

Neutrophils promote T-cell-mediated inflammation in allergy



To the Editor:

We recently proposed a novel role for neutrophils in allergy, namely, serving as antigen-presenting cells (APCs) for allergen-specific CD4⁺ T cells in allergic late-phase reactions (LPRs).¹ In detail, we identified HLA-DR–positive neutrophils in allergen-induced cutaneous LPRs *in vivo*. *In vitro* we demonstrated that the cytokines GM-CSF, IL-3, and IFN- γ , which are present in allergic LPRs *in vivo*, promote neutrophil survival and their expression of HLA-DR and HLA-DM. Moreover, neutrophils rapidly internalized and degraded the major birch pollen allergen Bet v 1 and induced proliferation and cytokine production of Bet v 1–specific effector T cells, although less pronounced than monocytes.¹ Notably, even on incubation with GM-CSF/IFN- γ /IL-3 HLA-DR–positive neutrophils failed to express significant levels of CD80 and CD86, which represent the ligands for the primary costimulatory receptor CD28.¹ CD28 is the best-studied signal 2–inducing molecule on the T-cell surface and is relevant for optimal activation and production of IL-2, necessary to drive clonal expansion of CD4⁺ T lymphocytes. Signal 1 is delivered through the T-cell receptor (TCR) on encounter with antigen-derived peptides presented by HLA class II molecules. Signaling through the TCR complex in the absence of CD28–costimulation may result in a growth arrest state termed clonal anergy.² Anergic T cells poorly proliferate to subsequent TCR stimulation even in the presence of costimulation. Clonal anergy can be prevented by the addition of agonistic antibodies to CD28 (α CD28) during the induction phase and is reversed by IL-2. All these findings tempted us to investigate whether activation of allergen-specific effector T cells by neutrophils resulted in clonal anergy. In this case, neutrophils might contribute to suppression rather than potentiation of the inflammatory response in allergic LPRs.

Following a previously published protocol for anergy induction of antigen-experienced T cells in the absence of B7 costimulation,³ we incubated a Bet v 1–specific T-cell clone (see this article's Methods section in the Online Repository at www.jacionline.org) with either a synthetic 12-mer peptide containing its T-cell epitope or with autologous cytokine-activated, Bet v 1–pulsed, irradiated (60 Gray) neutrophils in the absence or presence of α CD28. After 48 hours, T cells were washed and rested for 72 hours to permit reexpression of the TCR complex before rechallenge with Bet v 1 or Bet v 1 plus rIL-2 and irradiated autologous PBMCs as APCs. Proliferation was determined after 48 hours by [³H]-labeled thymidine incorporation. After primary stimulation with peptide, the clone responded significantly less to rechallenge with Bet v 1 than the clone initially stimulated with peptide plus α CD28 (Fig 1, A). After primary stimulation with Bet v 1–pulsed, activated neutrophils, the clone proliferated clearly stronger as compared with primary stimulation with peptide. The addition of α CD28 during primary stimulation with neutrophils did not enhance the response to secondary stimulation. The addition of IL-2 promoted strong proliferation in all cultures (Fig 1, A). Thereafter, we used another Bet v 1–specific T-cell clone and 5 T-cell lines (TCLs; see this article's Methods section in the Online Repository) from different birch pollen–allergic donors (Fig 1, B). T-cell cultures that had been first stimulated with peptides showed significantly

lower secondary responses than those first stimulated with autologous PBMCs plus allergen (Fig 1, B; $P = .043$, Wilcoxon signed rank test) that contain APCs that provide natural CD28 ligands. T cells first stimulated with autologous cytokine-activated neutrophils plus Bet v 1 demonstrated significantly stronger secondary responses as those initially exposed to peptides alone ($P = .018$) or PBMCs plus Bet v 1 ($P = .018$; Wilcoxon signed rank test). IL-2 enhanced allergen-specific proliferation in all cultures (Fig 1, B). Together, these experiments indicate that despite their limited expression of CD80 and CD86, neutrophils do not induce clonal anergy of CD4⁺ T cells.

To elucidate a possible explanation for this unexpected observation, we assessed the expression of various costimulatory molecules on the surface of neutrophils cultured in the absence and presence of GM-CSF/IL-3/IFN- γ (see Fig E1 in this article's Online Repository at www.jacionline.org). Using flow cytometry we observed a slight upregulation of 4-1BBL (CD137 L) on cytokine-activated neutrophils whereas the expression of CD70 (ligand for CD27), CD275 (ligand for inducible costimulator), CD80, and CD86 remained insignificant. The constitutive expression of CD58 (ie, lymphocyte function–associated antigen-3) by neutrophils was not altered by GM-CSF/IL-3/IFN- γ .⁴ CD58 binds to CD2 on T cells and together with TCR activation induces proliferation.^{5–7} To assess its involvement in neutrophil-induced T-cell activation we stimulated 8 Bet v 1–specific TCLs with allergen-pulsed activated neutrophils in the presence of α CD58 or an isotype-matched control antibody. The presence of α CD58 significantly reduced allergen-induced proliferation of all TCLs (Fig 1, C; $P = .008$). Similar results were obtained with α CD2 antibodies, confirming that the interaction of CD58 and CD2 is involved in T-cell activation by neutrophils (Fig 1, C; $P = .008$). However, the addition of a stimulating α CD28 antibody did not amplify T-cell proliferation in Bet v 1–specific T-cell cultures from 6 different donors, indicating that T cells had been fully activated by neutrophils (Fig 1, D). Together, these results suggest that CD58 on neutrophils serves as signal 2 for productive T-cell activation, possibly resulting in the amplification of T-cell–mediated allergic inflammation. To confirm the latter, we used a chimeric human/mouse model of birch pollen allergy recently established in our laboratory.⁸ Nonobese diabetic severe combined immunodeficient γ c^{–/–} (NSG) mice engrafted with PBMCs from allergic individuals develop airway hyperresponsiveness (AHR) to intranasal challenge with allergen. This allergic airway inflammation is mediated by CD4⁺ T cells as their depletion from PBMCs before engraftment abolished allergen-induced lung inflammation.⁹ In the present study, NSG mice engrafted with PBMCs from birch pollen–allergic individuals received autologous neutrophils after overnight activation with GM-CSF/IL-3/IFN- γ before intranasal challenge with birch pollen extract or PBS (Fig 2, A). Then, AHR was assessed by invasive measurement of murine lung function (Fig 2, B). The presence of neutrophils significantly enhanced allergen-induced AHR. NSG mice that had received neutrophils and were challenged with PBS showed significantly lower AHR than did those challenged with allergen. No difference was observed between PBS-challenged mice with and without neutrophils, indicating that mediators released by activated neutrophils alone had no effect on AHR.

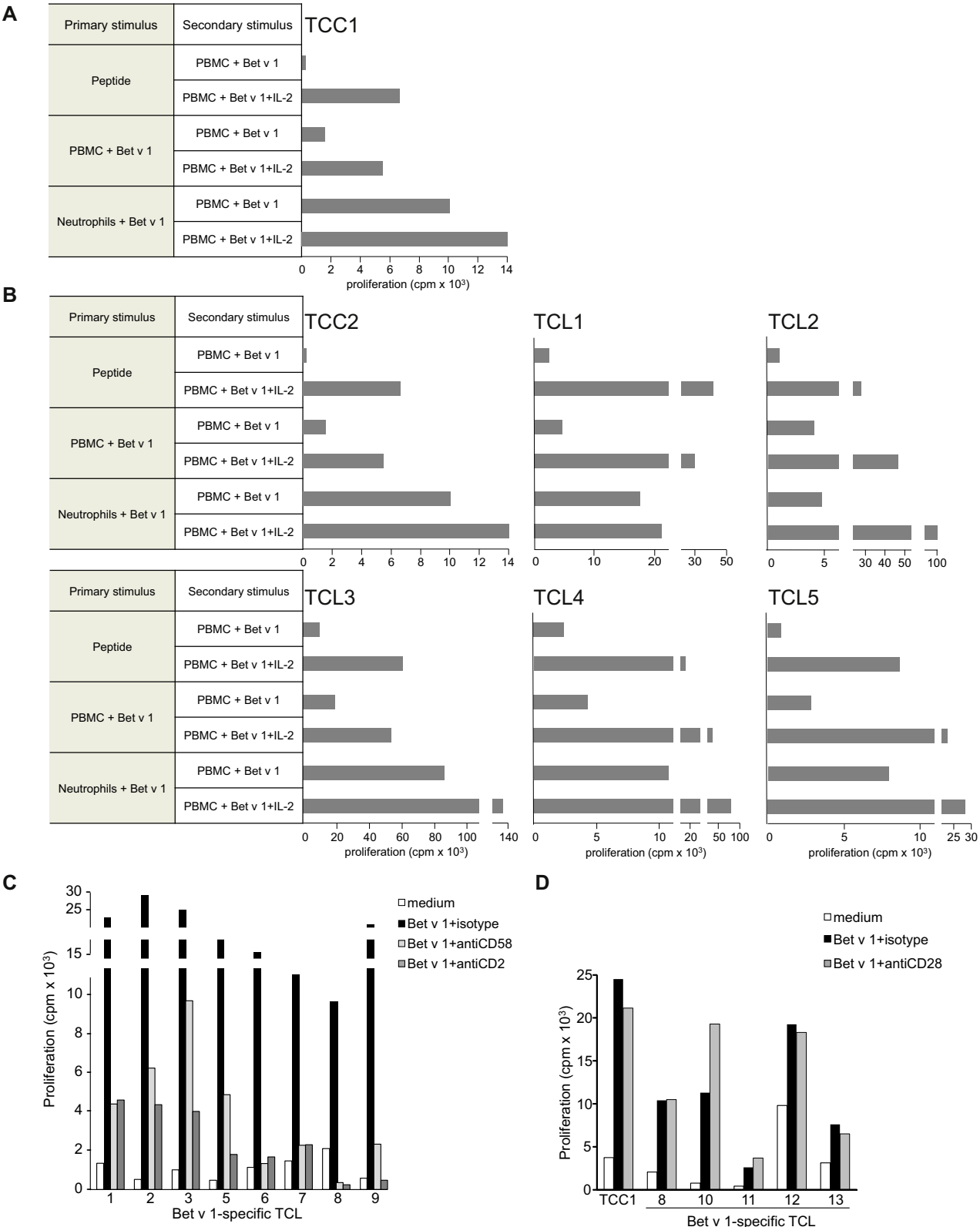


FIG 1. Neutrophils do not induce T-cell anergy. Allergen-specific proliferation of (A) a Bet v 1-specific T-cell clone (TCC1) after primary stimulation with a synthetic 12-mer peptide containing its epitope or activated neutrophils plus Bet v 1 ± αCD28; (B) a clone and 5 different Bet v 1-specific TCLs after primary stimulation with peptide, allergen-pulsed PBMCs, or neutrophils; (C) 8 TCLs to activated neutrophils in the presence of αCD58 or αCD2; (D) 1 TCC and 5 TCLs to activated neutrophils in the presence of αCD28. *cpm*, Counts per minute.

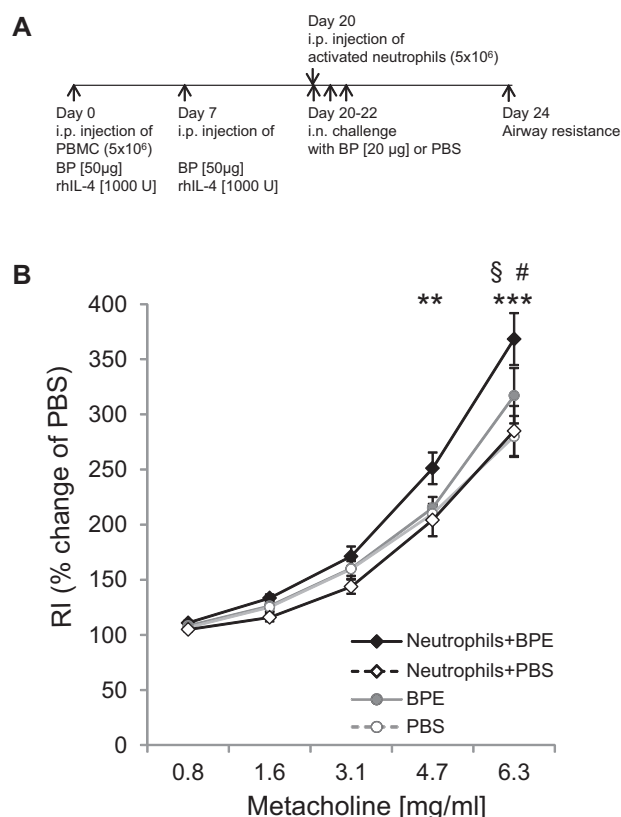


FIG 2. Neutrophils aggravate T-cell-mediated allergic inflammation *in vivo*. **A**, Experimental design. **B**, Airway resistance (RI, means $\pm$ SEM) in PBMC-engrafted mice ($n = 14$ -16 per group) without or with injection of neutrophils and subsequent intranasal challenge with birch pollen extract (BPE) or PBS. *i.n.*, Intranasal; *i.p.*, intraperitoneal. $^{\#}P < .05$ of BPE-challenged mice with/without neutrophils; $^{\$}P < .05$ of BPE/PBS-challenged mice. $^{**}P < .01$ and $^{***}P < .001$ of BPE/PBS-challenged mice with activated neutrophils, 2-way ANOVA.

Complementing our previous introduction of human neutrophils as APCs in allergic LPR, the results presented here provide insights in their mechanisms of T-cell activation. Our findings indicate that neutrophils fully activate T cells by costimulation of CD2 with CD58 and promote T-cell-mediated inflammation. We conclude that neutrophils, which are recruited in high numbers to inflammatory sites, also contribute to allergic inflammatory diseases through the activation of allergen-specific T cells.

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Association between rhinovirus species and nasopharyngeal microbiota in infants with severe bronchiolitis

To the Editor:

Bronchiolitis is the leading cause of hospitalization in US infants.¹ Rhinovirus (RV) is the second most common cause of severe bronchiolitis (ie, bronchiolitis requiring hospitalization) following respiratory syncytial virus (RSV).¹ RVs are RNA viruses consisting of more than 160 genotypes that are classified into 3 species (RV-A, RV-B, and RV-C).² RV-A and RV-C are more frequently found than RV-B in children with acute respiratory infections (ARIs) and wheezing illnesses.^{3,4} Emerging evidence suggests a complex interplay between viral infection, airway microbes, and host immune response in the pathobiology of ARI. Studies have shown that RV infection in children is associated with increased detection of pathogenic bacteria in the airways.^{5,6} Furthermore, detection of RV together with specific airway pathogens (eg, *Moraxella catarrhalis*) is associated with increased ARI and asthma symptoms.⁶ Recently, RV-A and RV-C were reported to differentially associate with detection of pathogenic bacteria in school-age children.⁷ However, no study has investigated the relationships between rhinovirus species and airway microbiota in infants, let alone infants with bronchiolitis. To address the knowledge gap, we examined the association between rhinovirus species and the nasopharyngeal airway microbiota determined by 16S rRNA gene sequencing in 774 infants with severe bronchiolitis.

This is a post hoc analysis of data from the 35th Multicenter Airway Research Collaboration (MARC-35) cohort study—a multicenter prospective cohort study of infants hospitalized for bronchiolitis. The details of the study design, setting, virus and microbiota measurements, and analysis are described in this article's Online Repository at www.jacionline.org. Briefly, 1016 infants (age <1 year) hospitalized for bronchiolitis were enrolled in 17 sites across 14 US states (see Table E1 in this article's Online

METHODS

Patients with allergy

Birch pollen-allergic patients showed rhinoconjunctivitis in spring, positive skin prick test results to birch pollen extract (ALK-ABELLÓ, Hørsholm, Denmark), and birch pollen-specific IgE of more than 0.35 kU_A/L (ImmunoCAP; Thermo Fisher Scientific, Uppsala, Sweden). The study was approved by the ethics committees of the Medical University of Vienna and conducted in accordance with the Declaration of Helsinki. Patients gave written informed consent.

Neutrophils

Neutrophils were isolated according to published protocols.^{E1} 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), osmotic red cell lysis of the granulocyte pellet, and a final immunomagnetic negative selection step using the human MACSxpress Neutrophil Isolation Kit (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) and resulted repeatedly in more 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) overnight before being used in the different experiments.^{E2}

Flow cytometry

Analyses were performed with a FACS Canto II system using FACS Diva (BD Biosciences, San Jose, Calif) and FlowJo software (TreeStar, Inc, Ashland, Ore). The following anti-human antibodies were used: CD16-phycoerythrin (PE) (clone 3G8), CD58-PE, CD70-PE, CD137L-PE, CD275-PE, HLA-DR-APC (clone L243), CD14-fluorescein isothiocyanate (FITC), CCR3-APC (all from Biolegend, San Diego, Calif), and CD66b-PerCP, CD3-V500, CD19-FITC, CD80-PE, and CD86-PE (all from BD Biosciences).

Allergen-specific T-cell cultures

Allergen-specific T cell clones were expanded from PBMCs of patients with allergy as described.^{E3} Briefly, PBMCs (1.5×10^6) were stimulated with recombinant Bet v 1 (5 μ g/mL, Biomay, Vienna, Austria) and expanded with recombinant human IL-2 (Roche, Basel, Switzerland). Cryopreserved HLA-DR-restricted Bet v 1-specific T-cell clones with known epitope specificity were thawed and expanded. T cells (2.5×10^4) were stimulated with irradiated (60 Gray) purified neutrophils (0.5×10^5) that had been cultured in the absence or presence of Bet v 1 (5 μ g/mL) in GM-CSF/IFN- γ /IL-3 for 24 hours. Low endotoxin azide-free anti-CD58 (Biolegend, 50 μ g/mL), anti-CD28 (CLB-CD28/1, clone 15EB, Sanquin, Amsterdam, The Netherlands, 30 μ g/mL), anti-CD2 (clone RPA-2.10, Biozym Scientific GmbH, Hessisch Oldendorf, Germany), and the respective isotype controls were added. Experiments were performed in triplicate. After 48 hours proliferative responses were determined by ³H-thymidine incorporation assay.

To test the induction of clonal anergy, allergen-specific T-cell cultures were first incubated with either 12-mer peptides (5 μ g/mL) or Bet v 1 (5 μ g/mL) without APC, PBMCs with Bet v 1 (5 μ g/mL), or neutrophils incubated in GM-CSF/IFN- γ /IL-3 with and without Bet v 1 (5 μ g/mL) for 24 hours. In some experiments, anti-CD28 (Sanquin) or isotype control was added. After

48 hours, proliferative responses were assessed to control the antigen-presenting activity of neutrophils (see Table E1). The remaining T cells were washed and allowed to rest for 72 hours in medium. Thereafter, PBMCs alone or with Bet v 1 (5 μ g/mL) or Bet v 1 plus IL-2 (4 U/mL, Roche) were added and proliferation was assessed after 48 hours by ³H-thymidine incorporation assay.

Mouse experiments

NSG mice were kept in animal facilities of the Medical University of Vienna under specific pathogen-free conditions. Six- to 8-week-old female mice were used. All animal procedures were reviewed by the Institutional Review Board of the Medical University of Vienna and approved by the Ministry of Sciences (BMWF-66.009/0040-WF/V/3b/2015).

NSG mice received 1 intraperitoneal injection of PBMCs (5×10^6) from a single patient plus birch pollen extract (BPE, 50 μ g) and human rIL-4 (1000 U, Miltenyi Biotec). The intraperitoneal administration of BPE and rIL-4 was repeated on day 7. On days 20 to 22, mice were anesthetized with isoflurane (Abbott, Wiesbaden, Germany) and challenged by intranasal application of BPE (20 μ g) or PBS. Autologous cytokine-activated neutrophils (5×10^6) were intraperitoneally injected on day 20 approximately 2 hours before intranasal challenges. On day 24, invasive measurement of AHR was performed on anesthetized, intubated, and mechanically ventilated mice in response to increasing doses of inhaled methacholine (1.6, 3.1, 4.8, 6.3, 12.5 mg/mL) with FinePointe Series Resistance and Compliance (Buxco, Data Sciences International, St Paul, Minn). Residual capacity was measured every 15 seconds following nebulization. The response to each single dose of methacholine was analyzed until a plateau phase was reached. The response to PBS was set to 100%.

Statistical analysis

The software SPSS10.01 (SPSS, Chicago, Ill) was applied to test the statistical significance of the results. Differences were considered significant if *P* was less than .05.

RESULTS

Allergen-specific T-cell clones and lines were incubated with Bet v 1 in the absence of APCs, GM-CSF/IFN- γ /IL-3-activated neutrophils, or PBMCs in the absence or presence of Bet v 1. Table E1 shows mean values of duplicates. No significant differences were found comparing counts per minute in T cells incubated with Bet v 1 without APC and those from cells incubated with neutrophils or PBMCs without allergen (*P* = .236, Friedman Test).

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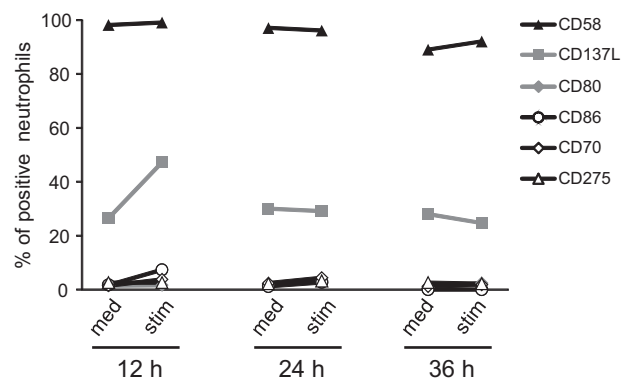


FIG E1. Expression of different costimulatory molecules on neutrophils. Neutrophils were stimulated with GM-CSF/IFN- γ /IL-3 (stim) or cultured in medium alone (med). Flow cytometry analyses were performed at indicated time points. *Symbols* indicate median values of 5 allergic individuals.

TABLE E1. T-cell responses in the 1° round of stimulation

APC Stimulus	Bet v 1	Neutrophils		PBMCs	
		Medium	Bet v 1	Medium	Bet v 1
TCC1	3,242	9,781	24,380	5,720	18,410
TCC2	148	243	9,662	182	14,850
TCL1	1,647	1,326	23,356	1,110	39,741
TCL2	1,456	415	28,483	1,720	27,850
TCL3	1,254	250	26,715	4,488	45,237
TCL4	2,511	3,934	12,475	3,128	50,618
TCL5	765	772	19,066	241	23,350
TCL6	304	1,437	13,598	881	26,034
TCL7	1,368	2,059	10,327	5,534	25,993

cpm, Counts per minute; TCC, T-cell clone.
Values are in *cpm*.