Subcutaneous immunotherapy using modified Phl p5a-derived peptides efficiently alleviates allergic asthma in mice

To the Editor,

Allergen-specific immunotherapy (AIT) is a treatment for allergic airway disease that induces long-term tolerance by repeated allergen injections and induced regulatory T (reg) cells at the expense of Th2 cells. Nonetheless, AIT requires large amounts of allergens that need to be administered over prolonged periods of time and treatment can induce severe side effects. Allergen-derived peptides, encoding the dominant T-cell epitopes, lack the capacity to bind IgE and are a safe alternative. Unfortunately, treatment response to peptide AIT is suboptimal for most allergens. Peptides may have a short half-life after administration and need to be phagocytosed by DCs for presentation to T cells to exert their tolerogenic activity. We have previously designed a novel strategy to increase uptake and presentation of peptides by DCs, while also influencing their tolerogenic phenotype.

Dendritic cells (DCs) express sialic acid-binding Ig-like lectins (siglecs), which function as endocytic receptors. In mice, sialylation of antigens has been shown to instruct DCs to manifest an anti-(siglecs), which function as endocytic receptors. In mice, sialylation of antigens has been shown to instruct DCs to manifest an anti-

In this study, we provide evidence that the use of Phl-p5a peptides can achieve a significant decrease in eosinophils in BALF and lung tissue compared with controls, the use of sialylated peptides did achieve a significant decrease in eosinophils in both BALF and lung tissue compared with sham-treated mice (Figure 2A). We observed a relative suppression of eosinophil numbers by Sia-peptide SCIT of 7-fold for BALF and 6-fold for lung compared with controls (Figure 2B).

Next, we assessed T-cell responses in the peptide SCIT-treated mice. Although ILC2s numbers were unaffected by peptide SCIT in our model, the number of Th2 (GATA3⁺) cells was significantly decreased in lung tissue after both GP-SCIT and Sia-peptide SCIT treatment as compared to controls (Figure S2B, 1E). Interestingly, Foxp3⁺ Tregs were increased in lung tissue only after Sia-peptide SCIT, as compared to both controls and mice receiving the unmodified peptide (Figure 1E). We observed decreased levels of IL-5 in BALF of GP-SCIT mice, while IL-10 and IL-13 were not affected (Figure S1E). Moreover, we found significantly decreased levels of IL-4, IL-13, IL-33, and IL-17 in lung tissue from GP-SCIT and/ or (Sia)-peptide SCIT-treated mice (Figures 2C, S2D).

Last, we evaluated the Th2 activity by measuring cytokine production in GP-pulsed ex vivo cultured lung cell suspensions and observed a trend toward decreased levels of IL-5, but not IL-13, in cells from mice treated with GP-SCIT or Sia-peptide SCIT (Figures 2D, S2C). In addition, levels of IL-10 were increased in cells from GP-SCIT and unmodified peptide SCIT mice (Figure 2D). In contrast, TGF-β1 levels were only significantly increased in lung cell suspensions from mice that had received unmodified peptide SCIT, although differences between groups were small (Figure S2C).

In this study, we provide evidence that the use of Phl-p5a peptides for SCIT is effective in suppressing asthmatic manifestations
Pollen Allergen Phleum pratense 5a

**Sequence**

| MW (g/mol) | Pep/g415de 1 P188-202 | CVTEAAFNDEIKAST |
|------------|------------------------|------------------|
| 1687       |                        |                  |

| MW (g/mol) | Pep/g415de 2 P206-220 | CVYKIPALEAARK |
|------------|------------------------|----------------|
| 1832       |                        |                |

**In vitro Co-cultures**

- BMDCs: $5 \times 10^5$/mL
- Refresh medium: 500 µL
- Harvest BMDCs + loading: CC 1:3
- CD4+ T cells: flow cytometry
- CFSE labeled: ELISA

**In vivo Animal model**

| Group | Sensitization | SCIT | Challenge |
|-------|---------------|------|-----------|
| NC    | 5 kSQ/ Alum   | PBS  | PBS       |
| PC    | 5 kSQ/ Alum   | PBS  | 25 kSQ    |
| GP    | 5 kSQ/ Alum   | 300kSQ Alum | 25 kSQ |
| 10pep | 5 kSQ/ Alum   | 10 µg peptide | 25 kSQ |
| 10Spep| 5 kSQ/ Alum   | 10 µg Sia-peptide | 25 kSQ |

**Resistance (cmH₂O s/mL)**

- Metacholine (µg/kg)
- GATA3+ in lung (%CD4+ Tcells)
- FoxP3+ in lung (%CD4+ Tcells)
FIGURE 1  A, Outline of (Sia-) peptides 1 and 2 and co-cultures of GP- or (Sia)-peptide stimulated BMDCs with CFSE-labeled CD4⁺ T cells. B, FoxP3⁺ T cells and CFSELow T cells (both as % of total single living CD3⁺CD4⁺ T cells) and levels of TGF-β1 and IL-5 pg/mL, n = 12 (mean ± SEM). C, Outline of the SCIT protocol and treatment groups. D, Airway resistance (R in cmH₂O.s/mL) at day 51. E, GATA3⁺ and FoxP3⁺ T cells in lung single cells (% live cells) (mean ± SEM). *P < 0.05, **P < 0.01, and ***P < 0.005 compared to unsialylated-peptide.
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shown that AIT modifies CD4+ T cells in an epitope-specific manner, as the reference GP-SCIT using crude extracts. It has recently been suggested that sialylation of the peptides used in SCIT resulted in increased T-cell activation, enhanced numbers of FoxP3+ T cells both in vitro and in vivo, and achieved increased suppression of Th2 cells and eosinophilic inflammation in lung tissue compared to unmodified peptides.

Whereas the GP-SCIT model is based on the whole GP-extract encompassing all allergens, our peptide SCIT uses two short synthetic peptides based on the major T-cell epitopes in Phl p5a,7 which might explain why peptide SCIT is not as effective on all parameters as the reference GP-SCIT using crude extracts. It has recently been shown that AIT modifies CD4+ T cells in an epitope-specific manner, resulting in depletion of those T-cell clones that were specifically increased in allergic patients.8 Therefore, optimal peptide SCIT might require peptide sequences from all major GP allergens. Moreover, since T-cell epitopes are dependent on MHC use, a wider variety of T-cell epitopes will be needed to obtain a formulation that can be applied in most GP allergic individuals, while keeping the peptides as short as possible (20 AA) to prevent IgE cross-linking and adverse events.7 Consequently, the net dosage of each individual peptide in the mixture used will be relatively low. We postulate that sialylation of the peptides used in such formulations is a valuable approach to increase efficiency of peptide SCIT.

In conclusion, the use of sialylated allergen-derived peptides encoding T-cell epitopes is a promising approach toward efficient and safe AIT treatment regimens.

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CONFLICTS OF INTEREST

The authors LH, RF, MA, WAdJ, AP, HV, WWJU, YvK, and MCN confirm that there are no conflicts of interest to disclose.

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Subsetting reveals CD16<sup>high</sup> CD62L<sup>dim</sup> neutrophils in chronic rhinosinusitis with nasal polyps

To the Editor:

Neutrophils are continuously present in the nasal mucosa, and frequently overlooked when listing potential factors involved in the pathology of chronic rhinosinusitis with nasal polyps (CRSwNP). The recent discovery of different neutrophil subsets, with diverse roles during inflammatory conditions, motivates a re-examination of their role in this disease. In the present investigation, the manifestation of neutrophil subsets was determined in CRSwNP patients, and the ability of CD16<sup>high</sup> CD62L<sup>dim</sup> neutrophils to phagocytose and form reactive oxygen species (ROS) was examined in vitro (Methods in the online Supporting Information).

An increase in eosinophil fractions in blood and nasal tissue from patients with CRSwNP was confirmed (Figure S1 A,B). This was not seen for neutrophil fractions (Figure S1 C,D). The remaining CD45<sup>+</sup> cells constituted mostly of monocytes, lymphocytes, macrophages, and basophils (data not shown). When the neutrophil population was subdivided according to the individual cellular expression of FcyRIII (CD16) and L-selectin (CD62L), three distinct sets of neutrophils could be demonstrated as previously described: CD16<sup>dim</sup> CD62L<sup>high</sup> (recently released from the bone marrow, regarded immature), CD16<sup>high</sup> CD62L<sup>high</sup> (mature neutrophils), and CD16<sup>high</sup> CD62L<sup>dim</sup> (thought to be more activated, displaying a hyper-segmented nuclear morphology accordingly) (Figure 1 A,B) (see full gating procedures shown in Figure S2 A,B).<sup>1</sup> The neutrophil subset CD16<sup>high</sup> CD62L<sup>dim</sup> was significantly higher in polyp mucosa (Figure 1C). The mature neutrophil subset, CD16<sup>high</sup> CD62L<sup>high</sup>, dominated in control nasal mucosa (Figure 1C). Nasal polyps exhibited a higher ratio of CD16<sup>high</sup> CD62L<sup>dim</sup> / CD16<sup>high</sup> CD62L<sup>high</sup> neutrophils compared with control nasal mucosa (Figure 1D). The normal mature neutrophil subset, CD16<sup>high</sup> CD62L<sup>high</sup>, dominated in peripheral blood in both CRSwNP patients and healthy controls (Figure S2 C).

The protein integrin CD11b forms a heterodimer with CD18 and mediates inflammation by regulating leukocyte adhesion and migration through binding to the cell surface molecule ICAM-1. CD11b is therefore often used as a marker of neutrophil activation and was presently shown to be upregulated on systemic and local neutrophils from CRSwNP patients (Figure S3). The expressed levels of CD11b were markedly increased on CD16<sup>high</sup> CD62L<sup>high</sup> and CD16<sup>high</sup> CD62L<sup>dim</sup> neutrophils in patient blood, nasal mucosa, and polyps (Figure 1 E-G). No such shift was seen in healthy controls (Figure S4 A-C). Correspondingly, ICAM-1 (full gating procedures shown in Figure S5) was upregulated on epithelial cells from CRSwNP patients, suggesting a mechanism for a neutrophil-epithelial cell interaction in nasal polyps (Figure 1 H,I). Recent use of topical steroids did not seem to affect the expression of CD16, CD62L, CD11b, or ICAM-1 (data not shown).

Neutrophils isolated from blood from CRSwNP patients were treated in vitro with LPS for 1 hour for activation (Figure 2A) resulting in neutrophils highly differentiating to the CD16<sup>high</sup> CD62L<sup>dim</sup> subset (Figure 2A, LPS(+), S aureus BP(-)). S aureus bioparticles (ie, biomolecules from killed bacteria) were subsequently added. LPS-treated neutrophils displayed a greater capacity to phagocytose S aureus bioparticles (Figure 2B). The levels were comparable with positive control assays with ionomycin (data not shown). Assays with neutrophils and DHR-123 revealed more ROS formation with LPS-treated neutrophils (constituting mostly of CD16<sup>high</sup> CD62L<sup>dim</sup> neutrophils, Figure 2A) (Figure 2C). These data indicate that CD16<sup>high</sup> CD62L<sup>dim</sup> neutrophils are prone to phagocytose bacterial particles and induce oxidative burst (Figure 2D).

In 2012, Pillay et al<sup>2</sup> identified the existence of different neutrophil subsets. The CD16<sup>high</sup> CD62L<sup>dim</sup> subset, regarded activated, exhibited T cell-suppressing capacities and decreased adherence to the endothelium during induced acute inflammatory conditions.<sup>2</sup> The same subset has been shown to enhance bradykinin-dependent contraction of smooth muscle cells in airways, suggesting a role for neutrophils in asthmatic airway hyperactivity.<sup>3</sup> More than 10% of the Western population suffers from CRS, and recent findings have stressed the immunological complexity of the disease, making it obvious that the concept of Th1/Th2 inflammation is not sufficient to explain the pathology. S aureus is found intramucosally, and colonizing the mucosal surface and its products has been demonstrated within the polyp tissue-stimulating immune responses.<sup>4</sup> Neutrophils generate ROS during phagocytosis and in response to soluble agonists. This functional response, termed oxidative burst, contributes to host defense and is together with phagocytosis a critical antimicrobial mechanism.