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## A novel role for neutrophils in IgE-mediated allergy: evidence for antigen-presentation in late-phase reactions

Dominika Polak, MSc<sup>1</sup>, Christine Hafner, MD<sup>2</sup>, Peter Briza, PhD<sup>3</sup>, Claudia Kitzmüller, PhD<sup>1</sup>, Adelheid Elbe-Bürger, PhD<sup>4</sup>, Nazanin Samadi, MSc<sup>1</sup>, Maria Gschwandtner, MSc<sup>5</sup>, Wolfgang Pfützner, MD<sup>6</sup>, Gerhard J. Zlabinger, MD<sup>7</sup>, Beatrice Jahn-Schmid, PhD<sup>1</sup>, Barbara Bohle, PhD<sup>1</sup>

<sup>1</sup>Department of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

<sup>2</sup>Department of Dermatology, University Hospital St. Pölten, Karl Landsteiner University of Health Sciences, St. Pölten, Austria

<sup>3</sup>Department of Molecular Biology, University Salzburg, Salzburg, Austria

<sup>4</sup>Department of Dermatology, Medical University of Vienna, Vienna, Austria

<sup>5</sup>Department of Dermatology, Research Division of Biology and Pathobiology of the Skin, Medical University of Vienna, Vienna, Austria

<sup>6</sup>Department of Dermatology and Allergology, Philipps University Marburg, Marburg, Germany

<sup>7</sup>Institute of Immunology, Medical University of Vienna, Vienna, Austria

### Abstract

**Background**—Neutrophils and allergen-specific T-cells accumulate in allergic late-phase reactions (LPR). Their presence is associated with severe inflammation. Cytokines like GM-CSF, IFN- $\gamma$ , and IL-3, typically found in allergic LPR, have been proposed to convert neutrophils into antigen-presenting cells (APC).

**Objective**—To assess the antigen-processing and presenting capacity of neutrophils from allergic patients.

**Methods**—Neutrophils were isolated from peripheral blood of birch pollen-allergic donors and stimulated with GM-CSF, IFN- $\gamma$ , and IL-3. Viability and expression of HLA-DR, CD80, and CD86 were assessed by flow cytometry. HLA-DM expression was analysed by immunoblotting. Allergen-uptake was studied with fluorescence-labelled major birch pollen allergen Bet v 1. Bet v

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**Corresponding author:** Barbara Bohle.

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1 was digested with neutrophilic endolysosomal extracts and resulting fragments were sequenced using mass spectrometry. Neutrophils were used as APC in co-culture experiments with autologous HLA-DR-restricted Bet v 1-specific T-cell clones reactive with epitopes in different regions of the allergen. In all experiments, monocytes were used for comparison. Fluids from suction blisters formed on top of LPR induced by intradermal allergen injection were assessed for HLA-DR-positive neutrophils by flow cytometry.

**Results**—The cytokines significantly enhanced the survival, allergen uptake and expression of HLA-DM and HLA-DR of neutrophils. Neutrophils rapidly degraded Bet v 1 into fragments containing all relevant T-cell epitopes. Cytokine-activated, allergen-pulsed neutrophils induced proliferative and cytokine responses of Bet v 1-specific T-cells irrespective of epitope specificity confirming that they fully processed and presented the allergen. HLA-DR-positive neutrophils were detected in cutaneous allergic LPR.

**Conclusion**—Neutrophils may serve as APC for local allergen-specific effector T-cells in allergic LPR.

### Keywords

antigen-presentation; neutrophils; IgE-mediated allergy; allergic inflammation; allergic late-phase response; T-cells; T-cell-activation

### Introduction

Re-exposure of allergic individuals to either the sensitizing or a cross-reactive allergen causes an acute response, seen in the skin as wheal and flare reaction, or, after inhalation, as rhinitis and airway constriction<sup>1,2</sup>. This immediate reaction is considered to result from the release of histamine and other preformed inflammatory mediators from mast cells upon cross-linking of adjacent FcεRI-bound IgE molecules on their surface<sup>3</sup>. The late-phase reaction (LPR) follows 3-48 h after allergen challenge, resulting in the skin as swelling and induration, in the nose as continued nasal blockage, and in the lung as late asthmatic reaction (LAR)<sup>1,2,4</sup>. LPR are accompanied by inflammatory cell infiltration, in particular by eosinophils and Th2 cells<sup>5-9</sup>, and have been used as models of chronic allergic inflammation<sup>10,11</sup>. The pivotal role of allergen-specific CD4<sup>+</sup> T-cells in LPR has been underlined by the finding that LAR can be provoked by T-cell-activation independently of the IgE response after administration of peptides containing T-cell epitopes but incapable of cross-linking IgE<sup>12</sup>.

Neutrophils are the first innate immune cells that migrate to inflammatory sites and rapidly exert manifold effector functions<sup>13</sup>. They also accumulate in allergic LPR<sup>9,14</sup> and their presence has been linked to more severe inflammation<sup>15-17</sup>. Bronchoalveolar lavage fluids of patients with allergic LPR contain larger numbers of neutrophils than those of individuals experiencing no LPR<sup>18,19</sup>. Furthermore, severe asthma has been associated with elevated numbers of neutrophils in sputum, tracheal aspirates and in the bronchial mucosa<sup>20-22</sup>. Activated neutrophils account for enzymes and other cytotoxic agents and release chemokines and cytokines that attract immature dendritic cells (DC), T-cells, monocytes and

macrophages and instruct the interaction of these cells types<sup>23, 24</sup>. Additionally, evidence for a direct cross-talk of neutrophils with T-cells has been provided<sup>25</sup>.

Recently, it was reported that human neutrophils acquire HLA-DR-dependent antigen-presenting capacity when co-cultured with autologous antigen-specific CD4<sup>+</sup> memory T-cells plus antigen<sup>26</sup>. Evidence for the antigen-presenting capacity of human neutrophils had already been indicated before, mainly because these professional phagocytes express HLA-DR when exposed to GM-CSF, IFN- $\gamma$ , and IL-3, both *in vitro*<sup>27, 28</sup> and *in vivo*<sup>29, 30</sup>. These cytokines are released by specific CD4<sup>+</sup> T-cells and sensitized mast cells in response to natural allergen exposure or the injection of allergy vaccines<sup>3, 5, 31–33</sup>. This tempted us to speculate that the cytokine milieu in allergic LPR may convert neutrophils into antigen-presenting cells (APC). To address this hypothesis, we stimulated neutrophils from allergic patients with a combination of GM-CSF, IFN- $\gamma$ , and IL-3, and characterized key features of antigen-presentation, namely upregulation of HLA-DR, HLA-DM, CD80, and CD86, as well as antigen internalization, processing, and presentation to T-cells by utilizing the major birch pollen allergen Bet v 1 as model protein. This clinically highly relevant allergen has been thoroughly characterized regarding its T-cell-reactivity<sup>34–36</sup>. Neutrophils were compared to monocytes which represent another type of phagocytes and are professional APC. Finally, we explored whether HLA-DR-positive neutrophils are actually found in allergen-induced LPR in the skin of allergic individuals.

## Material and Methods

### Allergic patients

Birch pollen-allergic patients showed rhinoconjunctivitis in spring, positive skin prick tests to birch pollen extract (ALK-ABELLO, Hørsholm, Denmark) and birch pollen-specific IgE of >0.35 kU<sub>A</sub>/l (ImmunoCAP; Thermo Fisher Scientific, Uppsala, Sweden). Grass pollen-allergic patients showed rhinoconjunctivitis in summer, positive skin prick tests to grass pollen extract (ALK-ABELLO) and grass pollen-specific IgE of >0.35 kU<sub>A</sub>/l (ImmunoCAP). The study was approved by the ethics committees of the Medical University of Vienna (EK-1488/2017) and Lower Austria (GS1-EK-4/317-2015) and conducted in accordance with the Declaration of Helsinki. Patients gave written informed consent.

### Allergens

Recombinant Bet v 1 and Hev b 3 were purchased from Biomay (Vienna, Austria). Bet v 1 was conjugated to pHrodo-succinimidyl ester (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Birch pollen extract was prepared as described<sup>37</sup>. Grass pollen extract for intradermal administration was purchased from ALK-ABELLO.

### Cell preparations

Neutrophils were isolated as described<sup>38</sup>. The procedure included a density gradient centrifugation step of whole blood on Ficoll-Hypaque (Pharmacia Diagnostics, Uppsala, Sweden), dextran sedimentation (4% dextran T 500, Carl ROTH, Germany), red cell osmotic lysis of the granulocyte pellet, and a final immunomagnetic negative selection step using the human MACSxpress<sup>®</sup> Neutrophil Isolation Kit (Miltenyi Biotec GmbH, Bergisch-Gladbach,

Germany) and resulted repeatedly in >99% pure CD16<sup>+</sup>CD66b<sup>+</sup>CCR3<sup>-</sup>HLA-DR<sup>-</sup>CD19<sup>-</sup>CD3<sup>-</sup>CD14<sup>-</sup> cells. Neutrophils (1x10<sup>6</sup>/ml) were cultured in RPMI 1640 (Sigma Aldrich, Darmstadt, Germany) supplemented with 10% autologous plasma alone or stimulated with GM-CSF (100 pg/ml), IFN- $\gamma$  (10 ng/ml) and IL-3 (30 ng/ml; all from Peprotech, Rocky Hill, USA) as described<sup>28</sup>. Monocytes (>98% CD14<sup>+</sup>CCR3<sup>-</sup>CD19<sup>-</sup>CD3<sup>-</sup>) were obtained by using CD14 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions.

### Flow cytometry

Analyses were performed with a FACS Canto II using FACS Diva (BD Biosciences, San Jose, CA, USA) and FlowJo software (TreeStar, Inc., Ashland, OR, USA). The following anti-human antibodies were used: HLA-DR-BV421<sup>TM</sup>, HLA-DR-APC (clone L243), CD80-Alexa647, CD3-APC-Cy7, CD16-PE (clone 3G8), CD15-PE-Cy7, CD15-PerCP-Cy5, CCR3-APC and PerCP-labelled CD14, CD19, CD56, CD123, and CCR3 (all from Biolegend, San Diego, CA), CD4-PE and CD86-PE, CD45-FITC (all from BD Biosciences) and CD3-PerCP and CD8-PerCP (both from eBioscience Inc., San Diego, CA). Viability was determined by using the Fixable Viability Dye eFluor<sup>®</sup> 780 (eBioscience Inc.). Annexin V-FITC (BD Pharmingen, San Jose, CA, USA) was used to determine cells undergoing apoptosis. For internalization experiments, cells (1x10<sup>6</sup>/ml) were incubated with and without pHrodo-labelled Bet v 1 (1.5  $\mu$ g/ml) in the presence of GM-CSF/IFN- $\gamma$ /IL-3.

### Detection of HLA-DM

Cell lysates were prepared from neutrophils and monocytes (each 10x10<sup>6</sup>) as described<sup>35</sup> immediately after their isolation and from HLA-DR-negative (3x10<sup>6</sup>) and HLA-DR-positive (1.8x10<sup>6</sup>) neutrophils sorted by a FACSaria III (BD Biosciences) after stimulation with GM-CSF/IFN- $\gamma$ /IL-3 for 48 h. Cell lysates (30  $\mu$ l) were separated by 15% SDS-PAGE and transferred onto nitrocellulose membranes. After saturation with PBS/Tween 20 supplemented with 5% skim milk overnight at 4°C immunoblotting was performed with a monoclonal mouse anti-HLA-DM antibody (Novus Biological, Abingdon, UK) and a horseradish peroxidase-conjugated anti-mouse IgG (CST, Massachusetts, USA). As loading control GAPDH was detected with a rabbit anti-GAPDH (Sigma, St. Louis, MO, USA) and a horseradish peroxidase-conjugated ECL anti-rabbit IgG (Amersham, Buckinghamshire, UK). After developing with the ECL Prime Western blotting Detection Reagent, the nitrocellulose was exposed to a high performance autoradiography film (both GE Healthcare, Buckinghamshire, UK).

### Endocytosis and endolysosomal degradation assays of Bet v 1

Endolysosomal microsomes were isolated from neutrophils and monocytes as described<sup>36</sup>. Microsomes (2  $\mu$ g) were incubated with Bet v 1 (2  $\mu$ g) at 37°C in a final volume of 20  $\mu$ l containing 100 mM citrate buffer (pH 4.8) and 2 mM dithiothreitol. At indicated time points samples were collected, denatured for 5 min at 95°C, loaded on a 15% SDS gel and visualized by Coomassie staining. Remaining peptides were analyzed using an ESI-QTOF mass spectrometer fitted with a capillary reversed phase HPLC (Waters, Milford, MA, USA).

The quantity of intact Bet v 1 was determined by an in-house capture ELISA. Briefly, microtiter plates (Maxisorp, Nunc, Denmark) were coated with the Bet v 1-specific monoclonal antibody BIP1 (20 µg/ml) in carbonate buffer (pH 9.6) overnight at 4°C. After saturation with 1% BSA in PBS/0.05% Tween 20 for 6 h at room temperature, samples were incubated overnight at 4°C. Non-digested Bet v 1 was used to generate a standard curve. After washing, sera from birch pollen-allergic patients containing Bet v 1-specific IgE were incubated for 1 h at 37°C and 1 h at 4°C. Bound IgE antibodies were detected with alkaline phosphatase-labelled anti-human IgE Ab (BD Biosciences). All samples were tested in duplicate at various dilutions.

### Allergen-specific T-cell cultures

Allergen-specific T-cell lines (TCL) were expanded from PBMC of allergic patients as described<sup>34</sup>. Cryopreserved HLA-DR-restricted Bet v 1-specific T-cell clones (TCC) with known epitope specificity were thawed and expanded. T-cells ( $2-5 \times 10^4$ ) were stimulated with purified irradiated (60 Gray) autologous neutrophils or monocytes ( $5 \times 10^4$ ), cultured in the absence or presence of Bet v 1 (5 µg/ml), birch pollen extract (20 µg/ml) or Hev b 3 (5 µg/ml) in GM-CSF/IFN- $\gamma$ /IL-3 for 24 h. All experiments were performed in duplicate. After 48 h supernatants were assessed for cytokine levels by bead array using the Luminex System 100 (Luminex, Austin, TX, USA). After 48 h proliferative responses were determined by <sup>3</sup>H-thymidine incorporation assay.

### Allergic LPR and suction blisters

Grass pollen extract (ALK-ABELLO) was injected intradermally into the left volar forearm of grass pollen-allergic individuals according to the manufacturer's instructions. The diluent was administered into the right volar forearm as negative control. All individuals developed immediate reactions within 15 min. Provided the individuals developed visible cutaneous LPR, skin suction blisters were formed on top of the injection sites after 20-24 h by using a low pressure instrument from Electronic Diversities (Ridge Road, Finksburg, MD, USA) by mounting 2 sterile, 5-hole (5 mm diameter per hole) skin suction plates. Low pressure (150–200 mmHg) was applied over a time period of 2 h, and adjusted according to blister size to prevent blood vessel trauma through excessive mechanical stress. Fluids from intact and hemoglobin-free blisters were collected using a Micro-Fine syringe (BD Biosciences), pooled, centrifuged at 1000 x g for 5 min at 4°C, and analyzed by flow cytometry. The total numbers of neutrophils detected in the blister fluids after injection of allergen ranged from 282-2646. No neutrophils were detected in blister fluids after injection of the diluent.

### Statistics

Data were analyzed using IBM SPSS 20.0 (SPSS, Chicago, IL, USA). Statistical differences were determined by the Wilcoxon signed ranks test and considered statistically significant for  $P < 0.05$ .

## Results

### Neutrophils from allergic donors acquire features of APC upon stimulation with GM-CSF, IFN- $\gamma$ , and IL-3

Neutrophils from six allergic individuals were incubated with either titrated concentrations of GM-CSF, IFN- $\gamma$ , or IL-3, or their combination<sup>28</sup> for up to 48 h and HLA-DR expression was assessed subsequently (see Figure E1A in the Online Repository). Similar to what has been reported for neutrophils from non-allergic individuals<sup>27</sup>, GM-CSF was the most potent HLA class II stimulus, followed by IFN- $\gamma$  and IL-3. All three cytokines in combination induced highest percentages of HLA-DR<sup>+</sup> neutrophils when compared to medium control (see Figure E1B in the Online Repository). As all three cytokines are present in the microenvironment of allergic LPR we used the cytokine mix for further *in vitro* experiments.

First, we monitored the effect of GM-CSF/IFN- $\gamma$ /IL-3 on the survival of neutrophils from allergic individuals for up to 72 h. The cytokines significantly delayed apoptosis and prolonged neutrophil survival and after 48 h around 50% of the cells were still alive (Fig. 1A). Next, we assessed the expression of HLA class II and key costimulatory molecules on neutrophils and monocytes in the presence of GM-CSF/IFN- $\gamma$ /IL-3 after 3, 6, 12, 24 and 48 h. None of these molecules were detected after 3 and 6 h, respectively (data not shown). After 12 h, activated neutrophils significantly upregulated HLA-DR but neither CD80 nor CD86 (Fig. 1B). The constitutive expression of HLA-DR on monocytes was not affected by GM-CSF/IFN- $\gamma$ /IL-3. However, monocytes significantly upregulated CD80 and slightly downregulated CD86 in the presence of the cytokines (Fig. 1B). The cytokine-induced expression of HLA-DM was detected in lysates from HLA-DR<sup>+</sup> neutrophils and absent in cytokine-stimulated HLA-DR<sup>-</sup> and freshly isolated neutrophils (Fig. 1C). As expected, monocytes showed strong HLA-DM expression.

### Neutrophils internalize and rapidly degrade Bet v 1 to fragments that contain relevant T-cell epitopes of the major birch pollen allergen

To study allergen uptake, neutrophils and monocytes were incubated with GM-CSF/IFN- $\gamma$ /IL-3 and Bet v 1 labelled with pHrodo, a dye that emits fluorescence only when it reaches the acidic endolysosomes. The percentage of pHrodo<sup>+</sup> cells was assessed during a time course of 3-48 h of culture. Both cell types efficiently internalized Bet v 1 (Fig. 2A). Co-staining of HLA-DR revealed that after 24 h more than 60% of HLA-DR<sup>+</sup> neutrophils had internalized Bet v 1 (Fig. 2A and Figure E2 in the Online repository). To evaluate antigen-processing capacity, lysosomal extracts were prepared from either cell type and incubated with Bet v 1. SDS-PAGE analysis revealed that neutrophilic proteases fully degraded Bet v 1 within 6 h whereas monocytic enzymes required more than 24 h for this process (Fig. 2B). The more rapid proteolysis of Bet v 1 by neutrophilic extracts was confirmed by means of capture ELISA. No intact allergen was found after 2 h (Fig. 2C) while 10% of intact Bet v 1 were still detected after 24 h of incubation with monocytic proteases. The Bet v 1-fragments resulting from degradation by either cell type were characterized by tandem mass spectrometry (Fig. 2D). Already after 2 minutes of incubation with neutrophilic proteases, several peptide clusters derived from the N-terminus Bet v 1<sub>1-38</sub>, the central region Bet v 1<sub>39-79</sub> and the C-terminus Bet v 1<sub>103-159</sub> were identified. At the same time point, only N-

terminal fragments located within the region Bet v 1<sub>1-79</sub> were found in samples digested by monocytes. After 1 hour, additional peptides originating from the region Bet v 1<sub>80-103</sub> were detected in neutrophilic samples. A similar fragmentation was achieved by monocytic extracts with a delayed kinetics. Importantly, the peptide clusters matched frequently recognized T-cell-activating regions such as Bet v 1<sub>4-15</sub>, Bet v 1<sub>19-33</sub>, Bet v 1<sub>112-123</sub>, and Bet v 1<sub>142-153</sub> that have been identified in birch pollen-allergic donors<sup>34</sup> (Fig. 2D).

### Neutrophils fully activate allergen-specific effector T-cells

To assess whether neutrophils are actually able to present the processed peptides to specific effector T-cells, we first co-cultured Bet v 1-specific TCL from different birch pollen-allergic individuals with autologous neutrophils or monocytes that had been stimulated with GM-CSF/IFN- $\gamma$ /IL-3 plus allergen. These polyclonal T-cell cultures proliferated in response to activated neutrophils that had been incubated with Bet v 1 but not with the control allergen Hev b 3 (Table 1). Neutrophils also induced T-cell proliferation when incubated with birch pollen extract containing native Bet v 1. Bet v 1-specific TCL also proliferated in response to allergen-pulsed APC, i.e. neutrophils and monocytes that had been washed after stimulation with GM-CSF/IFN- $\gamma$ /IL-3 plus allergen (Table 1). As expected, the proliferative responses were more pronounced when monocytes served as APC.

Bet v 1 contains multiple T-cell epitopes distributed over its entire sequence<sup>34</sup> and thus was also used to test whether neutrophils were able to present peptides derived from all parts of the protein. To this end, we stimulated HLA-DR-restricted Bet v 1-reactive TCC specific for different epitopes spread over the entire amino acid sequence with cytokine-activated neutrophils or monocytes incubated with either Bet v 1 or birch pollen extract. All TCC proliferated in the presence of either cell type irrespective of their epitope recognition (Fig. 3). Again, monocytes were in general more potent APC than neutrophils. The control allergen Hev b 3 did not induce proliferative responses in the tested clones. We also determined the cytokine response of TCC3 and TCC5, both showing a Th2-phenotype with production of high amounts of IL-4, IL-13 and IL-5 and low IFN- $\gamma$ , to stimulation by neutrophils. Both clones released Th2 cytokines comparably to stimulation by Bet v 1-pulsed monocytes (Table 2) indicating their full activation by allergen-pulsed neutrophils. In line with the reduced proliferative response (Fig. 3A), the levels of the individual cytokines produced by TCC3 in response to allergen-pulsed neutrophils were lower than those induced by allergen-pulsed monocytes.

A series of control experiments was performed to prove that activated neutrophils were indeed the responsible APC. First, we confirmed that Bet v 1-specific TCC cultured with Bet v 1 in the absence of APC did not proliferate (see Figure E3A in the Online Repository). Second, we employed autologous eosinophils (CD66b<sup>+</sup>CCR3<sup>+</sup>CD16<sup>-</sup>) as APC, as these cells were detected as the sole contaminating cell type in our 99% pure neutrophil preparations. Eosinophils did not induce T-cell proliferation under the chosen conditions (see Figure E3A in the Online Repository). Third, neutrophils incubated with Bet v 1 in the absence of GM-CSF/IFN- $\gamma$ /IL-3 failed to induce T-cell-proliferation (see Figure E3B in the Online Repository). Finally, the addition of chloroquine, an inhibitor of acidification of endosomal/lysosomal compartments and antigen-processing, to cytokine-stimulated neutrophils during

the priming with Bet v 1 prevented T-cell proliferation (see Figure E3B in the Online Repository).

### HLA-DR-positive neutrophils are present in cutaneous allergic LPR

To assess whether HLA-DR-positive neutrophils are present in allergic LPR *in vivo*, we employed suction blister technology<sup>39</sup>. To achieve a reliable induction of LPR we intradermally injected extract from grass pollen because it contains more major allergens than birch pollen. Suction blisters were induced at the initial intradermal injection sites after 20-24 h. While blister fluids at sites of diluent-injection contained T lymphocytes but neither granulocytes nor monocytes, blisters at sites of allergen-injection contained CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, monocytes, eosinophils, and neutrophils, but were devoid of B cells (Fig. 4). We analyzed the neutrophils *ex vivo* for HLA-DR expression. In five independent experiments we detected 11-25% HLA-DR-positive neutrophils in the blister fluids from allergen-induced LPR (Fig. 4). In addition, neutrophils from two individuals were isolated from peripheral blood collected at the day of suction blistering. As expected, freshly isolated blood neutrophils were HLA-DR-negative (data not shown). However, when these neutrophils were incubated with GM-CSF/IFN- $\gamma$ /IL-3 for 24 h, similar percentages of HLA-DR-positive neutrophils to those in suction blister fluids were detected (Fig. 4). Neutrophils cultured in medium alone remained HLA-DR-negative.

### Discussion

Large numbers of neutrophils infiltrate the airways and skin during allergen-induced LPR<sup>5, 18, 32, 40, 41</sup>. In addition to this recruitment which is mainly mediated by IgE-activated mast cells<sup>3</sup>, certain pollen species have been reported to attract neutrophils to the airways in a toll-like receptor<sup>4</sup>, myeloid differentiation protein-2, and chemokine (C-X-C motif) receptor (CXCR) 2-dependent manner<sup>42</sup>. Recruited neutrophils are generally considered to contribute to the allergic inflammation by producing compounds with pathogenic potential, e.g. reactive oxygen species, elastase, leukotrienes, and cytokines (reviewed in<sup>15</sup> and<sup>17</sup>). Herein, we propose a novel role for neutrophils in allergic inflammation: in the local cytokine environment they convert into functional APC and activate allergen-specific effector CD4<sup>+</sup> T-cells.

Evidence for the upregulation of HLA class II molecules on human neutrophils in the presence of GM-CSF, IFN- $\gamma$  and IL-3 *in vitro* and *in vivo* had already been indicated in the past (reviewed in<sup>43</sup>). We confirm that also neutrophils from allergic donors show prolonged survival, suppressed apoptosis and HLA-DR expression in the presence of these cytokines. Beyond that, we demonstrate that these neutrophils express HLA-DM, an intracellular molecular chaperone that orchestrates the loading of peptides into HLA class II molecules. As the capacity to fully degrade antigens and properly load antigenic peptides onto HLA II molecules is essential for antigen presentation, we addressed these features by a sophisticated experimental approach involving highly purified neutrophils, a clinically relevant and immunologically well-characterized allergen, and allergen-specific HLA-DR-restricted TCC with defined epitope reactivity. Neutrophils efficiently internalized and fully cleaved the major birch pollen allergen. The resulting proteolytic fragments encompassed its



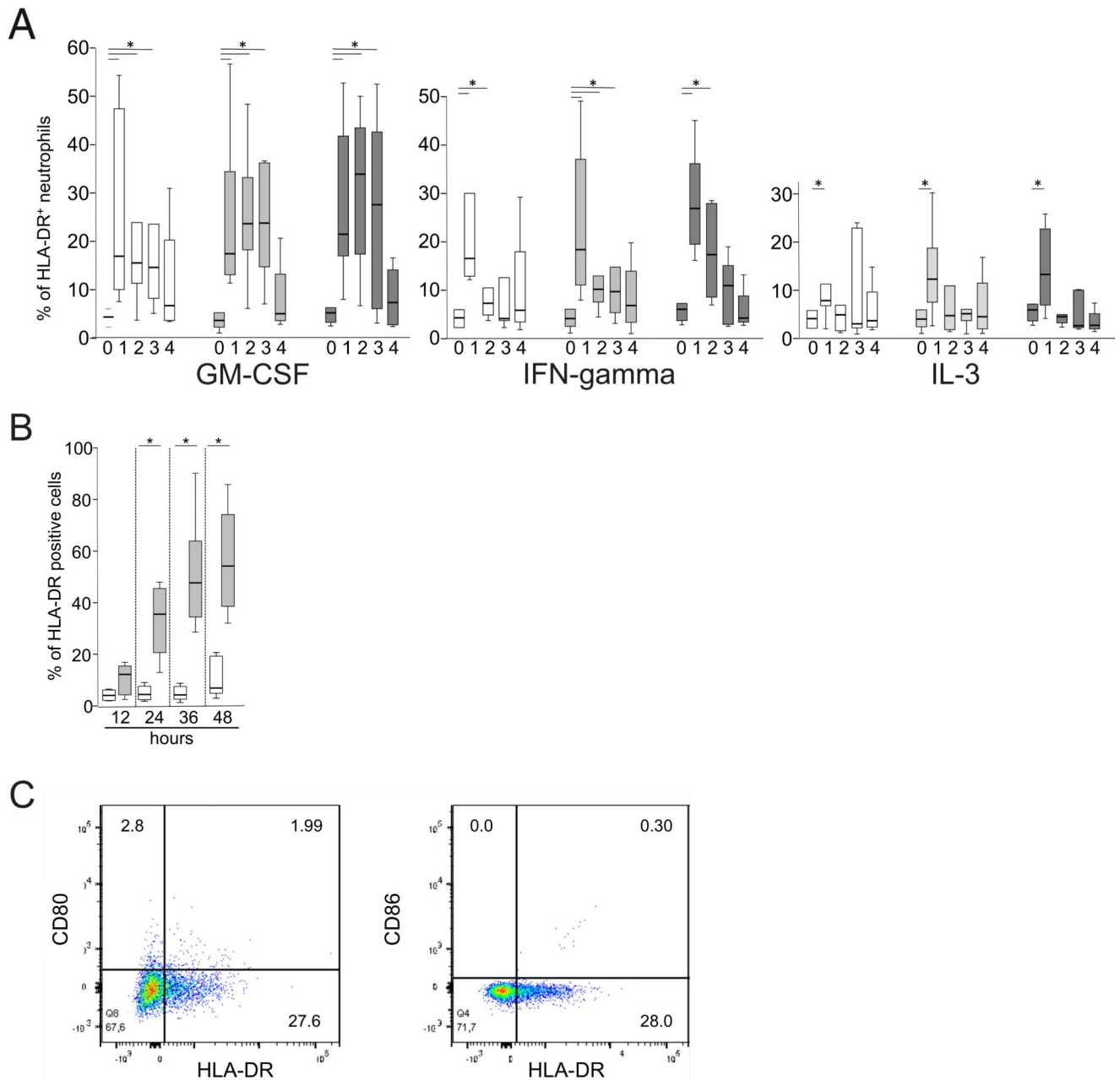
relevant T-cell-activating regions. Proper loading and presentation of the peptides was confirmed in T-cell-activation assays. Notably, T-cells specific for epitopes spreading over the entire amino acid sequence of Bet v 1 and restricted by different HLA-DR alleles responded to allergen-loaded neutrophils. These results strongly indicate that neutrophils show no limitation regarding antigen-processing and subsequent T-cell activation. Collectively, our *in vitro* findings provide strong evidence for the genuine antigen-presenting capacity of neutrophils in the cytokine environment of allergic LPR. This evidence was further supported by the *in vivo* identification of HLA-DR-positive neutrophils in cutaneous allergic LPR.

The comparison of endolysosomal processing by neutrophils and monocytes revealed no major differences in the quality of the peptides of Bet v 1. However, remarkable differences in the kinetics of antigen proteolysis by both types of phagocytes became evident. The rapid cleavage of Bet v 1 by neutrophils resembled the degradation behaviour described for macrophages<sup>44</sup>. Hev b 3 was also rapidly degraded (data not shown). In contrast, significant amounts of Bet v 1 remained intact during endolysosomal proteolysis by monocytes and resembled the processing behaviour of DC<sup>35</sup>. The slow degradation of internalized antigens has been considered to guarantee that DC can still generate and present proteolytic fragments to naïve T-cells after migration to the lymph nodes<sup>44</sup>. Thus, efficient priming of naïve T-cells may depend on the prevention of premature intracellular antigen degradation. This ability would not apply to neutrophils which rapidly fully digest internalized antigens, even though they have been shown to transport antigen to lymphatic compartments<sup>26, 45</sup>. Furthermore, even upon optimum stimulation with GM-CSF, IFN- $\gamma$  and IL-3, neutrophils did not express high levels of CD80 and CD86 which are relevant for full activation of naïve T lymphocytes. In support of these results, Vono *et al* demonstrated that human neutrophils failed to activate allogeneic naïve T-cells<sup>26</sup>. In contrast to naïve T cells, previously activated T cells also respond to APC lacking costimulatory molecules, although less strongly<sup>46</sup>. Together, we conclude that human neutrophils upon appropriate stimulation are highly efficient in collecting antigens and subsequent activation of effector and memory CD4<sup>+</sup> T-cells that do not require high expression of HLA class II and costimulatory molecules.

In summary, we propose a compelling new role for neutrophils in the pathophysiology of IgE-mediated allergy. Upon natural exposure or injection of allergens during allergen-specific immunotherapy, activated mast cells induce an influx of leukocytes with neutrophil infiltration as relatively early event<sup>8</sup>. The local cytokine microenvironment prolongs their lifespan and fosters them to internalize, process and present allergen to specific CD4<sup>+</sup> effector T-cells. Activated T-cells then execute various effector mechanisms, for example release IL-5 which recruits and activates eosinophils, that further contribute to the allergic inflammation<sup>5</sup>. Indeed, eosinophil infiltration of asthmatic lungs after allergen challenge has been reported to be delayed compared to the accumulation of CD4<sup>+</sup> T-cells and neutrophils<sup>8</sup>. Elevated expression of HLA-DR in cutaneous cellular infiltrates at sites of allergen-induced LPR has been described<sup>47</sup>. Here, we show that HLA-DR-positive neutrophils are present at such inflammatory sites as well as CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, monocytes, and eosinophils. These results match the cell populations that have been detected in biopsies of late local skin reactions obtained 24 h after subcutaneous injections of allergen vaccines for specific immunotherapy<sup>48</sup>. In contrast to human basophils<sup>49</sup> and eosinophils

(see Figure E3B in the Online Repository) neutrophils are able to present allergens and activate T-cells. Hence, it is tempting to speculate that neutrophils act as magnifiers of the local allergic T-cell-mediated inflammation following natural exposure or vaccination during allergen-specific immunotherapy.

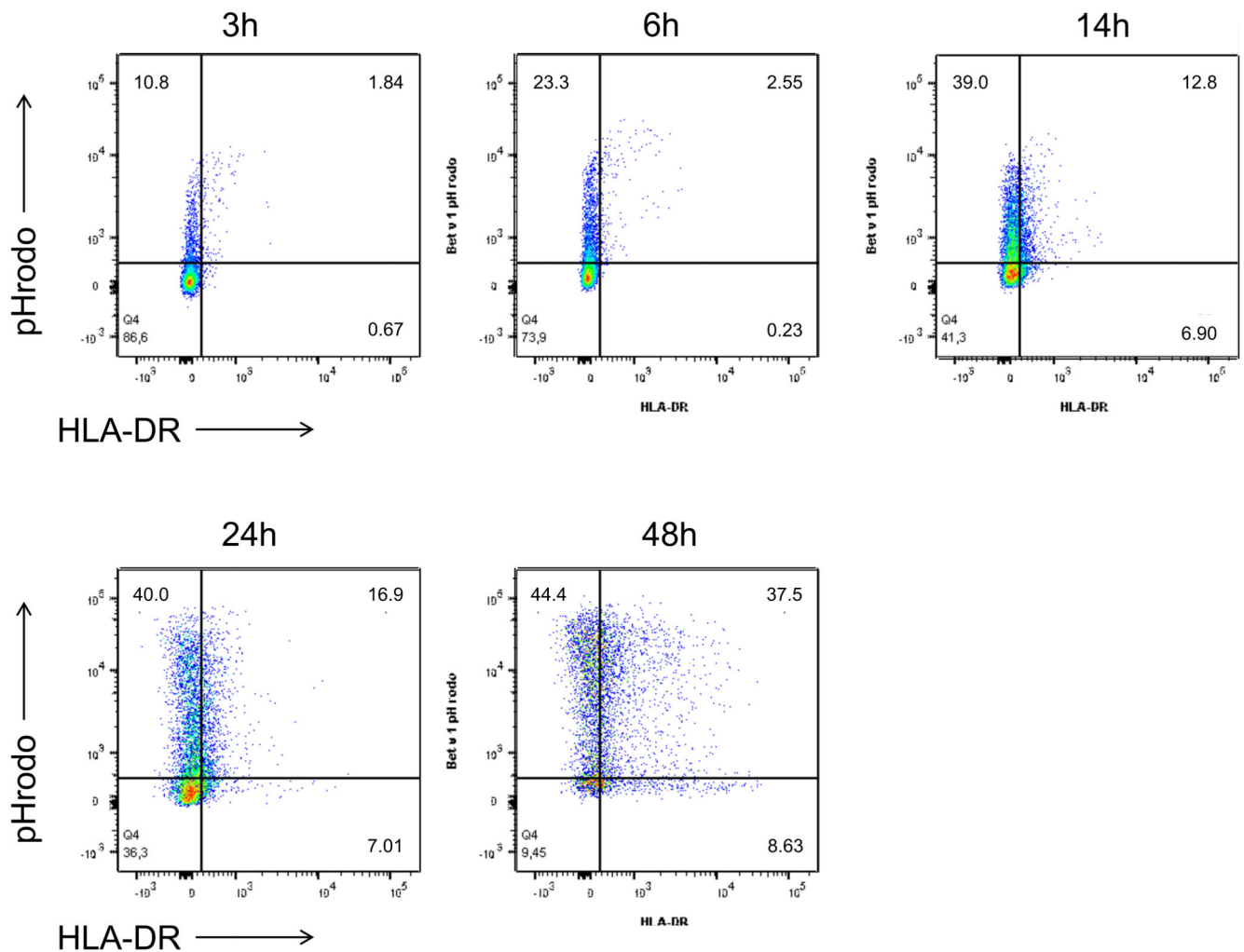
## Extended Data



**Figure E1. Expression of HLA-DR on cytokine-activated neutrophils.**

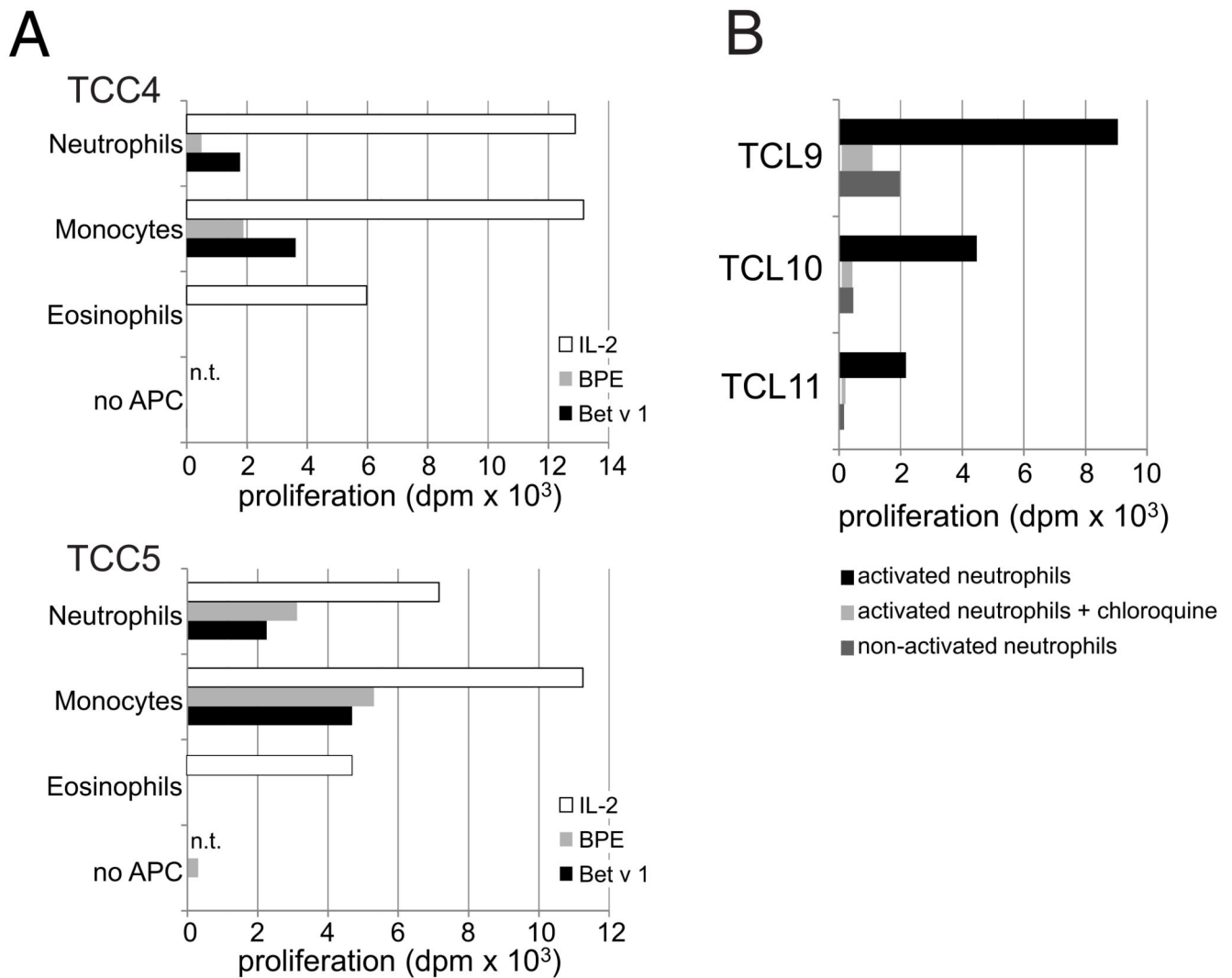
(A) Purified neutrophils from six birch pollen-allergic individuals were cultured in medium alone (0) or stimulated with titrated concentrations 10(1), 1(2), 0.1(3), and 0.01(4) ng/ml of GM-CSF, IFN- $\gamma$ , and IL-3. The percentage of HLA-DR-positive cells was assessed by flow cytometry at 24 (white), 36 (light gray) and 48 h (dark gray box plots); (B) Neutrophils were left in medium alone (white) or stimulated with a cocktail of GM-CSF (100 pg/ml), IFN- $\gamma$  (10 ng/ml) and IL-3 (30 ng/ml, gray) according to published protocols<sup>1</sup>. The percentage of HLA-DR-positive cells was assessed at indicated time points; (C) The percentage of HLA-

DR-positive cells expressing CD80 and CD86 at 24 h, the dot blot shows one representative experiment. \* $P < 0.05$ , Wilcoxon Signed Ranks test;



**Figure E2. HLA-DR expression of Bet v 1-positive neutrophils.**

Purified neutrophils from birch pollen-allergic individuals were incubated with pHrodo-labelled Bet v 1 in a cocktail of GM-CSF (100 pg/ml), IFN- $\gamma$  (10 ng/ml) and IL-3 (30 ng/ml, gray). At the indicated time points the percentage of HLA-DR<sup>+</sup> neutrophils that had internalized Bet v 1 was assessed using flow cytometry; the dot blot shows one representative experiment of six.



**Figure E3. Allergen-induced proliferation of Bet v 1-specific T cells in the presence of different cell types.**

(A) Autologous neutrophils ( $CD66b^+CD16^+CCR3^-$ ) and eosinophils ( $CD66b^+CCR3^+CD16^-$ ) were sorted from peripheral blood cells by FACS Aria III (BD Biosciences). Monocytes were isolated by using CD14 MicroBeads (Miltenyi Biotec). All three cell types (purity >99%) were incubated with GM-CSF/IFN- $\gamma$ /IL-3 plus/minus Bet v 1 (5  $\mu$ g/ml), birch pollen extract (20  $\mu$ g/ml), or IL-2 (2 U/ml) for Online Repository: Polak et al 3 24 h, irradiated (60 Gray) and co-cultured with two Bet v 1-specific T cell clones (TCC); (B) neutrophils (purity >99%) were incubated without (non-activated) or with GM-CSF/IFN- $\gamma$ /IL-3 plus/minus Bet v 1 (5  $\mu$ g/ml) in the presence or absence of chloroquine diphosphate salt (100  $\mu$ g/ml, Sigma Aldrich, Darmstadt, Germany), washed, irradiated (60 Gray) and added to three different Bet v 1-specific T cell lines (TCL). In all experiments proliferation was assessed after 48 h, dpm = cpm in response to stimulus - cpm in medium controls; n.t., not tested.

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## Abbreviations

<b>aa</b>	amino acid
<b>Ab</b>	antibody
<b>APC</b>	antigen-presenting cell
<b>BPE</b>	birch pollen extract
<b>cpm</b>	counts per minute
<b>DC</b>	dendritic cell
<b>dpm</b>	delta-cpm
<b>GM-CSF</b>	granulocyte macrophage colony-stimulating factor
<b>h</b>	hour
<b>IL</b>	interleukin
<b>LAR</b>	late asthmatic reaction
<b>LPR</b>	late-phase response
<b>PBMC</b>	peripheral blood mononuclear cells
<b>TCC</b>	T-cell clone
<b>TCL</b>	T-cell line
<b>TCR</b>	T-cell receptor
<b>Th</b>	T helper
<b>TNF</b>	tumor necrosis factor

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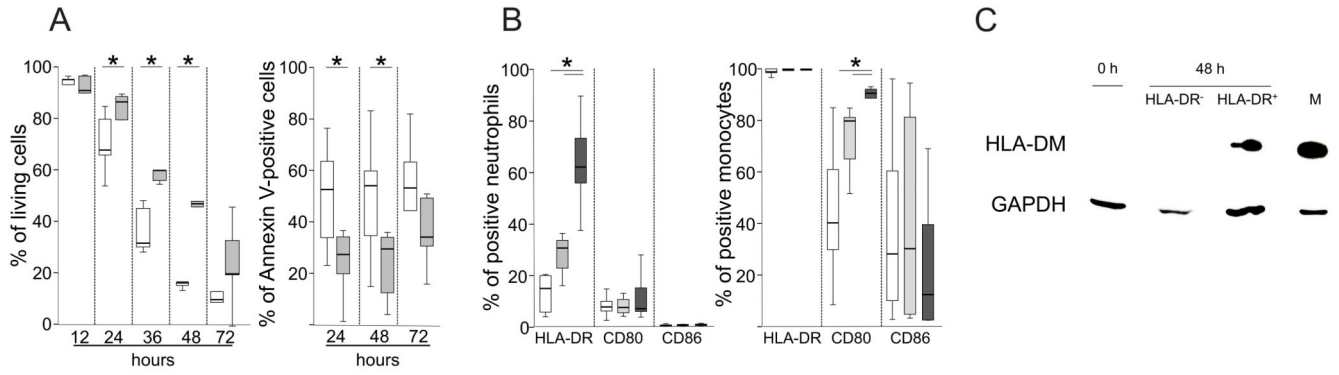
1. Smith WB, Guida L, Sun Q, Korpelainen EI, van den Heuvel C, Gillis D, et al. Neutrophils activated by granulocyte-macrophage colony-stimulating factor express receptors for interleukin-3 which mediate class II expression. *Blood.* 1995; 86:3938–44. [PubMed: 7579364]

### Key messages

- Cytokine-stimulated neutrophils activate allergen-specific T-cells to proliferate and synthesize cytokines
- HLA-DR-positive neutrophils are found in cutaneous allergic late-phase reactions *in vivo*

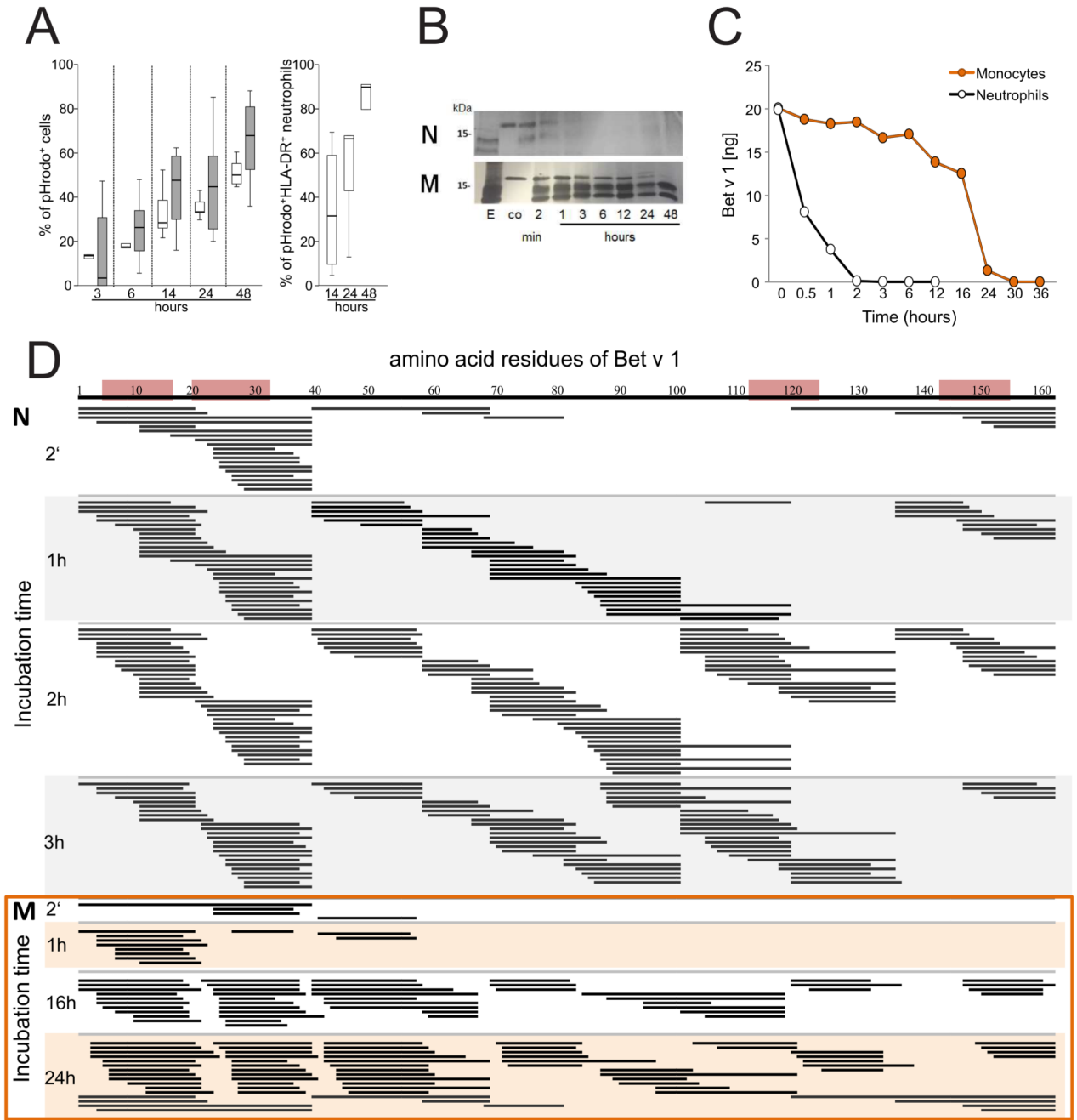
**Capsule summary**

Neutrophils may serve as antigen-presenting cells in the allergic late-phase reaction.



**Figure 1. Survival and key antigen-presentation molecules of activated neutrophils from allergic individuals.**

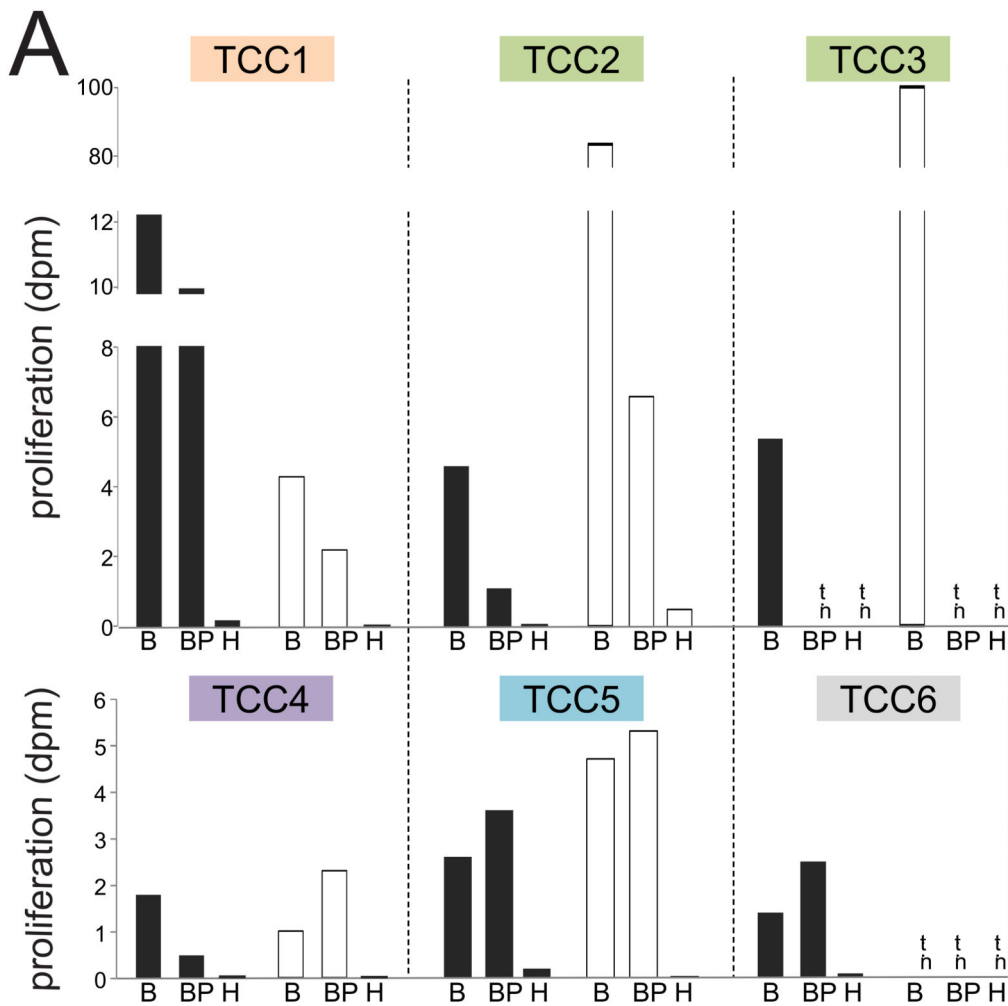
(A) Neutrophils from six birch pollen-allergic subjects were stimulated with GM-CSF/IFN- $\gamma$ /IL-3 (gray) or cultured in medium alone (white box plots). Vital and Annexin-V-positive cells were assessed at the indicated time points by flow cytometry; (B) Neutrophils or monocytes from seven birch pollen-allergic subjects were stimulated with GM-CSF/IFN- $\gamma$ /IL-3 and the expression of HLA-DR, CD80 and CD86 was assessed after 14 (white), 24 (light gray) and 48 h (dark gray); \* $P < 0.05$ , Wilcoxon Signed Ranks test; (C) Lysates of freshly isolated neutrophils (0 h), monocytes (M), or neutrophils stimulated with GM-CSF/IFN- $\gamma$ /IL-3 for 48 h sorted into HLA-DR<sup>+</sup> and HLA-DR<sup>-</sup> cells were assessed for HLA-DM and GAPDH by immunoblotting;



**Figure 2. Uptake and degradation of Bet v 1 by neutrophils.**

(A) Neutrophils (white box plots) and monocytes (gray) from six birch pollen-allergic individuals were incubated with pHrodo-labelled Bet v 1 in GM-CSF/IFN- $\gamma$ /IL-3. The percentage of pHrodo<sup>+</sup> cells and pHrodo<sup>+</sup>HLA-DR<sup>+</sup> neutrophils was assessed at the indicated time points using flow cytometry; (B) SDS-PAGE analysis of the degradation of Bet v 1 by lysosomal extracts from neutrophils (N) or monocytes (M), E, lysosomal extract, co: Bet v 1 without lysosomal extracts; (C) degradation of Bet v 1 quantified by capture ELISA; (D) peptides (black lines) generated over time by incubation of Bet v 1 with

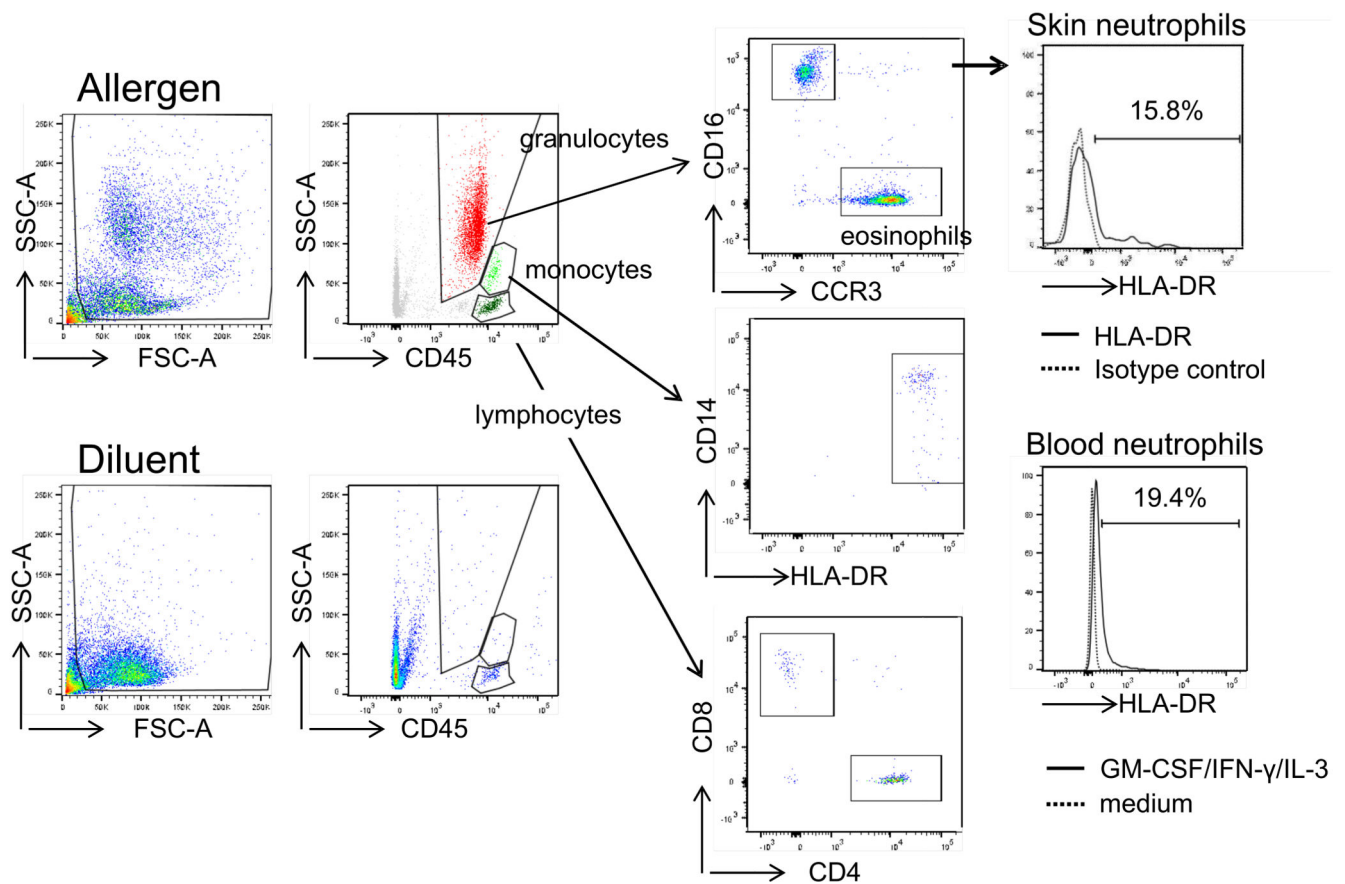
lysosomal extracts from neutrophils (N) or monocytes (M) were characterized by mass spectrometry. Frequently recognized T-cell-activating regions in the amino acid sequence of Bet v 1 on top are highlighted in red. The results in B and C are representative of two and three independent experiments, respectively.



**Figure 3. T-cell proliferation to allergen-pulsed neutrophils and monocytes.**

(A) Proliferation of six T-cell clones (TCC) to rBet v 1 (B), birch pollen extract (BP) or Hev b 3 (H) and either neutrophils (black bars) or monocytes (white bars) as APC; dpm x 10<sup>3</sup> are shown; n.t., not tested; (B) Epitopes recognized by the TCC are highlighted in different colours in the amino acid sequence of Bet v 1.





**Figure 4. Detection of HLA-DR-positive neutrophils in cutaneous allergic LPR.**

Allergen or diluent was injected intradermally and suction blisters were performed at the injection sites after 20–24 h. Fluids were analyzed for T-cells, monocytes, eosinophils and neutrophils by flow cytometry. Skin neutrophils were assessed for HLA-DR expression. One representative of five experiments is shown. In two patients, also blood neutrophils were isolated, cultured in medium or GM-CSF/IFN- $\gamma$ /IL-3 for 24 h and analyzed for HLA-DR expression by flow cytometry.

Table 1

## Proliferative responses of Bet v 1-specific T-cell cultures

APC Stimulus	Neutrophils			Monocytes			HLA-DR phenotype
	Bet v 1	Birch pollen	Hev b 3	Bet v 1	Birch pollen	Hev b 3	
TCL1	13.3 <sup>a</sup>	n.t.	1.8	21.6	n.t.	1.7	DRB1*0301/1401
TCL2	21.6	14.1	n.t.	6.7	4.4	n.t.	DRB1*0101/0301
TCL3	8.8	4.4	<0.01	16.2	14.9	<0.01	DRB1*0301/0801
TCL4	1.2	3.4	0.02	11.6	12.1	<0.01	n.t.
TCL5	3.6	0.1	0.04	17.7	4.7	0.08	DRB1*0701/1501
TCL6	1.2	n.t.	<0.01	9.9	n.t.	<0.01	DRB1*0701/1501
TCL7	1.6	n.t.	n.t.	12.1	n.t.	n.t.	n.t.
TCL8	1.8	n.t.	n.t.	10.7	n.t.	n.t.	DRB1*0701/1501
TCL9	7.2	n.t.	n.t.	33.2	n.t.	n.t.	DRB1*0301/1401

<sup>a</sup>dpm × 10<sup>3</sup> are shown; n.t., not tested; TCL7-9 were stimulated with allergen-pulsed APC

**Table 2**  
**Cytokine response of Bet v 1-specific Th2 clones to Bet v 1-pulsed APC**

TCC 3	APC	Stimulus	Cytokines [pg/ml]												
			IL-4	IL-5	IL-9	IL-10	IL-13	IL-22	IL-17A	IFN- $\gamma$	GM-CSF	TNF- $\alpha$			
	Neutrophils	Medium	62.8	1456	<4.5	0.1	1039	<4.5	<4.5	<4.5	<4.5	231	109	240	
	Neutrophils	Bet v 1	3483	4461	51.3	0.0	2716	<4.5	<4.5	<4.5	319	164	1866		
	Monocytes	Medium	476.2	1830	<4.5	<4.5	1074	<4.5	<4.5	<4.5	<4.5	<4.5	888		
	Monocytes	Bet v 1	16770	8450	395	20.6	23891	<4.5	<4.5	<4.5	726	13059	9644		
<b>TCC 5</b>	Neutrophils	Medium	483	4799	<4.5	5.6	3694	<4.5	<4.5	<4.5	52.4	<4.5	1715		
	Neutrophils	Bet v 1	14415	8796	184	1281	13939	184	39.9	1246	7957	5943			
	Monocytes	Medium	717	4816	<4.5	6.2	3843	<4.5	<4.5	<4.5	50.2	<4.5	369		
	Monocytes	Bet v 1	15073	8816	194	1297	15571	194	37.3	1246	7957	6004			