

Letter to the Editor

[DOI: 10.1002/eji.201848057]

T-cell-derived cytokines enhance the antigen-presenting capacity of human neutrophils

Allergen exposure of allergic individuals induces immediate reactions caused by histamine and other mediators released from sensitized mast cells as a consequence of IgE cross-linking. After 3–48 h, cell-mediated late-phase reactions may follow, characterized by early accumulation of neutrophils together with allergen-specific CD4⁺ T-cells and subsequent infiltration of eosinophils [1, 2]. Up to now, neutrophils were regarded to contribute to inflammation by released mediators. However, we detected HLA-DR-positive neutrophils in cutaneous allergic late-phase reactions in vivo and demonstrated in vitro that in the presence of a cocktail of GM-CSF, IFN- γ , and IL-3, neutrophils internalized, processed, and presented allergen via HLA-DR resulting in proliferation and cytokine production of specific T cells [3]. In turn, anti-CD3-activated CD4⁺ and particularly CD8⁺ T cells have been shown to modulate apoptosis and activation of neutrophils [4, 5]. As allergen-stimulated CD4⁺ T cells release several cytokines in addition to GM-CSF, IFN- γ , and IL-3, we sought to assess whether key cytokines of allergen-specific Th2- and Th1-cells affect the antigen-presenting activity of neutrophils.

To estimate the amount of cytokines released by allergen-specific T cells, we specifically stimulated 16 clones from different allergic patients and determined the major Th2- and Th1-like cytokines IL-4, IL-5, IL-13, IL-2, and IFN- γ , GM-CSF, IL-3, the inflammatory cytokines TNF- α and IL-6, the immunosuppressive cytokine IL-10, as well as IL-17, which contributes to the recruitment of neutrophils. We classified Th1- or Th2-cells according to IL-4/IFN- γ ratios and found extremely heterogeneous cytokine patterns released by either subset (Supporting Information Table 1). In addition to the anticipated high levels of IL-4, IL-5, and IL-13, several Th2-clones produced remarkable levels of IL-2, IL-3, IL-10, and/or IL-17. In addition to IFN- γ , some Th1-clones produced IL-3 and IL-10. With the exception of very few Th2-clones, both subsets synthesized considerable levels of GM-CSF, TNF- α , and IL-6.

To assess the expression of HLA-DR, neutrophils from eight birch pollen-allergic individuals were cultured in medium or stimulated with recombinant IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFN- γ , TNF- α , or GM-CSF. In accordance with neutrophils from nonallergic individuals [4, 5], HLA-DR was upregulated by GM-CSF, IFN- γ , and high levels of IL-3 (Supporting Information Fig. 1A). However, even high levels (10 ng/mL) of the remaining cytokines had no effect (Fig. 1A). Furthermore, none of the cytokines notably enhanced surface expression of CD80 and CD86 nor decreased the expression of CD58, which we recently demonstrated to be involved in functional activation of T-cells by neutrophils [6] (Fig. 1A). Simultaneous analyses of cell viability revealed that GM-CSF, IFN- γ , and high doses of TNF- α enhanced the percentage

of viable neutrophils. Weaker dose-dependent effects were observed for IL-2, IL-3, and interestingly, also for IL-4 (Supporting Information Fig. 1B). None of the remaining cytokines showed an effect (data not shown).

To study allergen uptake, we employed β -lactoglobulin (Bos d 5) labeled with pHrodoTM Red dye that emits fluorescence when phagosomal and granular vesicles fuse and the pH value is reduced. GM-CSF, IFN- γ , TNF- α , IL-2, and IL-3 dose dependently enhanced the percentage of pHrodo⁺ neutrophils (Fig. 1B). Notably, even low doses of IL-10 significantly reduced allergen uptake as did low amounts of IL-4. No impact of the remaining cytokines was found (data not shown). As several allergen-stimulated Th2- and Th1-clones produced IL-10 (Supporting Information Table 1), we wondered whether this immunosuppressive cytokine still diminishes allergen uptake of neutrophils in combination with other cytokines. Therefore, we incubated neutrophils from six birch pollen-allergic donors with pHrodo-labeled Bos d 5 in the presence of cytokine cocktails corresponding to the cytokines released by the clones Th2-1, Th2-2, Th2-3, Th1-1, Th1-2, or Th1-3 (Supporting Information Table 1). All cocktails significantly upregulated allergen uptake, however, cocktail Th2-1, containing very high levels of IL-10 and no IFN- γ , and to a lesser extent also cocktail Th2-3, showed a lower impact than the other cocktails (Fig. 2A). In parallel, all cocktails induced a comparable upregulation of HLA class II molecules (Fig. 2A). Next, we incubated neutrophils with pHrodo-labeled Bos d 5 in variants of cocktail Th2-1 lacking GM-CSF, TNF- α , or IL-3 or variants of cocktail Th2-3 lacking IFN- γ and TNF- α ,

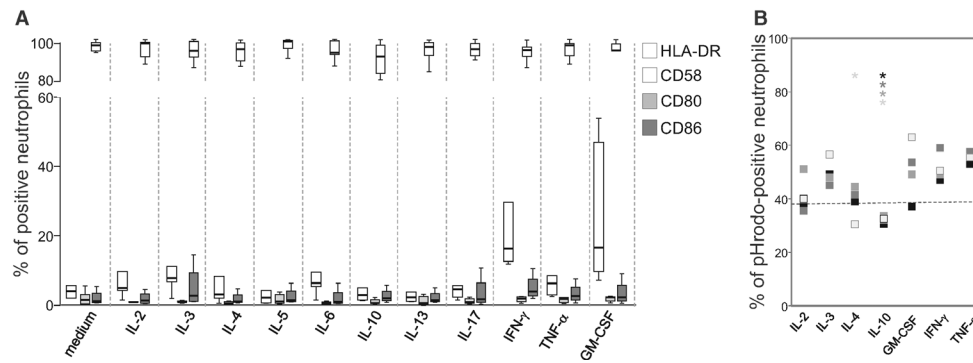


Figure 1. Effects of T-cell-derived cytokines on the expression of HLA-DR, CD80, CD86, and CD58 of neutrophils. (A) The percentage of HLA-DR⁺, CD80⁺, CD86⁺, and CD58⁺ neutrophils from eight different birch pollen-allergic individuals after incubation in medium or with 10 ng/mL of recombinant cytokines for 24 h depicted as box plots: boxes show the values within the first and third quartile divided by the median. The whiskers indicate the minimum and maximum of all data. (B) Neutrophils from five different allergic individuals were incubated with pHrodo-labeled Bos d 5 in the presence of 10 ng (black), 1 ng (dark gray), 0.1 ng (medium gray), and 0.01 ng/mL (light gray) of recombinant cytokines. Squares represent the median values of the percentage of pHrodo⁺ neutrophils, and the dotted line represents the medium control. A single experiment per individual was performed. * $p < 0.05$, Wilcoxon-signed ranks test.

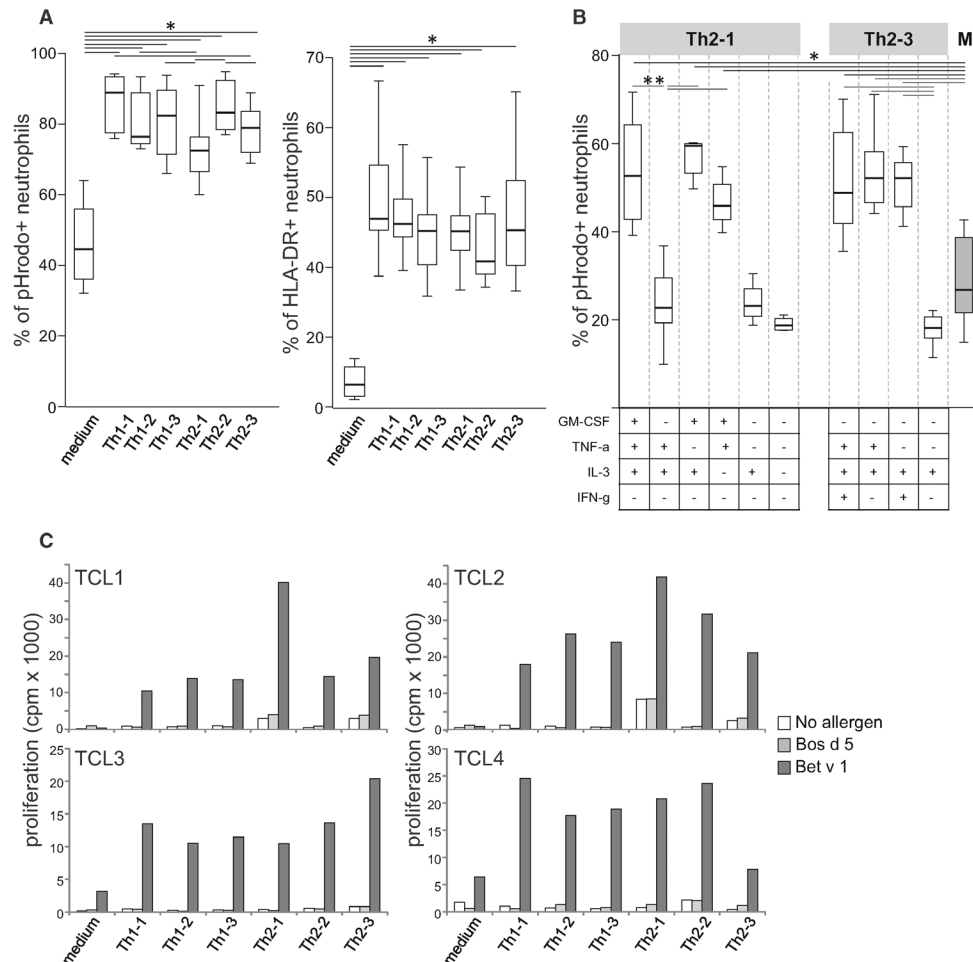


Figure 2. T-cell-derived cytokines induce antigen-presenting activity of neutrophils. (A) The percentage of pHrodo⁺ and HLA-DR⁺ neutrophils of six allergic individuals after incubation with pHrodo-labeled Bos d 5 in cytokine mixes as produced by Th1- and Th2-clones (Supporting Information Table 1). (B) The percentage of pHrodo⁺ neutrophils from seven allergic individuals after incubation with pHrodo-labeled Bos d 5 in the cytokine mixes Th2-1 and Th2-3, their variants lacking GM-CSF, TNF- α , IL-3, and IFN- γ , or medium (M). (A, B) A single experiment per individual was performed. Box plots are shown; the box includes the first and third quartile divided by the median. The whiskers indicate the minimum and maximum of all data. * $p < 0.05$, Wilcoxon signed-ranks test. (C) Proliferation of four Bet v 1-specific T-cell lines (TCL) in response to neutrophils incubated without or with Bet v 1 or Bos d 5 in the absence or presence of cytokine mixes; cpm, counts per minute; bars show the mean value of duplicates.

respectively. Figure 2B shows that GM-CSF counteracted the reduced allergen uptake in the presence of > 12 ng/mL of IL-10 contained in cocktail Th2-1, whereas IL-3 and TNF- α were not effective. In cocktail Th2-3, per se lacking GM-CSF and containing 0.4 ng/mL of IL-10, the presence of either IFN- γ or TNF- α was sufficient to rescue allergen uptake (Fig. 2B). To confirm their function as APC, we stimulated neutrophils with the different cytokine cocktails in the absence or presence of the major birch pollen allergen Bet v 1 or Bos d 5, and added them to autologous Bet v 1-specific T-cell lines. The proliferative response to Bet v 1 was similar irrespective of the cytokine cocktail used to stimulate neutrophils (Fig. 2C). None of the T-cell lines proliferated to the negative control Bos d 5. These results indicate that neutrophils may become functional APC in a Th2- as well as a Th1-like cytokine milieu irrespective of the presence of IL-10.

In summary, GM-CSF most potently transformed neutrophils from allergic individuals into functional APC [4, 5, 7]. Similar to phagocytosis of bacteria [8], allergen uptake by neutrophils was reduced by IL-10. However, this inhibitory effect was overruled by the presence of GM-CSF, IFN- γ , and TNF- α and did not diminish their T-cell-activating activity. We conclude that cytokines released from both allergen-specific effector Th1 and Th2-cells enhance the life-span and antigen-presenting activity of neutrophils. In turn, neutrophils activate co-localized allergen-specific effector

T cells [1, 2] and thereby, amplify allergen-specific cell-mediated inflammation [6].

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Acknowledgments: This work was supported by the Austrian Science funds (FWF), projects W1248 and SFB F4610, and the Medical University of Vienna.

Author Contributions: N.S., D.P., and C.K. performed the experiments and analyzed the data; P.S. provided antibodies; G.J.Z. helped with cytokine measurements; and N.S., D.P., C.K., B.J.S., and B.B. wrote the manuscript.

Conflict of interest: The authors declare no commercial or financial conflict of interest.

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Keywords: Allergen-specific T cells • Allergy • Neutrophils • Th1 • Th2

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The peer review history for this article is available at <https://publons.com/publon/10.1002/eji.201848057>

Received: 13/12/2018

Revised: 12/4/2019

Accepted: 19/7/2019

Accepted article online: 22/7/2019



The detailed *Materials and methods* for Letters to the Editor are available online in the Supporting information

*Both authors contributed equally to this work.

European Journal of Immunology

Supporting Information for

DOI 10.1002/eji.201848057

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**T-cell-derived cytokines enhance the antigen-presenting capacity of human
neutrophils**

SUPPORTING INFORMATION

The impact of T cell-derived cytokines on the antigen-presenting capacity of human neutrophils

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Running head: Activation of neutrophils by Th2 and Th1 cytokines

This work was supported by the Austrian Science Fund, projects W1248 and SFB F4610, and by the Medical University of Vienna.

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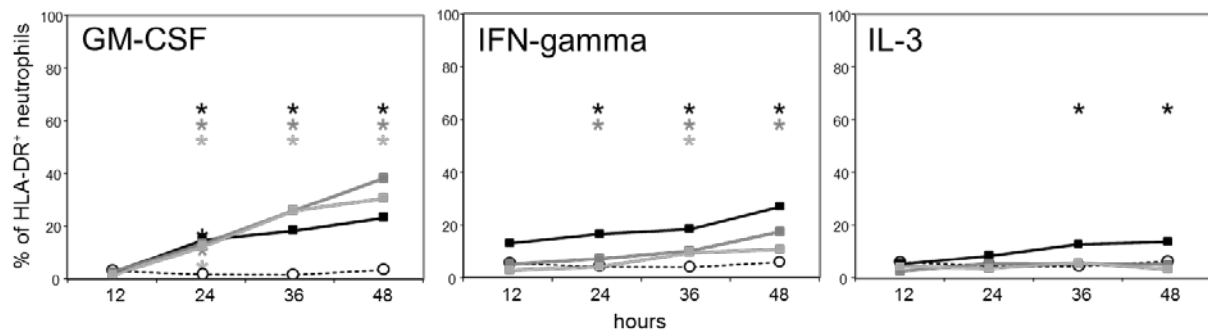
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Table 1. Cytokine patterns of allergen-specific CD4⁺ T cells in response to allergen

TCC	Allergen	IL-2*	IL-3	IL-4	IL-5	IL-13	IL-6	IL-10	IL-17	IFN-γ	TNF-α	GM-CSF
Th2-1**	Amb a 1	480	93	197	>8000	>33600	<6	>12000	<6.0	<6	688	642
Th2-2	Art v 1	514	18	>4500	>8000	>33600	10612	897	38.2	191	12695	3271
Th2-3	Bet v 1	>50000	<6	>4500	6977	13853	601	441	17922	408	>83000	<6.0
Th2-4	Phl p 1	<6	92	218	>8000	>33600	583	21	<6	<6	1118	1321
Th2-5	Amb a 1	63	173	245	1206	15286	>11000	103	<6	21	1591	1204
Th2-6	Amb a 1	374	31	279	1206	20300	52	3714	<6	26	395	488
Th2-7	Bet v 1	22	n.t.	15300	5100	>33600	1950	9200	150	60	2080	9300
Th2-8	BP	3546	170	>4500	236	12923	3683	551	<6	<6	5439	1415
Th1-1	Art v 1	734	224	<6	117	n.t.	778	2179	<6	6648	2420	1032
Th1-2	Bet v 1	12	234	<6	9	304	3147	121	<6	976	2895	3147
Th1-3	Bet v 1	20	<6	<6	80.2	182	>11000	9	<6	130	2344	>11000
Th1-4	Art v 1	382	30.2	<6	138	1194	1866	4234	<6	765	2505	335
Th1-5	BP	n.t.	<6	25	217	n.t.	8919	n.t.	n.t.	11659	8341	492
Th1-6	BP	18	<6	32	<6	397	803	25	<6	290	638	233
Th1-7	BP	38	<6	71	11	278	2393	142	6	1769	2985	482
Th1-8	BP	n.t.	n.t.	500	156	560	8300	31	58	10300	7100	1200

*pg/ml are shown; cytokine levels in supernatants from T-cell clones (TCC) plus PBMC without allergen were subtracted from levels in supernatants from TCC plus PBMC plus allergen; **Th2 cells produce 10 x IL-4 than IFN-γ; Th1 cells produce 10 x IFN-γ than IL-4; BP, birch pollen extract

A



B

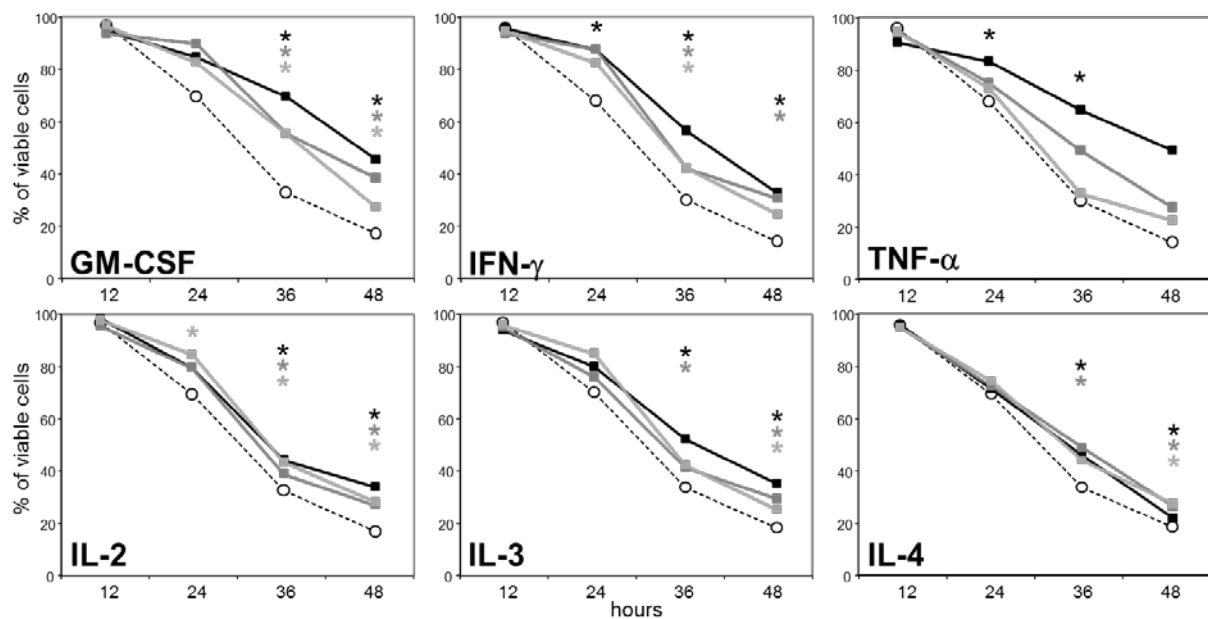


Figure E1. Effects of cytokines on HLA-DR expression and viability of neutrophils from allergic individuals. Neutrophils were kept in medium alone (dotted line) or incubated with recombinant cytokines at a concentration of 10 ng (black line), 1 ng (dark gray), or 0.1 ng (light gray). HLA-DR expression (A) and cell viability (B) were assessed over time by flow cytometry. Squares represent median values of 8 different birch pollen-allergic individuals. * $P < 0.05$, Wilcoxon Signed Ranks test.

Birch pollen-allergic individuals

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 $>0.35 \text{ kU}_A/\text{l}$ (ImmunoCAP; Thermo Fisher Scientific, Uppsala, Sweden). The study was approved by the ethics committees of the Medical University of Vienna (EK-1344/2018) and conducted in accordance with the Declaration of Helsinki. Patients gave written informed consent.

Isolation and stimulation of neutrophils

Neutrophils were isolated from heparinized peripheral blood by density gradient centrifugation using Ficoll-Hypaque (Pharmacia Diagnostics, Uppsala, Sweden), dextran sedimentation (4% dextran T 500, Carl ROTH, Germany), and osmotic lysis of erythrocytes. The resulting cell population was analyzed by flow cytometry performed with a FACS Canto II using FACS Diva (BD Biosciences, San Jose, CA, USA) and FlowJo software (TreeStar, Inc., Ashland, OR, USA) employing PE-CD14, PerCP-CD16 (3G8), APC-CCR3, BV421-HLA-DR (L243), BV510-CD3, PE-CD19, FITC-CD66b, PE-CD80 and PE-CD86 antibodies (all BD Bio-sciences, San Jose, CA, USA). Viability was determined with the Fixable Viability Dye eFluor® 780 (eBioscience Inc.). Cells (1×10^6) were cultured in 500 μl of RPMI 1640 supplemented with 10% autologous plasma without or with recombinant cytokines (Peprotech, Rocky Hill, NJ, USA) at concentration ranges of 0.01-10 ng/ml for up to 48 h. In some experiments, cytokine cocktails corresponding to Th1-1, Th1-2, Th1-3, Th2-1, Th2-2 and Th2-3 (Table E1) were used. For internalization experiments, pHrodo-labelled Bos d 5 (10 $\mu\text{g}/\text{ml}$) was added. In adherence to the 'Guidelines for the use of flow cytometry and cell sorting in immunological studies' the gating strategy for the analysis of neutrophils is shown in Figure E2.

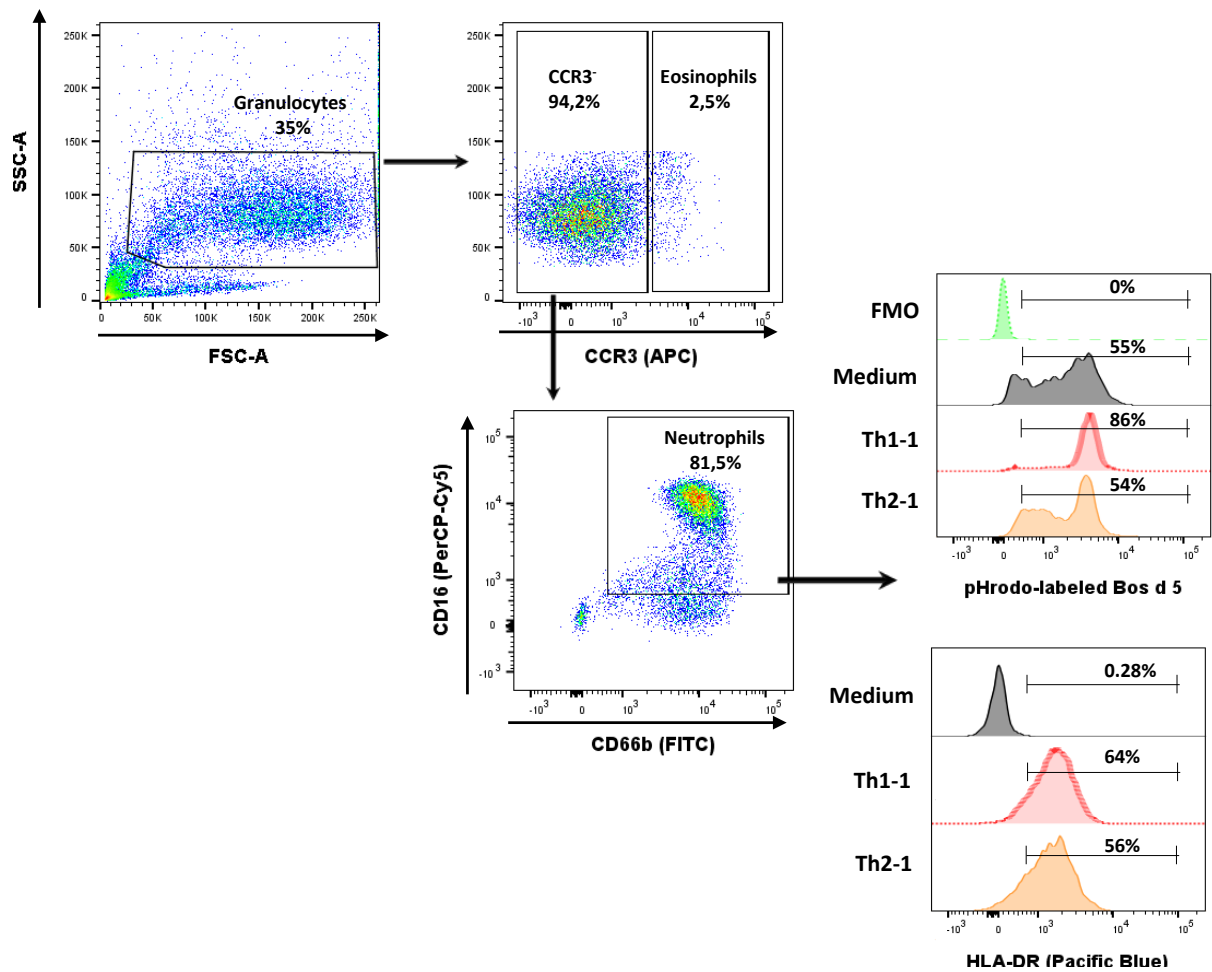


Figure E2. Characterization of neutrophils. Cells were cultured for 20 h in medium in the absence or presence of pHrodo-labelled Bos d 5 and the cytokine cocktails Th1-1 and Th2-1. Viable granulocytes were defined by SSC-A/FSC-A properties. Eosinophils were excluded from the population based on CCR3 expression. Neutrophils were identified as CD66b⁺CD16⁺ cells and the percentage of pHrodo⁺ and HLA-DR⁺ cells in response to the different stimuli was assessed. The Fluorescence Minus One (FMO) control was used in cultures incubated in medium without pHrodo-labelled Bos d 5.

Stimulation of Bet v 1-specific T cells

Before being used as antigen-presenting cells, neutrophils were applied to an immunomagnetic negative selection step using the human MACSxpress[®] Neutrophil Isolation Kit (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) [1]. This approach resulted repeatedly in >99% pure CD16⁺CD66b⁺CCR3⁻HLA-DR⁻CD19⁻CD3⁻CD14⁻ cells.

Allergen-specific T cell clones (TCC) were stimulated with autologous PBMC in the absence or presence of allergen (5 µg/ml). After 24 h, supernatants were harvested and cytokines were assessed by bead array (Luminex, Austin, TX, USA). Allergen-specific T-cell lines were expanded from PBMC of birch pollen-allergic patients stimulated with recombinant Bet v 1.0101 (Bet v 1) purchased from Biomay AG, Vienna, Austria as described [2]. Bet v 1-reactive T cells ($2-5 \times 10^4$, viability >85% as assessed with Bürker chamber of trypan blue-stained cells) were stimulated with purified autologous neutrophils (5×10^4) that had been cultured without or with Bet v 1 or Bos d 5 (5 µg/ml each) in the absence or presence of the different cytokine cocktails for 24 h, washed three times with PBS, and irradiated with 60 Gray. All experiments were performed in duplicate. After 48 h proliferative responses were measured by ³[H]thymidine incorporation during 16 h as counts per minute (cpm).

Statistics

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

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