

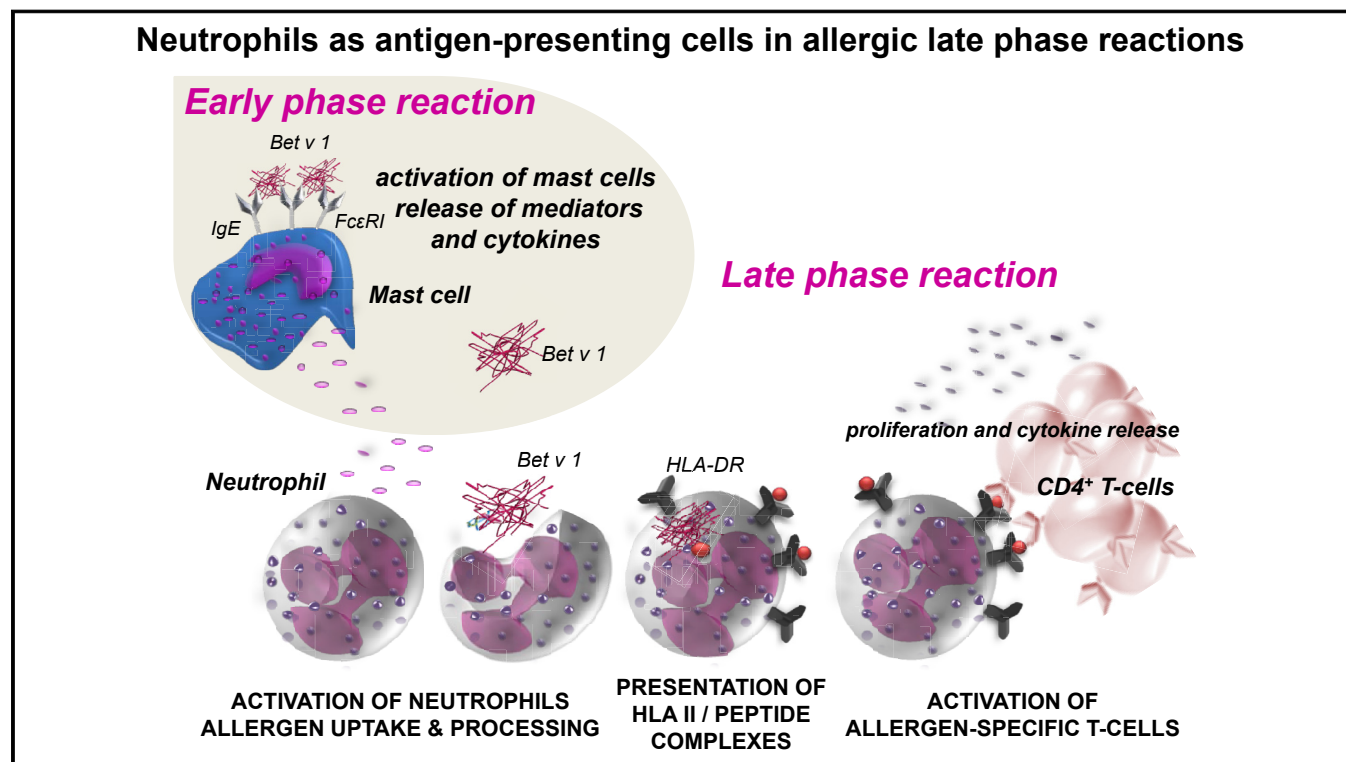
A novel role for neutrophils in IgE-mediated allergy: Evidence for antigen presentation in late-phase reactions



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GRAPHICAL ABSTRACT



Background: Neutrophils and allergen-specific T cells accumulate in patients with allergic late-phase reactions (LPRs). Their presence is associated with severe inflammation. Cytokines, such as GM-CSF, IFN- γ , and IL-3, which are typically found in patients with allergic LPRs, have been

proposed to convert neutrophils into antigen-presenting cells (APCs).

Objective: We sought to assess the antigen-processing and antigen-presenting capacities of neutrophils from allergic patients.

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Methods: Neutrophils were isolated from peripheral blood of donors with birch pollen allergy and stimulated with GM-CSF, IFN- γ , and IL-3. The viability and expression of HLA-DR, CD80, and CD86 were assessed by using flow cytometry. HLA-DM expression was analyzed by means of immunoblotting. Allergen uptake was studied after fluorescence labeling of the major birch pollen allergen Bet v 1. Bet v 1 was digested with neutrophilic endolysosomal extracts, and the resulting fragments were sequenced by using mass spectrometry.

Neutrophils were used as APCs in coculture experiments with autologous HLA-DR-restricted and 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 LPRs induced by using intradermal allergen injection were assessed for HLA-DR⁺ neutrophils by using flow cytometry.

Results: The cytokines significantly enhanced the survival, allergen uptake, and expression of HLA-DM and HLA-DR on 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⁺ neutrophils were detected in patients with cutaneous allergic LPRs.

Conclusion: Neutrophils can serve as APCs for local allergen-specific effector T cells in patients with allergic LPRs. (*J Allergy Clin Immunol* 2019;143:1143-52.)

Key words: Antigen presentation, neutrophils, IgE-mediated allergy, allergic inflammation, allergic late-phase reaction, T cells, T-cell activation

Re-exposure of allergic patients to either the sensitizing allergen or a cross-reactive allergen causes an acute response, which is seen in the skin as a wheal-and-flare reaction or, after inhalation, as rhinitis and airway constriction.^{1,2} This immediate reaction is considered to result from release of histamine and other preformed inflammatory mediators from mast cells on cross-linking of adjacent Fc ϵ R1-bound IgE molecules on their surfaces.³ The late-phase reaction (LPR) follows 3 to 48 hours after allergen challenge, resulting in the skin as swelling and induration, in the nose as continued nasal blockage, and in the lung as a late asthmatic reaction.^{1,2,4} LPRs are accompanied by inflammatory cell infiltration, particularly by eosinophils and T_H2 cells,⁵⁻⁹ and have been used as models of chronic allergic inflammation.^{10,11} The pivotal role of allergen-specific CD4⁺ T cells in patients with LPRs has been underlined by the finding that late asthmatic reactions 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.¹²

Neutrophils are the first innate immune cells that migrate to inflammatory sites and rapidly exert manifold effector functions.¹³ They also accumulate in patients with allergic LPRs,^{9,14} and their presence has been linked to more severe inflammation.¹⁵⁻¹⁷ Bronchoalveolar lavage fluid of patients with allergic LPRs contain larger numbers of neutrophils than that of patients experiencing no LPRs.^{18,19} Furthermore, severe asthma has been associated with increased numbers of neutrophils in sputum, tracheal aspirates, and the bronchial mucosa.²⁰⁻²² Activated

Abbreviations used

APC:	Antigen-presenting cell
DC:	Dendritic cell
LPR:	Late-phase reaction
PE:	Phytoerythrin
PerCP:	Peridinin-chlorophyll-protein complex
TCC:	T-cell clone
TCL:	T-cell line

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 cell types.^{23,24} Additionally, evidence for direct cross-talk of neutrophils with T cells has been provided.²⁵

Recently, it was reported that human neutrophils acquire HLA-DR-dependent antigen-presenting capacity when cocultured with autologous antigen-specific CD4⁺ memory T cells plus antigen.²⁶ Evidence for the antigen-presenting capacity of human neutrophils had been indicated previously, mainly because these professional phagocytes express HLA-DR when exposed to GM-CSF, IFN- γ , and IL-3 both *in vitro*^{27,28} and *in vivo*.^{29,30} These cytokines are released by specific CD4⁺ T cells and sensitized mast cells in response to natural allergen exposure or injection of allergy vaccines.^{3,5,31-33} This tempted us to speculate that the cytokine milieu in patients with allergic LPRs can convert neutrophils into antigen-presenting cells (APCs).

To address this hypothesis, we stimulated neutrophils from allergic patients with a combination of GM-CSF, IFN- γ , 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 using the major birch pollen allergen Bet v 1 as a model protein. This clinically highly relevant allergen has been characterized thoroughly regarding its T-cell reactivity.³⁴⁻³⁶ Neutrophils were compared with monocytes, which represent another type of phagocyte and are professional APCs. Finally, we explored whether HLA-DR⁺ neutrophils are actually found in the skin of allergic patients with allergen-induced LPRs.

METHODS

Allergic patients

Patients with birch pollen allergy had rhinoconjunctivitis in the spring, positive skin prick test responses to birch pollen extract (ALK-Abelló, Hørsholm, Denmark), and birch pollen-specific IgE levels of greater than 0.35 kU_A/L (ImmunoCAP; Thermo Fisher Scientific, Uppsala, Sweden). Patients with grass pollen allergy had rhinoconjunctivitis in summer, positive skin prick test responses to grass pollen extract (ALK-Abelló), and grass pollen-specific IgE levels of greater than 0.35 kU_A/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 provided 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, Calif), according to the manufacturer's instructions. Birch pollen

extract was prepared, as previously described.³⁷ Grass pollen extract for intradermal administration was purchased from ALK-Abelló.

Cell preparations

Neutrophils were isolated, as previously described.³⁸ 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, Karlsruhe, Germany), red cell osmotic lysis of the granulocyte pellet, and a final immunomagnetic negative selection step using the human MACSxpress Neutrophil Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), which resulted repeatedly in greater than 99% pure CD16⁺CD66b⁺CCR3⁺HLA-DR⁺CD19⁺CD3⁺CD14⁺ cells. Neutrophils (1×10^6 /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- γ (10 ng/mL), and IL-3 (30 ng/mL; all from PeproTech, Rocky Hill, NJ), as previously described.²⁸ Monocytes (>98% CD14⁺CCR3⁺CD19⁺CD3⁺) were obtained by using CD14 MicroBeads (Miltenyi Biotec), according to the manufacturer's instructions.

Flow cytometry

Analyses were performed with a FACSCanto II by using FACSDiva (BD Biosciences, San Jose, Calif) and FlowJo (TreeStar, Ashland, Ore) software. The following anti-human antibodies were used: HLA-DR–Brilliant Violet 421, HLA-DR–allophycocyanin (clone L243), CD80–Alexa Fluor 647, CD3–allophycocyanin-Cy7, CD16–phycoerythrin (PE; clone 3G8), CD15–PE-Cy7, CD15–peridinin-chlorophyll-protein complex (PerCP)–Cy5, CCR3–allophycocyanin, and PerCP-labeled CD14, CD19, CD56, CD123, and CCR3 (all from BioLegend, San Diego, Calif), CD4-PE and CD86-PE, CD45–fluorescein isothiocyanate (all from BD Biosciences), and CD3-PerCP and CD8-PerCP (both from eBioscience, San Diego, Calif). Viability was determined by using the Fixable Viability Dye eFluor 780 (eBioscience). Annexin V–fluorescein isothiocyanate (BD PharMingen, San Jose, Calif) was used to determine cells undergoing apoptosis. For internalization experiments, cells (1×10^6 /mL) were incubated with and without pHrodo-labeled Bet v 1 (1.5 μ g/mL) in the presence of GM-CSF/IFN- γ /IL-3.

Detection of HLA-DM

Cell lysates were prepared from neutrophils and monocytes (each 10×10^6), as previously described,³⁵ immediately after their isolation and from HLA-DR⁺ (3×10^6) and HLA-DR⁺ (1.8×10^6) neutrophils sorted by using a FACSAria III (BD Biosciences) after stimulation with GM-CSF/IFN- γ /IL-3 for 48 hours. Cell lysates (30 μ L) were separated by using 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 mouse anti-HLA-DM mAb (Novus Biological, Abingdon, United Kingdom) and a horseradish peroxidase–conjugated anti-mouse IgG (CST, Danvers, Mass). As a loading control, glyceraldehyde-3-phosphate dehydrogenase was detected with a rabbit anti–glyceraldehyde-3-phosphate dehydrogenase (Sigma, St Louis, Mo) and a horseradish peroxidase–conjugated ECL anti-rabbit IgG (Amersham, Buckinghamshire, United Kingdom). After development with the ECL Prime Western blotting detection reagent, the nitrocellulose was exposed to a high-performance autoradiography film (both from GE Healthcare, Buckinghamshire, United Kingdom).

Endocytosis and endolysosomal degradation assays of Bet v 1

Endolysosomal microsomes were isolated from neutrophils and monocytes, as previously described.³⁶ Microsomes (2 μ g) were incubated with Bet v 1 (2 μ g) at 37°C in a final volume of 20 μ L containing 100 mmol/L citrate buffer (pH 4.8) and 2 mmol/L dithiothreitol. At indicated time points, samples were collected, denatured for 5 minutes at 95°C, loaded onto a 15% SDS gel, and visualized by using Coomassie staining. Remaining peptides were

analyzed by using an ESI-QTOF mass spectrometer fitted with a capillary reversed-phase HPLC (Waters, Milford, Mass).

The quantity of intact Bet v 1 was determined by using an in-house capture ELISA. Briefly, microtiter plates (Maxisorp, Nunc, Denmark) were coated with the Bet v 1–specific mAb BIP1 (20 μ g/mL) in carbonate buffer (pH 9.6) overnight at 4°C. After saturation with 1% BSA in PBS/0.05% Tween 20 for 6 hours at room temperature, samples were incubated overnight at 4°C. Nondigested Bet v 1 was used to generate a standard curve. After washing, sera from patients with birch pollen allergy containing Bet v 1–specific IgE were incubated for 1 hour at 37°C and 1 hour at 4°C. Bound IgE antibodies were detected with alkaline phosphatase–labeled anti-human IgE antibody (BD Biosciences). All samples were tested in duplicates at various dilutions.

Allergen-specific T-cell cultures

Allergen-specific T-cell lines (TCLs) were expanded from PBMCs of allergic patients, as previously described.³⁴ Cryopreserved HLA-DR–restricted Bet v 1–specific T-cell clones (TCC) with known epitope specificity were thawed and expanded. T cells (2.5×10^4) were stimulated with purified irradiated (60 Gr) autologous neutrophils or monocytes (5×10^4) cultured in the absence or presence of Bet v 1 (5 μ g/mL), birch pollen extract (20 μ g/mL), or Hev b 3 (5 μ g/mL) in GM-CSF/IFN- γ /IL-3 for 24 hours. All experiments were performed in duplicates. After 48 hours, supernatants were assessed for cytokine levels by using a bead array with the Luminex System 100 (Luminex, Austin, Tex). After 48 hours, proliferative responses were determined by using the tritiated thymidine incorporation assay.

Allergic LPRs and suction blisters

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

Statistics

Data were analyzed by using IBM SPSS 20.0 software (SPSS, Chicago, Ill). Statistical differences were determined by using the Wilcoxon signed-ranks test and considered statistically significant at a *P* value of less than .05.

RESULTS

Neutrophils from allergic donors acquire features of APCs on stimulation with GM-CSF, IFN- γ , and IL-3

Neutrophils from 6 allergic patients were incubated with either titrated concentrations of GM-CSF, IFN- γ , or IL-3 or their combination²⁸ for up to 48 hours, and HLA-DR expression was assessed subsequently (see Fig E1, A, in this article's Online Repository at www.jacionline.org). Similar to what has been reported for neutrophils from nonallergic subjects,²⁷ GM-CSF was the most potent HLA class II stimulus, followed by IFN- γ and IL-3. All 3 cytokines in combination induced the highest

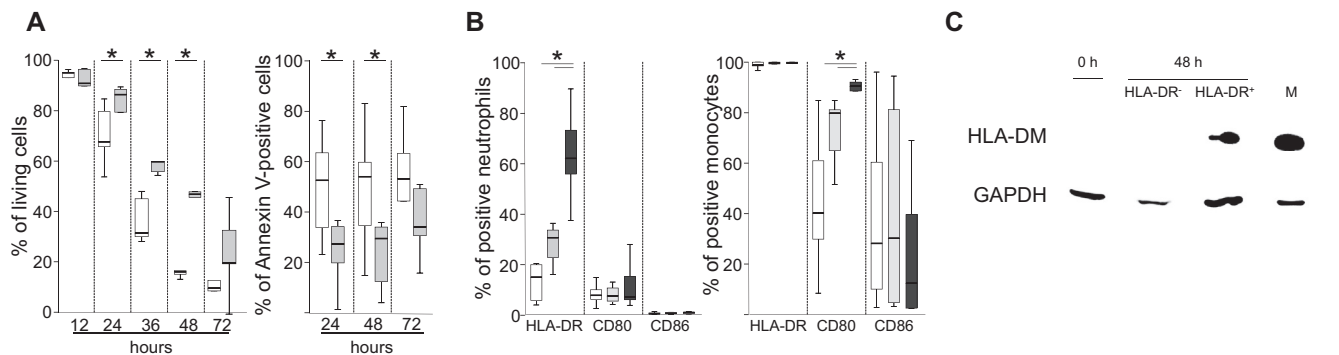


FIG 1. Survival and key antigen-presenting molecules of activated neutrophils from allergic patients. **A**, Neutrophils from 6 patients with birch pollen allergy were stimulated with GM-CSF/IFN- γ /IL-3 (gray box plots) or cultured in medium alone (white box plots). Vital and Annexin V⁺ cells were assessed at the indicated time points by using flow cytometry. **B**, Neutrophils or monocytes from 7 patients with birch pollen allergy were stimulated with GM-CSF/IFN- γ /IL-3, and expression of HLA-DR, CD80, and CD86 was assessed after 14 (white box plots), 24 (light gray box plots), and 48 (dark gray box plots) hours. * $P < .05$, Wilcoxon signed-rank test. **C**, Lysates of freshly isolated neutrophils (0 h), monocytes (M), or neutrophils stimulated with GM-CSF/IFN- γ /IL-3 for 48 hours sorted into HLA-DR⁺ and HLA-DR⁻ cells were assessed for HLA-DM and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by using immunoblotting.

percentages of HLA-DR⁺ neutrophils when compared with medium controls (see Fig E1, B). Because all 3 cytokines are present in the microenvironment of patients with allergic LPRs, we used the cytokine mix for further *in vitro* experiments.

First, we monitored the effect of GM-CSF/IFN- γ /IL-3 on neutrophil survival from allergic patients for up to 72 hours. The cytokines significantly delayed apoptosis and prolonged neutrophil survival, and after 48 hours, around 50% of the cells were still alive (Fig 1, A). Next, we assessed the expression of HLA class II and key costimulatory molecules on neutrophils and monocytes in the presence of GM-CSF/IFN- γ /IL-3 after 3, 6, 12, 24, and 48 hours. None of these molecules were detected after 3 and 6 hours, respectively (data not shown). After 12 hours, activated neutrophils significantly upregulated HLA-DR but neither CD80 nor CD86 (Fig 1, B). Constitutive expression of HLA-DR on monocytes was not affected by GM-CSF/IFN- γ /IL-3. However, monocytes significantly upregulated CD80 and slightly downregulated CD86 in the presence of the cytokines (Fig 1, B). Cytokine-induced expression of HLA-DM was detected in lysates from HLA-DR⁺ neutrophils and absent in cytokine-stimulated HLA-DR⁻ and freshly isolated neutrophils (Fig 1, C). 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- γ /IL-3 and Bet v 1 labeled with pHrodo, a dye that emits fluorescence only when it reaches the acidic endolysosomes. The percentage of pHrodo⁺ cells was assessed during a time course of 3 to 48 hours of culture. Both cell types efficiently internalized Bet v 1 (Fig 2, A). Costaining of HLA-DR revealed that after 24 hours, more than 60% of HLA-DR⁺ neutrophils had internalized Bet v 1 (Fig 2, A, and see Fig E2 in this article's Online Repository at www.jacionline.org).

Lysosomal extracts were prepared from either cell type and incubated with Bet v 1 to evaluate antigen-processing capacity. SDS-PAGE analysis revealed that neutrophilic proteases fully degraded Bet v 1 within 6 hours, whereas monocytic enzymes required more than 24 hours for this process (Fig 2, B). 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 hours (Fig 2, C), whereas 10% of intact Bet v 1 was still detected after 24 hours of incubation with monocytic proteases. The Bet v 1 fragments resulting from degradation by either cell type were characterized by using tandem mass spectrometry (Fig 2, D). After only 2 minutes of incubation with neutrophilic proteases, several peptide clusters derived from the N-terminus Bet v 1₁₋₃₈, the central region Bet v 1₃₉₋₇₉, and the C-terminus Bet v 1₁₀₃₋₁₅₉ were identified. At the same time point, only N-terminal fragments located within the region Bet v 1₁₋₇₉ were found in samples digested by monocytes. After 1 hour, additional peptides originating from the region Bet v 1₈₀₋₁₀₃ 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 1₄₋₁₅, Bet v 1₁₉₋₃₃, Bet v 1₁₁₂₋₁₂₃, and Bet v 1₁₄₂₋₁₅₃, which have been identified in donors with birch pollen allergy (Fig 2, D).³⁴

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 cocultured Bet v 1-specific TCLs from different patients with birch pollen allergy with autologous neutrophils or monocytes that had been stimulated with GM-CSF/IFN- γ /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 I). Neutrophils also induced T-cell proliferation when incubated with birch pollen extract

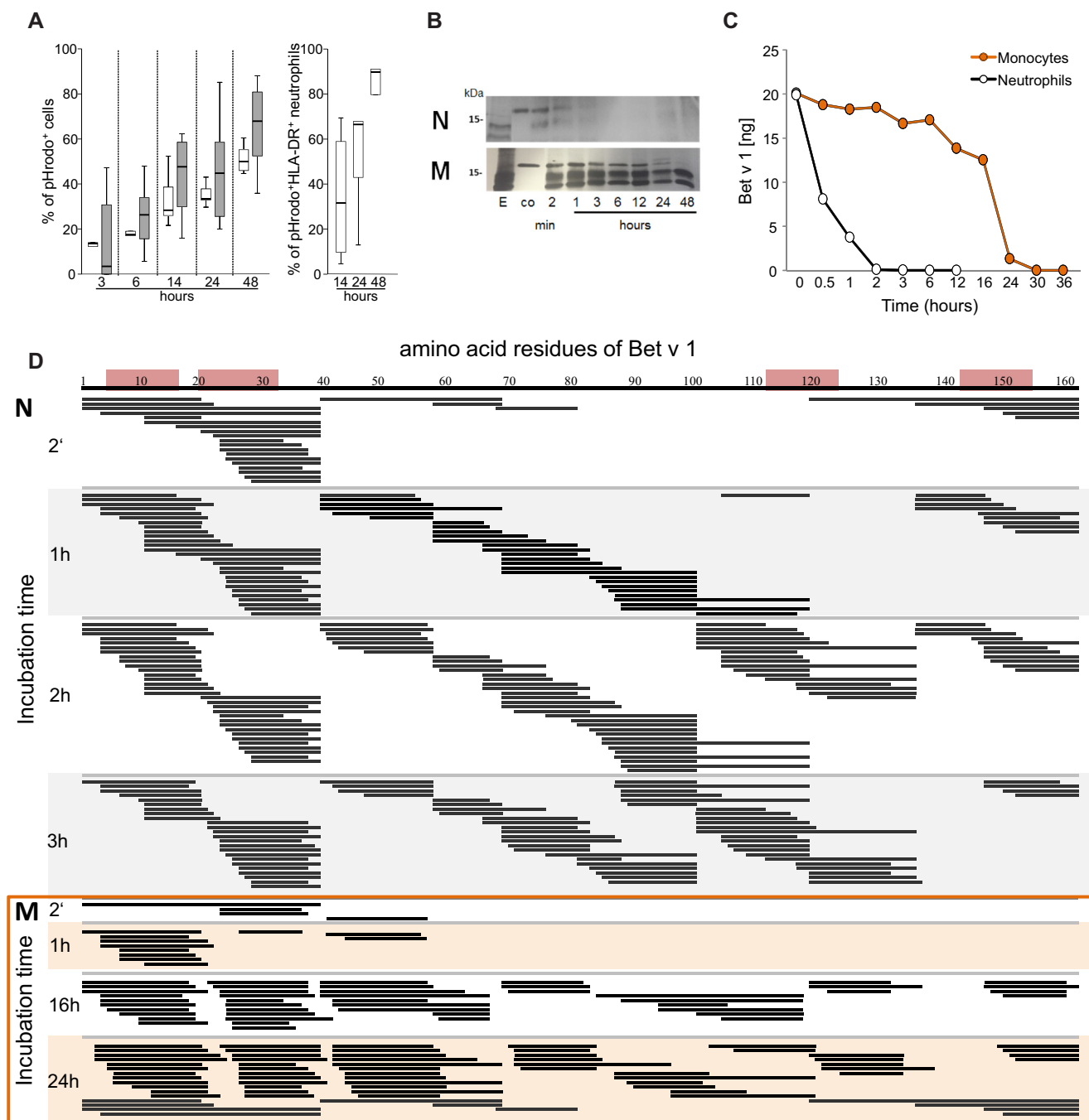


FIG 2. Uptake and degradation of Bet v 1 by neutrophils. **A**, Neutrophils (white box plots) and monocytes (gray box plots) from 6 patients with birch pollen allergy were incubated with pHrodo-labeled Bet v 1 in GM-CSF/IFN- γ /IL-3. The percentage of pHrodo⁺ cells and pHrodo⁺HLA-DR⁺ neutrophils was assessed at the indicated time points by using flow cytometry. **B**, SDS-PAGE analysis of the degradation of Bet v 1 by lysosomal extracts from neutrophils (N) or monocytes (M). co, Bet v 1 without lysosomal extracts; E, lysosomal extract. **C**, Degradation of Bet v 1 quantified by using capture ELISA. **D**, Peptides (black lines) generated over time by means of incubation of Bet v 1 with lysosomal extracts from neutrophils (N) or monocytes (M) were characterized by using mass spectrometry. Frequently recognized T cell-activating regions in the amino acid sequence of Bet v 1 at the top are highlighted in red. The results in Fig 2, B and C, are representative of 2 and 3 independent experiments, respectively.

TABLE I. Proliferative responses of Bet v 1-specific T-cell cultures

APCs 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*	NT	1.8	21.6	NT	1.7	DRB1*0301/1401
TCL2	21.6	14.1	NT	6.7	4.4	NT	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	NT
TCL5	3.6	0.1	0.04	17.7	4.7	0.08	DRB1*0701/1501
TCL6	1.2	NT	<0.01	9.9	NT	<0.01	DRB1*0701/1501
TCL7	1.6	NT	NT	12.1	NT	NT	NT
TCL8	1.8	NT	NT	10.7	NT	NT	DRB1*0701/1501
TCL9	7.2	NT	NT	33.2	NT	NT	DRB1*0301/1401

TCL7 to TCL9 were stimulated with allergen-pulsed APCs.

NT, Not tested.

*Values of Δ cpm $\times 10^3$ are shown.

containing native Bet v 1. Bet v 1-specific TCLs also proliferated in response to allergen-pulsed APCs (ie, neutrophils and monocytes that had been washed after stimulation with GM-CSF/IFN- γ /IL-3 plus allergen; Table I). As expected, the proliferative responses were more pronounced when monocytes served as APCs.

Bet v 1 contains multiple T-cell epitopes distributed over its entire sequence³⁴ 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 TCCs 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 TCCs proliferated in the presence of either cell type, irrespective of their epitope recognition (Fig 3). Again, monocytes were in general more potent APCs 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, with both showing a T_H2 phenotype with production of high amounts of IL-4, IL-13, and IL-5 and low amounts of IFN- γ to stimulation by neutrophils. Both clones released T_H2 cytokines comparably with stimulation by Bet v 1-pulsed monocytes (Table II), indicating their full activation by allergen-pulsed neutrophils. In line with the reduced proliferative response (Fig 3, A), 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 APCs. First, we confirmed that Bet v 1-specific TCCs cultured with Bet v 1 in the absence of APCs did not proliferate (see Fig E3, A, in this article's Online Repository at www.jacionline.org). Second, we used autologous eosinophils (CD66b⁺CCR3⁺CD16⁺) as APCs because 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 Fig E3, A). Third, neutrophils incubated with Bet v 1 in the absence of GM-CSF/IFN- γ /IL-3 did not induce T-cell proliferation (see Fig E3, B). Finally, 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 Fig E3, B).

HLA-DR⁺ neutrophils are present in patients with allergic cutaneous LPRs

To assess whether HLA-DR⁺ neutrophils are present in allergic LPR *in vivo*, we used suction blister technology.³⁹ To achieve a reliable induction of LPRs, 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 to 24 hours. Although blister fluids at sites of diluent injection contained T lymphocytes but neither granulocytes nor monocytes, blisters at sites of allergen injection contained CD4⁺ and CD8⁺ 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 5 independent experiments we detected 11% to 25% HLA-DR⁺ neutrophils in blister fluids from patients with allergen-induced LPRs (Fig 4). In addition, neutrophils from 2 subjects were isolated from peripheral blood collected at the day of suction blistering. As expected, freshly isolated blood neutrophils were HLA-DR⁺ (data not shown). However, when these neutrophils were incubated with GM-CSF/IFN- γ /IL-3 for 24 hours, similar percentages of HLA-DR⁺ neutrophils to those in suction blister fluids were detected (Fig 4). Neutrophils cultured in medium alone remained HLA-DR⁺.

DISCUSSION

Large numbers of neutrophils infiltrate the airways and skin during allergen-induced LPRs.^{5,18,32,40,41} In addition to this recruitment, which is mainly mediated by IgE-activated mast cells,³ certain pollen species have been reported to attract neutrophils to the airways in a Toll-like receptor 4-, myeloid differentiation protein 2-, and CXCR2-dependent manner.⁴² Recruited neutrophils are generally considered to contribute to the allergic inflammation by producing compounds with pathogenic potential, such as reactive oxygen species, elastase, leukotrienes, and cytokines (reviewed in Monteseirin¹⁵ and Hosoki et al¹⁷). Here we propose a novel role for neutrophils in allergic inflammation: in the local cytokine environment they convert into functional APCs and activate allergen-specific effector CD4⁺ T cells.

Evidence for the upregulation of HLA class II molecules on human neutrophils in the presence of GM-CSF, IFN- γ , and IL-3 *in vitro* and *in vivo* had already been indicated in the past, as reviewed by Takashima and Yao.⁴³ Also, we confirm that neutrophils from allergic donors show prolonged survival, suppressed

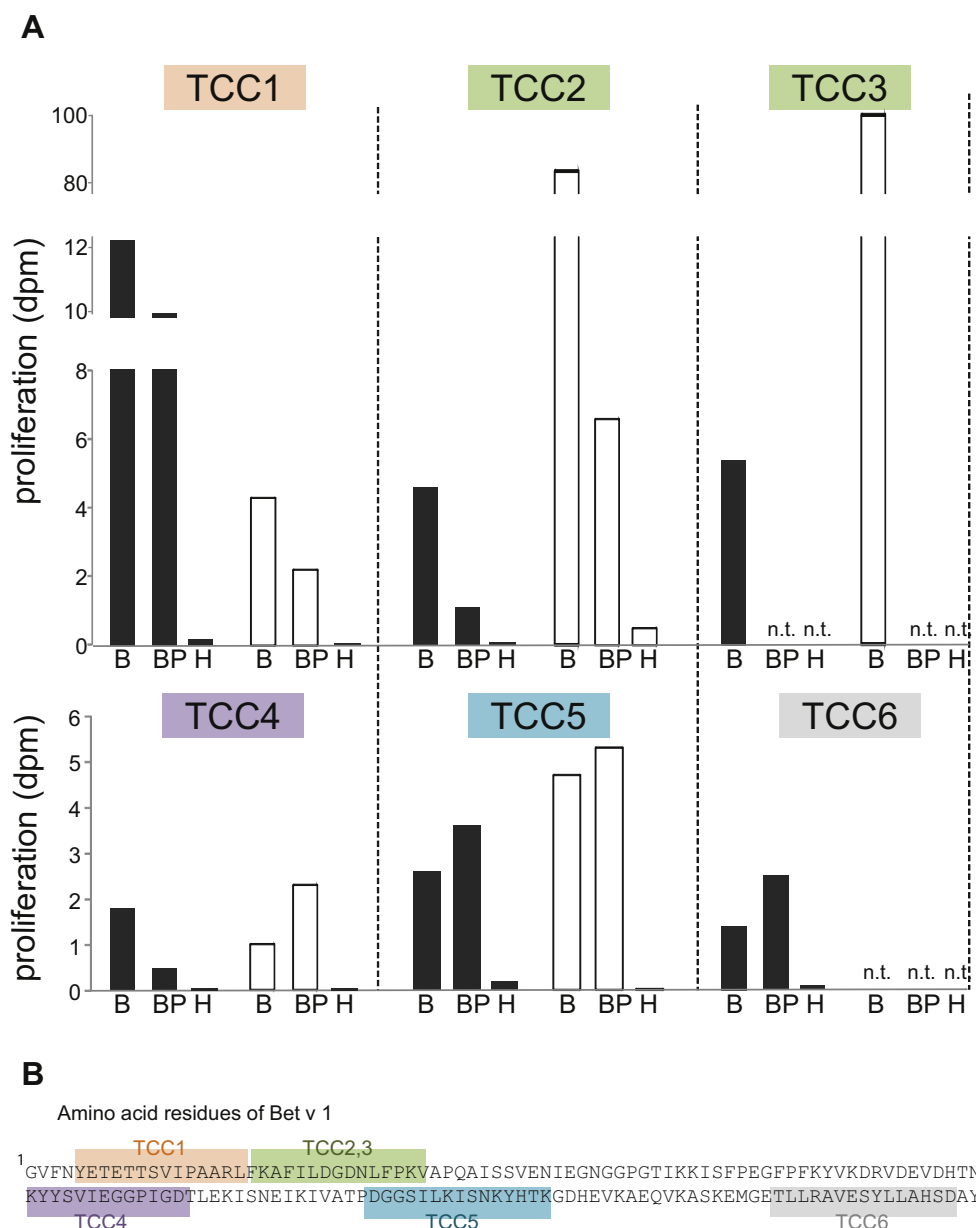


FIG 3. T-cell proliferation to allergen-pulsed neutrophils and monocytes. **A**, Proliferation of 6 TCCs to rBet v 1 (B), birch pollen extract (BP) or Hev b 3 (H) and either neutrophils (black bars) or monocytes (white bars) as APCs; Δ cpm $\times 10^3$ values are shown. *n.t.*, Not tested. **B**, Epitopes recognized by TCCs are highlighted in different colors in the amino acid sequence of Bet v 1.

TABLE II. Cytokine response of Bet v 1-specific T_H2 clones to Bet v 1-pulsed APCs

			Cytokines (pg/mL)									
	APC	Stimulus	IL-4	IL-5	IL-9	IL-10	IL-13	IL-22	IL-17A	IFN- γ	GM-CSF	TNF- α
TCC 3	Neutrophils	Medium	62.8	1456	<4.5	0.1	1039	<4.5	<4.5	231	109	240
	Neutrophils	Bet v 1	3483	4461	51.3	0.0	2716	<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	888
	Monocytes	Bet v 1	16770	8450	395	20.6	23891	<4.5	<4.5	726	13059	9644
TCC 5	Neutrophils	Medium	483	4799	<4.5	5.6	3694	<4.5	<4.5	52.4	<4.5	1715
	Neutrophils	Bet v 1	14415	8796	184	1281	13939	184	39.9	1196	7957	5943
	Monocytes	Medium	717	4816	<4.5	6.2	3843	<4.5	<4.5	50.2	<4.5	369
	Monocytes	Bet v 1	15073	8816	194	1297	15571	194	37.3	1246	7977	6004

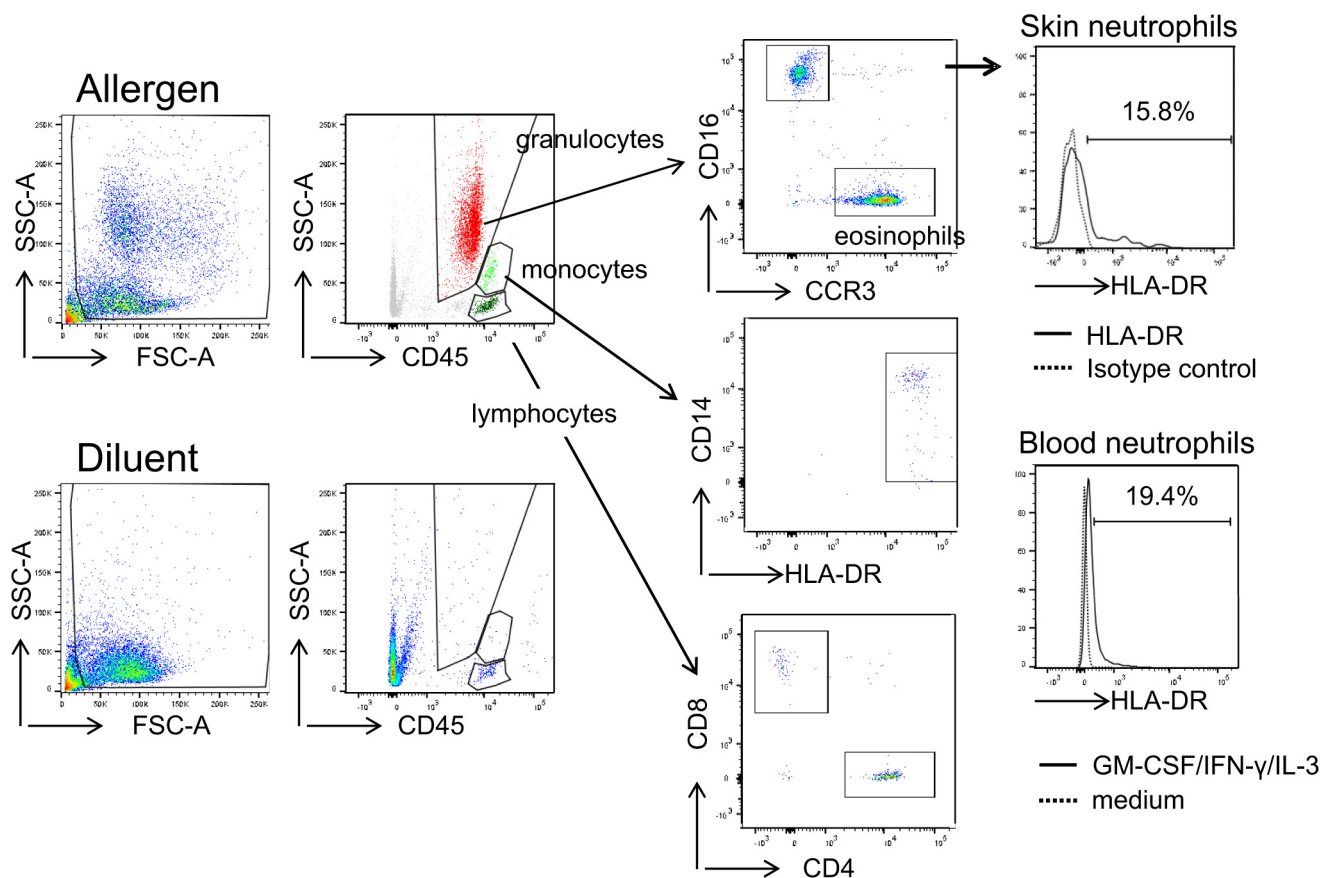


FIG 4. Detection of HLA-DR⁺ neutrophils in patients with allergic cutaneous LPRs. Allergen or diluent was injected intradermally, and suction blisters were performed at injection sites after 20 to 24 hours. Fluids were analyzed for T cells, monocytes, eosinophils, and neutrophils by using flow cytometry. Skin neutrophils were assessed for HLA-DR expression. One representative experiment of 5 is shown. In 2 patients blood neutrophils were isolated, cultured in medium or GM-CSF/IFN- γ /IL-3 for 24 hours, and analyzed for HLA-DR expression by using flow cytometry. FSC, Forward scatter; SSC, side scatter.

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. Because the capacity to fully degrade antigens and properly load antigenic peptides onto HLA II molecules is essential for antigen presentation, we addressed these features using a sophisticated experimental approach involving highly purified neutrophils, a clinically relevant and immunologically well-characterized allergen, and allergen-specific HLA-DR-restricted TCCs with defined epitope reactivity. Neutrophils internalized and cleaved fully 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 indicate strongly 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 patients with allergic LPRs. This

evidence was supported further by the *in vivo* identification of HLA-DR⁺ neutrophils in patients with cutaneous allergic LPRs.

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 behavior described for macrophages.⁴⁴ Hev b 3 was also degraded rapidly (data not shown). In contrast, significant amounts of Bet v 1 remained intact during endolysosomal proteolysis by monocytes and resembled the processing behavior of DCs.³⁵ The slow degradation of internalized antigens has been considered to guarantee that DCs can still generate and present proteolytic fragments to naive T cells after migration to the lymph nodes.⁴⁴ Thus efficient priming of naive T cells might depend on the prevention of premature intracellular antigen degradation. This ability would not apply to neutrophils, which digest internalized antigens rapidly and fully, even though they have been shown to transport antigen to lymphatic compartments.^{26,45} Furthermore, even on optimum stimulation with GM-CSF, IFN- γ , and IL-3, neutrophils did not express high levels of CD80 and CD86, which are relevant for full activation of naive T lymphocytes. In support of these results, Vono et al²⁶

demonstrated that human neutrophils did not activate allogeneic naive T-cells. In contrast to naive T cells, previously activated T cells also respond to APCs lacking costimulatory molecules, although less strongly.⁴⁶ Together, on appropriate stimulation, we conclude that human neutrophils are highly efficient in collecting antigens and subsequent activation of effector and memory CD4⁺ T cells, which 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. On natural exposure or injection of allergens during allergen-specific immunotherapy, activated mast cells induce an influx of leukocytes with neutrophil infiltration as a relatively early event.⁸ The local cytokine microenvironment prolongs their lifespan and fosters them to internalize, process, and present allergen to specific CD4⁺ effector T cells. Activated T cells then execute various effector mechanisms, such as IL-5 release, which recruits and activates eosinophils and further contributes to the allergic inflammation.⁵ Indeed, eosinophil infiltration of the lungs of asthmatic patients after allergen challenge has been reported to be delayed compared with accumulation of CD4⁺ T cells and neutrophils.⁸ Increased expression of HLA-DR in cutaneous cellular infiltrates at sites of allergen-induced LPRs has been described.⁴⁷ Here we show that HLA-DR⁺ neutrophils are present at such inflammatory sites, as well as CD4⁺ and CD8⁺ T cells, monocytes, and eosinophils. These results match the cell populations that have been detected in biopsy specimens of patients with late local skin reactions obtained 24 hours after subcutaneous injection of allergen vaccines for specific immunotherapy.⁴⁸

In contrast to human basophils⁴⁹ and eosinophils (see Fig E3, B), 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 after natural exposure or vaccination during allergen-specific immunotherapy.

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Key messages

- Cytokine-stimulated neutrophils activate allergen-specific T cells to proliferate and synthesize cytokines.
- HLA-DR⁺ neutrophils are found in patients with cutaneous allergic LPRs *in vivo*.

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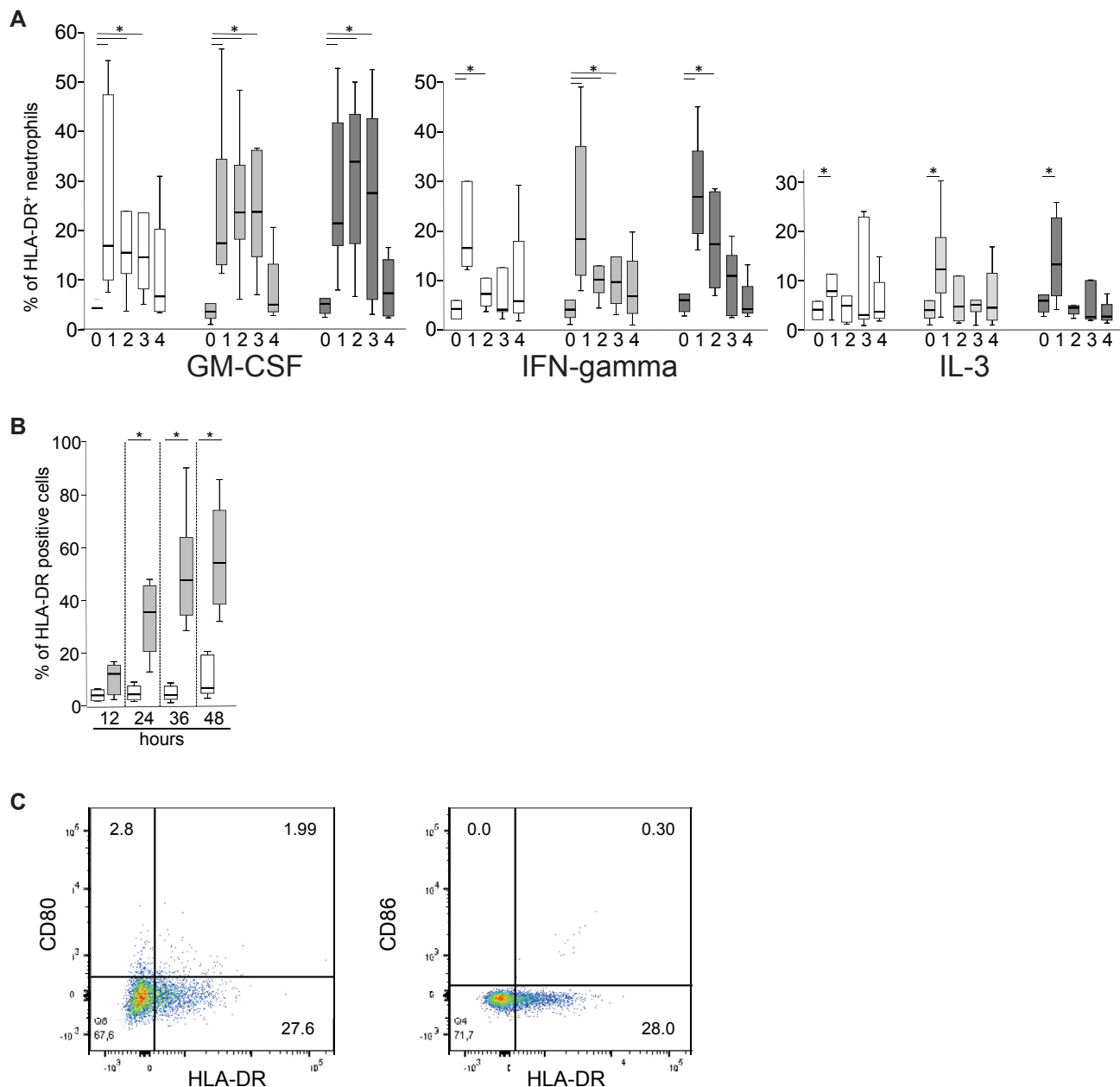


FIG E1. Expression of HLA-DR on cytokine-activated neutrophils. **A**, Purified neutrophils from 6 patients with birch pollen allergy were cultured in medium alone (0) or stimulated with titrated concentrations of 10 ng/mL (1), 1 ng/mL (2), 0.1 ng/mL (3), and 0.01 ng/mL (4) of GM-CSF, IFN- γ , and IL-3. Percentage of HLA-DR⁺ cells was assessed by using flow cytometry at 24 (white box plots), 36 (light gray box plots), and 48 (dark gray box plots) hours. **B**, Neutrophils were left in medium alone (white box plots) or stimulated with a cocktail of GM-CSF (100 pg/mL), IFN- γ (10 ng/mL), and IL-3 (30 ng/mL, gray box plots) according to published protocols.^{E1} The percentage of HLA-DR⁺ cells was assessed at indicated time points. **C**, Percentage of HLA-DR⁺ cells expressing CD80 and CD86 at 24 hours. The dot blot shows 1 representative experiment. * $P < .05$, Wilcoxon signed-rank test.

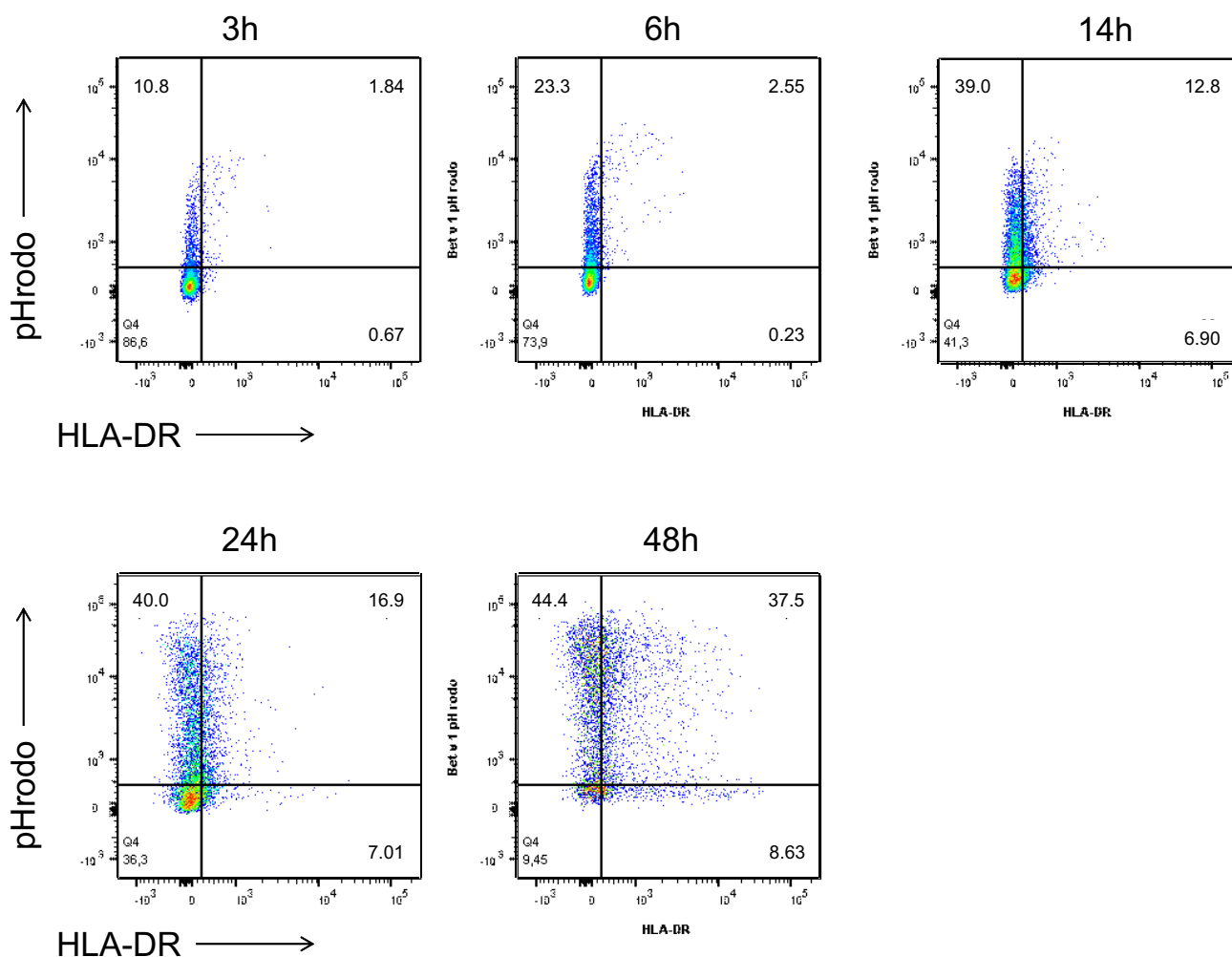


FIG E2. HLA-DR expression of Bet v 1⁺ neutrophils. Purified neutrophils from patients with birch pollen allergy were incubated with pHrodo-labeled Bet v 1 in a cocktail of GM-CSF (100 pg/mL), IFN- γ (10 ng/mL), and IL-3 (30 ng/mL, *gray box plots*). At the indicated time points, the percentage of HLA-DR⁺ neutrophils that had internalized Bet v 1 was assessed by using flow cytometry. The dot blot shows 1 representative experiment of 6.

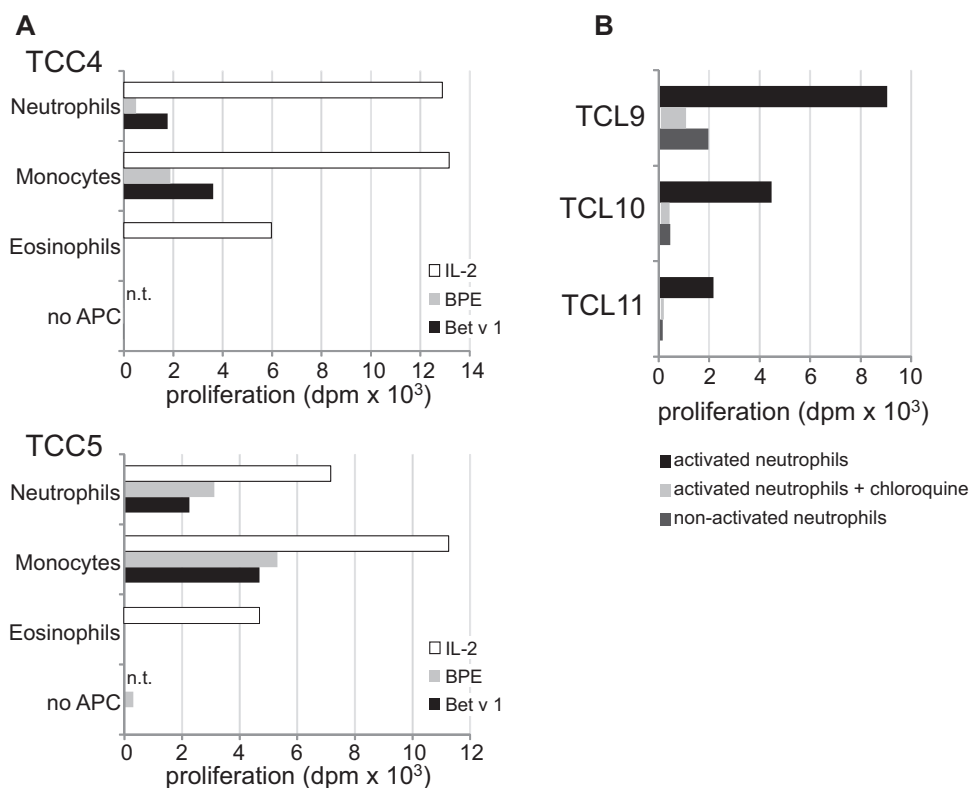


FIG E3. Allergen-induced proliferation of Bet v 1-specific T cells in the presence of different cell types. **A**, Autologous neutrophils (CD66b⁺CD16⁺CCR3⁻) and eosinophils (CD66b⁺CCR3⁺CD16⁻) were sorted from peripheral blood cells by using the FACSaria III (BD Biosciences). Monocytes were isolated with CD14 MicroBeads (Miltenyi Biotec). All 3 cell types (purity >99%) were incubated with GM-CSF/IFN- γ /IL-3 with or without Bet v 1 (5 μ g/mL), birch pollen extract (20 μ g/mL), or IL-2 (2 U/mL) for 24 hours, irradiated (60 Gr), and cocultured with 1 Bet v 1-specific TCCs. **B**, Neutrophils (purity >99%) were incubated without (nonactivated) or with GM-CSF/IFN- γ /IL-3 with or without Bet v 1 (5 μ g/mL) in the presence or absence of chloroquine diphosphate salt (100 μ g/mL; Sigma-Aldrich), washed, irradiated (60 Gr), and added to 3 different Bet v 1-specific TCLs. In all experiments proliferation was assessed after 48 hours. *dpm*, Counts per minute in response to stimulus – counts per minute in medium controls. *n.t.*, Not tested.