involvement of the transcription factor NF- κ B in eosinophils. TLR3 is the only TLR that does not use MyD88. Instead, the adaptor TRIF is utilized. MyD88-dependent signaling, which is used by the other virus-recognizing TLRs (TLR7/8 and TLR9), is the most common pathway. It results in the activation of MAPKs, PI3K and NF- κ B that regulate transcription of proinflammatory genes and type I interferons [32–34].

Also, eosinophils were cultured in the presence of IL-5, a cytokine that mediates eosinophil differentiation and survival [35, 36]. We have recently demonstrated higher levels of IL-5 in serum of patients with allergic rhinitis than of healthy subjects, and that IL-5 augments TLR7- and TLR9-induced responses [14]. IL-5 alone gave rise to a small IL-8 release, and in the presence of poly(I:C), a synergistic increase in IL-8 secretion was seen. These findings suggest that pretreatment with IL-5 makes eosinophils more responsive to TLR3 stimulation, and that the effects seen following poly(I:C) are not an indirect result of the differential induction of apoptosis in un-

stimulated and stimulated cells, but truly mediated via TLR3.

In a recent study, we have shown enhanced levels of TLR3 mRNA and protein in biopsies of nasal mucosa from patients with symptomatic AR [37]. The increase was most prominent in the epithelial cells, but TLR3-positive staining was also seen in scattered intraepithelial and subepithelial granulocytes. It is well known that eosinophils accumulate in the airways during periods of allergic inflammation [38], and in the present study, a trend towards an increased proportion of eosinophils was found in PB from allergic patients examined during the pollen season. In parallel, the expression of TLR3 was reduced in both BM and PB eosinophils during symptomatic AR. As the expression of TLR3 was downregulated in the presence of IL-4 and IL-5, one explanation might be an autoregulatory feedback loop initiated by Th2 cytokines as part of the allergic inflammation in the airways.

The present study has demonstrated the expression and function of TLR3 in human eosinophils by several methods. Data suggest that eosinophils activated via TLR3 might be more able to home to inflamed tissues, which in the context of AR correspond to the nose, and to direct leukocyte traffic into inflamed areas. Also, a decreased level of TLR3 was seen during the pollen season and in the presence of a Th2 cytokine milieu. In view of this and recent advances as to the role of respiratory infections in allergy and asthma, our findings further pinpoint that eosinophils might operate as a link between viral infection and exacerbations of allergic disease.

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