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Effector cell signature in peripheral blood following nasal allergen challenge in grass pollen allergic individuals

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basophils; IL-4; nasal allergen challenge; seasonal allergic rhinitis; T cells.

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Abstract

Background: Several studies have demonstrated the time course of inflammatory mediators in nasal fluids following nasal allergen challenge (NAC), whereas the effects of NAC on cells in the periphery are unknown. We examined the time course of effector cell markers (for basophils, dendritic cells and T cells) in peripheral blood after nasal grass pollen allergen challenge.

Methods: Twelve participants with seasonal allergic rhinitis underwent a control (diluent) challenge followed by NAC after an interval of 14 days. Nasal symptoms and peak nasal inspiratory flow (PNIF) were recorded along with peripheral basophil, T-cell and dendritic cell responses (flow cytometry), T-cell proliferative responses (thymidine incorporation), and cytokine expression (FluoroSpot assay).

Results: Robust increases in nasal symptoms and decreases in PNIF were observed during the early (0–1 h) response and modest significant changes during the late (1–24 h) response. Sequential peaks in peripheral blood basophil activation markers were observed (CD107a at 3 h, CD63 at 6 h, and $CD203c^{bright}$ at 24 h). T effector/memory cells ($CD4^+CD25^{lo}$) were increased at 6 h and accompanied by increases in $CD80^+$ and $CD86^+$ plasmacytoid dendritic cells (pDCs). *Ex vivo* grass antigen-driven T-cell proliferative responses and the frequency of $IL\text{-}4^+CD4^+$ T cells were significantly increased at 6 h after NAC when compared to the control day.

Conclusion: Basophil, T-cell, and dendritic cell activation increased the frequency of allergen-driven $IL\text{-}4^+CD4^+$ T cells, and T-cell proliferative responses are detectable in the periphery after NAC. These data confirm systemic cellular activation following a local nasal provocation.

Seasonal allergic rhinitis (SAR) affects up to 25% of the population in Westernized countries and represents a major socio-economic and health burden (1–3). SAR is a T helper 2 (Th2) cell-driven IgE-dependent inflammatory disease, characterized by the chronic inflammation of the lining of the nasal mucosa (4, 5). Following nasal allergen provocation, typical symptoms in the first 30 min include immediate pruritus and sneezing followed by clear watery nasal discharge and congestion with/without eye symptoms. In a proportion of individuals, this early response is followed by a late nasal response that manifests largely as ongoing modest nasal obstruction with persistent falls in PNIF from 1 to 24 h (6, 7).

During the initial sensitization phase in SAR, immature dendritic cells (DCs) that reside in the upper layers of the epithelium and lamina propria of the airways capture antigens, mature and migrate to the draining lymph nodes, where they prime and activate naïve T cells (8). This results in the production of Th2 cytokines, such as interleukin-4 (IL-4) and

Abbreviations
AUC, area under the curve; EPR, early-phase response; LPR, late-phase response; NAC, nasal allergen challenge; pDCs, plasmacytoid dendritic cells; PNIF, peak nasal inspiratory flow; SAR, seasonal allergic rhinitis; SQ-U, standard quality unit; TNSS, total nasal symptom scores; VAS, Visual Analog Scale.

interleukin-13 (IL-13), which lead to the differentiation and clonal expansion of naïve T cells to allergen-specific Th2 cells (9). IL-4 and IL-13 have been reported to act on memory B cells to produce IgE locally in the nasal mucosa (10). These cytokines upregulate vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, which results in the infiltration of eosinophils, T cells and basophils into the nasal mucosa. Plasmacytoid dendritic cells (pDCs) have been shown to efficiently drive allergen-dependent Th2 memory responses, suggesting that they play an active role in the allergic reaction (11–14) although this is somewhat controversial.

We (15) and others (7, 16–18) have described the time course of inflammatory mediators in nasal secretions following nasal allergen challenge (NAC) (15–18). However, the effects of NAC on peripheral blood cells (in terms of composition and activation states) have not been well delineated. In this study, human NAC was used to determine cellular changes in the peripheral blood. To control for diurnal variation in any of the end points, and also for possible effects of the slight trauma of repeated nasal sampling, we included a control day following nasal provocation with the allergen diluent alone. We tested the hypothesis that NAC is associated with priming and activation of accessory cells, including basophils and pDCs, and an increase in the frequency of grass pollen-specific IL-4$^+$ T cells during the late nasal response. Changes in immunologic parameters were related to changes in nasal symptoms and peak nasal inspiratory flow (PNIF) during both early (0–1 h)- and late (1–10 h)-phase responses (EPR and LPR) after challenge.

Methods

Subjects

Participants were selected on the basis of moderate–severe SAR symptoms according to the ARIA classification (19). Subjects had a positive skin prick test (wheal >5 mm) to *Phleum pratense,* timothy grass, pollen extract (ALK Soluprick®; ALK-Abelló, Hørsholm, Denmark) and positive timothy grass pollen-specific IgE (>0.70 kU/l). Exclusion criteria included a clinical history of other allergies (including symptoms during the tree pollen season), chronic asthma, a prebronchodilator FEV_1 <70% of predicted value at screening or baseline visit, or other significant medical illnesses. All subjects provided written informed consent. The study was approved by the South West London REC3 Research Ethics Committee and the Research offices of Imperial College London and the Royal Brompton and Harefield Hospitals NHS Foundation Trust.

Study design

This was an open single-center study conducted outside the pollen season. The demographic data and skin prick test, as well as total IgE and specific IgE levels to timothy grass for study participants (CAP FEIA system; Thermo-Fisher, Uppsala, Sweden), are included in Table 1. During a screening visit, a questionnaire was completed and skin prick tests were performed with a panel of common inhaled allergens (*D. pteronyssinus* [house dust mite], mixed grass pollens, timothy grass, mixed tree pollens, birch pollen, mixed weed pollens, dog hair, cat hair, horse hair, Aspergillus fumigatus, Cladosporium, and Alternaria species, Soluprick; ALK-Abelló, Horsholm, Denmark). After 7 ± 2 days, participants underwent a control nasal provocation with the allergen diluent only. After another 14 ± 2 days, participants returned for the active nasal allergen provocation. On each challenge day, participants rested and acclimatized for at least 30 min prior to the provocation. Nasal challenge with control or timothy grass pollen (Aquagen, *P. pratense*; ALK) was administered using a nasal Bidose applicator (Valois S.A., Marly le Roi, France), which delivers two 100-μl actuations. The challenge consisted of three allergen doses or matched diluent controls, given at 10-min intervals: 1500, 3000 and 10 000 BU (biological units)/ml, with one actuation of each dose applied to each nostril. This gave a total dose of 2900 BU/ml, equivalent to approximately 1.16 μg major allergen, *Phleum p5* (15). Total nasal symptom scores (TNSS, scale 0–12), visual analogue scale (VAS, scale 0–10 cm), and PNIF (liters/min) were recorded at baseline, 10 min after each dose, and then again at 3, 6, and 24 h after challenge. TNSS comprised the sum of the scores of 4 nasal symptoms (nasal itch, sneezing, discharge, and congestion/obstruction), scored from 0 to 3 (0 = none, 1 = mild, 2 = moderate, and 3 = severe). Peripheral blood (60 ml) was collected before nasal challenge and at 3, 6, and 24 h following challenge.

Table 1 Patient demographics

Parameter	Study 1, $n = 12$	Study 2, $n = 14$
Gender (M : F)	7 : 5	8 : 6
Age (mean, range)	39 (21, 66)	34 (29, 66)
Timothy grass-sIgE, kU_A/l (mean, SD)	32.9 ± 39.1	29.3 ± 35.6
Total IgE, kU/l (mean, SD)	238.3 ± 318.3	251.0 ± 259.0
Skin LPR to allergen (mm^2)	9.9	10.3

Distribution of age, gender, specific IgE, and skin late-phase response (LPR).

Immunological tests

The antibodies used in this study are listed in Appendix S1 (Supporting information). *In vivo* basophil activation status was determined using 8-color multiparameter flow cytometric analysis. Briefly, heparinized whole blood (100 μl) was immunostained with anti-human CD3, CD303, CD294 (CRTh2), CD203c, CD63, and CD107a antibodies or relevant isotype controls. Whole-blood basophils stimulated with anti-human IgE (100 ng/ml) or *P. pratense* extract (100 ng/ml) were used as positive controls. Standard BD Bioscience lysing, washing, and fixative procedures were used followed by acquisition on the BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). Nonactivated and activated basophils were

identified as $CD203c^{dim}$ and $CD203c^{bright}CRTh2^+$ cells, respectively. Additionally, activated basophils were also identified as $CD63^+$ and $CD107a^+$ $CRTh2^+$ cells. pDCs were immunostained with anti-human CD123, CD303 (BDCA2), HLA-DR, CD80, CD86 antibodies, or relevant isotype controls. T effector cells were immunostained with anti-human CD45, CD3, CD4, CD25 (IL-2 receptor), CD152 (CTLA-4), CD294 (CRTh2), and CD194 (CCR4) antibodies or relevant isotype controls. Peripheral blood mononuclear cells were isolated from fresh heparinized venous blood via centrifugation over Ficoll gradients (Histopaque-1077; Sigma-Aldrich, Gillingham, UK). Furthermore, to assess *in vitro* antigen-driven $CD4^+$ T-cell proliferation, negatively selected $CD4^+$ T cells from PBMCs (1×10^5 cells/well) were co-cultured at 1 : 1 ratio with irradiated antigen-presenting cells (non-$CD4^+$ cells) at 3000 rads in the presence of 3 μg/ml *P. pratense* (ALK-Abelló, Hørsholm, Denmark). Cells were cultured for 7 days at 37°C in 5% CO_2 and pulsed for the last 18 h with titrated methylthymidine (MP Biomedicals, Costa Mesa, CA, USA). Frequency of *P. pratense*-specific IL-4^+CD4^+ and IFN-γ^+CD4^+ T cells was measured by dual IL-4 and IFN-γ FluoroSpot assay (Diaclone, Besancon, France). For the measurements of T-cell proliferation (PerkinElmer, Groningen, Nederland) and cytokine secretion (FluroSPOT) only, we recruited a second cohort of 14 participants with seasonal rhinitis and performed these measurements before and at 6 h after both diluent and allergen challenges separated by an interval of 14 days, to observe the effects of diurnal variation on these measurements (Table 1, study 2). Further methodological details are available in the Supporting information.

Statistics

Within-group analyses were performed with the two-tailed Wilcoxon's matched pairs signed-rank test. Time course analyses were achieved by means of Friedman and Dunn's correction for repeated nonparametric measurements. The statistical software package used was GraphPad Prism, version 6 (GraphPad Software Inc., San Diego, CA, USA). P values < 0.05 were considered statistically significant.

Results

Nasal symptoms and peak inspiratory flow after NAC

Nasal allergen challenge resulted in a dose-dependent increase in nasal symptoms (TNSS), visual analog scores (VAS), nasal blockage and fall in PNIF. No changes were seen after diluent challenge. Area under the curve analyses showed significant differences between allergen and diluent challenges for TNSS ($P = 0.0005$) and PNIF ($P = 0.01$), VAS ($P = 0.001$) and nasal blockage ($P = 0.002$; Fig. 1A–D).

NAC is associated with increased *in vivo* peripheral blood basophil activation

Basophil activation status was assessed by the expression of the surface markers CD107a, CD63, and CD203c on $CRTH2^+$ (CD294) basophils in whole blood obtained before and at intervals after challenge (Fig. 2). On the NAC day, $CD107a^+CRTh2^+$ basophils were significantly elevated

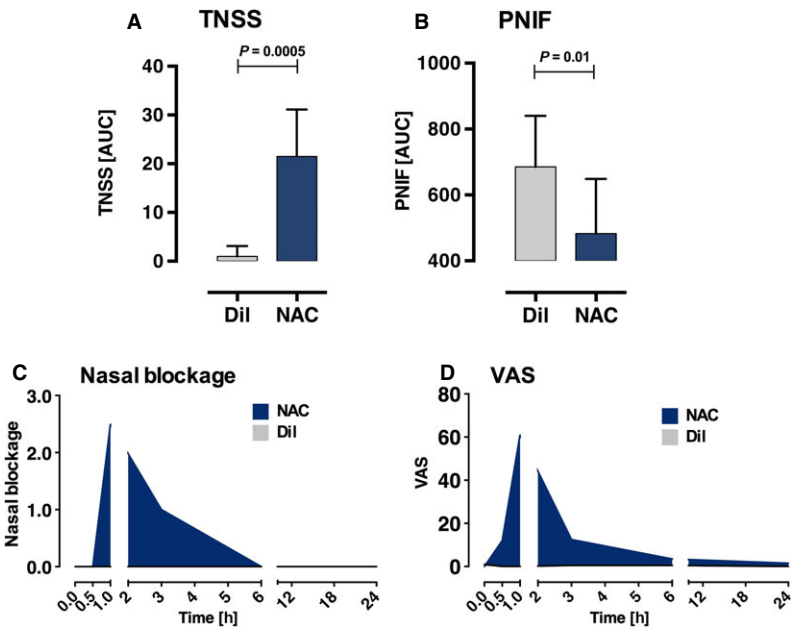

Figure 1 Effects of nasal allergen challenge on early and late allergic responses. (A) TNSS (AUC), (B) PNIF (AUC), (C) nasal blockage, and (D) VAS were measured following control (diluent, DIL) or nasal timothy grass pollen allergen challenge (NAC) in 12 patients with SAR. Antigen dose titration was performed for 10–30 min. Data are shown as median (IQR) for TNSS and PNIF and median for VAS and nasal blockage (AUC, area under the curve; NAC, nasal allergen challenge; PNIF, peak nasal inspiratory flow; TNSS, total nasal symptom scores; VAS, Visual Analog Scale).

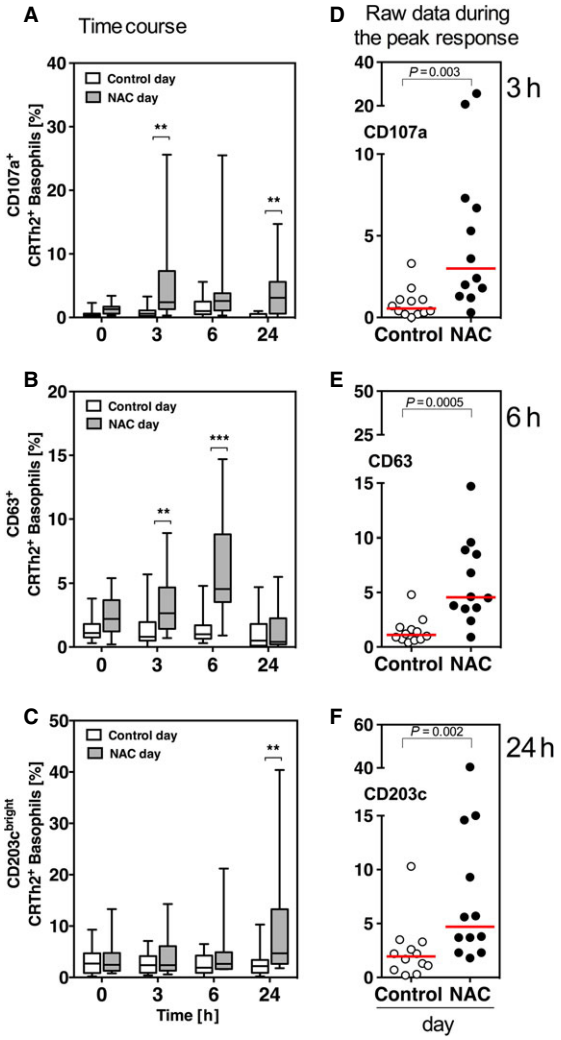

Figure 2 Increased expression of CD107a, CD63, and CD203c^{bright} on CRTH2$^+$ basophils after nasal allergen challenge. (A–C) Time course of basophil activation status in peripheral blood as measured by the surface markers CD107a, CD63, and CD203c^{birght} (*P* value represents increases from baseline). (D–F) Increased proportion of CD107a$^+$(3 h), CD63$^+$ (6 h), and CD203c^{bright} CRTH2$^+$ basophils (24 h) on nasal allergen challenge (NAC) day compared to control day. Bars represent medians. $^{**}P < 0.01$ and $^{***}P < 0.001$ (*P* value represents difference between control and NAC day).

compared to baseline peaking at 3 h after the challenge and remaining elevated at 6 and 24 h (3 h, $P = 0.0008$; 6 h, $P = 0.008$; 24 h, $P = 0.005$; Fig. 2A, D). CD63$^+$CRTh2$^+$ basophils peaked at 6 h ($P = 0.002$; Fig. 2), and an increase in CD203c^{bright} CRTh2$^+$ basophils was observed at 6 h ($P = 0.04$) and 24 h ($P = 0.0002$; Fig. 2) when compared to baseline. In contrast, no diurnal changes in the proportion of CD107a$^+$, CD63$^+$, nor CD203c^{bright}CRTh2$^+$ basophils were observed in venous samples obtained at the same intervals on the control day following provocation with the allergen diluent.

Activation of pDCs and CD4$^+$ T cells after NAC

We investigated the effects of NAC on peripheral CD123$^+$CD303$^+$pDC activation as measured by surface expression of CD80 and CD86 (Fig. 3). The proportions of CD80$^+$CD123$^+$CD303$^+$ pDCs ($P = 0.008$) and CD86$^+$CD123$^+$CD303$^+$ pDCs ($P = 0.02$) were increased at 6 h following NAC compared to baseline as was the proportion of CD80$^+$CD86$^+$ (double-positive) pDCs ($P = 0.001$). No changes in the proportion of CD80$^+$ and CD86$^+$ pDCs were observed following the control diluent challenge up to 24 h, and the changes from baseline in CD80$^+$, CD86$^+$, and CD80$^+$CD86$^+$ pDCs at 6 h after allergen challenge were significantly larger compared to those observed on the control day ($P = 0.0004$, $P = 0.05$, and $P = 0.009$; Fig. 3A, B). Th2 cells characterized as CD4$^+$CRTh2$^+$ T cells were increased at 6 h ($P = 0.002$) and remained elevated at 24 h ($P = 0.01$) compared to baseline, and these changes were significantly larger compared to those observed at 6 h on the control day ($P = 0.04$; Fig. 4). In contrast, CD4$^+$CCR4$^+$ T cells were significantly reduced at 6 h on the NAC day when compared to the control day ($P = 0.01$; Fig. 4). The increase in activated pDCs following NAC was observed to closely parallel increases in CD4$^+$CD25^{lo} and CD4$^+$CD152$^+$ T cells, which were significant at 6 h following NAC compared to baseline ($P < 0.0001$, $P = 0.0036$). Moreover, these increases in CD4$^+$CD25^{lo} and CD4$^+$CD152$^+$ T cells remained significant when compared to corresponding values at 6 h on the control day ($P = 0.03$; $P = 0.001$; Fig. 4).

Ex vivo grass pollen-induced CD4$^+$ T-cell proliferative response and frequency of IL-4$^+$CD4$^+$ T cells are increased following NAC

In addition to testing whether NAC was associated with *in vivo* phenotypically activated CD4$^+$ T cells, we explored the time course of *ex vivo* timothy grass pollen extract-driven CD4$^+$ T-cell proliferative responses using T cells sampled at baseline, 3, 6, and 24 h after *in vivo* NAC. *Ex vivo* T-cell proliferative responses were tested using thymidine incorporation after 6-day T-cell co-culture with allergen, and the frequency of allergen-driven IL-4$^+$CD4$^+$ T cells was tested by the use of FluoroSpot assays. We observed a significant increase in CD4$^+$ T-cell proliferative response at 6 h ($P = 0.01$) and IL-4$^+$CD4$^+$ T cells ($P = 0.003$) at 6 h but not at 24 h after allergen challenge when compared to baseline (Fig. 5A, B).

To exclude the possibility that these changes in T-cell proliferative responses and increased T-cell IL-4 secretion were due to diurnal variation, we repeated the study after an interval of 4–6 months and recruited 14 additional grass allergic volunteers who underwent nasal allergen or control challenge. This was because in view of the requirement for large volumes of blood to be sampled at several time points for CD4$^+$ T-cell isolation, it was not possible to take additional blood on the control day for these measurements to be performed. In the replication study, venous blood samples for T-cell proliferation and FluoroSpot assays were confined to prechallenge and 6 h postchallenge on allergen and control

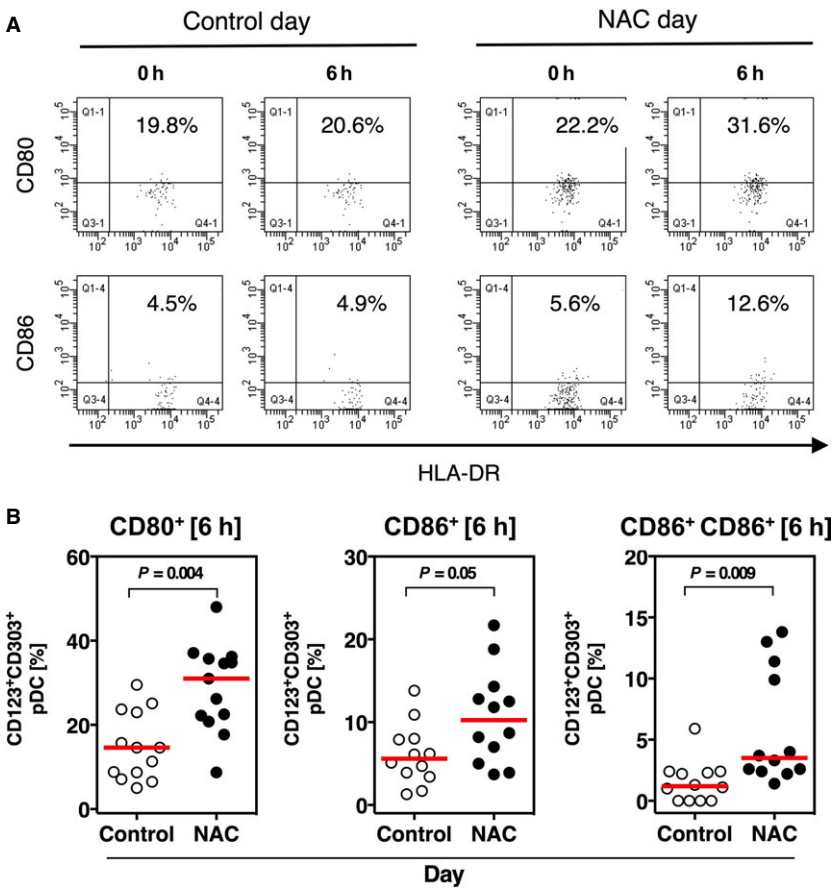

Figure 3 Priming and activation of plasmacytoid dendritic cells (pDCs) in the periphery following nasal allergen challenge. (A) Dot plot from representative patient sample (n = 12) showing increased $CD80^+$ and $CD86^+$ BDCA-2^+ ($CD303^+$) pDCs at 6 h on the nasal allergen challenge (NAC) but not on control day. (B) Increased proportion of $CD80^+$, $CD86^+$, and $CD80^+CD86^+$ BDCA-2^+ ($CD303^+$) pDCs (change from baseline) in peripheral blood on a NAC and control day. Bars represent medians.

days. In this study, we reproduced our previous findings. Thus, we showed that *ex vivo* grass pollen-driven $CD4^+$ T-cell proliferative responses were significantly increased at 6 h after allergen challenge when compared to baseline (P = 0.01; Fig. 5C, left hand panel). Similarly, IL-4^+CD4^+ T cells were also increased at 6 h after NAC compared to baseline (P = 0.002; Fig. 5C, middle panel), whereas no significant changes in T-cell proliferative responses nor IL-4^+CD4^+ T cells were observed on the control day after diluent challenge. Furthermore, we did not observe any significant change in the frequency of IFN-γ^+CD4^+ T cells either after nasal allergen or on the control day (Fig. 5C, right hand panel). These data show unequivocally that changes in allergen-driven $CD4^+$ T-cell proliferation and the frequency of IL-4^+CD4^+ cells were driven by nasal allergen provocation and independent of any effects of any diurnal variation in these parameters.

Discussion

We have shown that nasal grass allergen provocation conducted outside the grass pollen season is associated with changes in the activation status and phenotypic markers of peripheral blood cells in individuals with SAR. The data support the concept that exposure to allergen at the level of the respiratory mucosa induces systemic alterations to various components of the immune system, including cells that are responsible for antigen presentation, cells that direct immune polarization, and effector cells. The data further underline the notion that allergic rhinitis is both a local and a systemic disease. Moreover, we suggest that these changes can be monitored in a clinical setting and potentially used to monitor effects of therapy for SAR. Induction of nasal symptoms and falls in PNIF by NAC were followed by the activation of peripheral blood basophils and by priming and activation of both pDCs and $CD4^+$ $CD25^{lo}$ or memory T cells. The proliferative response of T cells and the frequency of Th2 (IL-4^+CD4^+) cells following *in vitro* allergen stimulation were increased 6 h after *in vivo* NAC. As no changes in T-cell proliferation nor cytokine production were observed at 6 h after the diluent challenge, the changes after NAC could not be explained by natural diurnal rhythm. In contrast, no change in the frequency of IFN-γ^+CD4^+ T cell was observed.

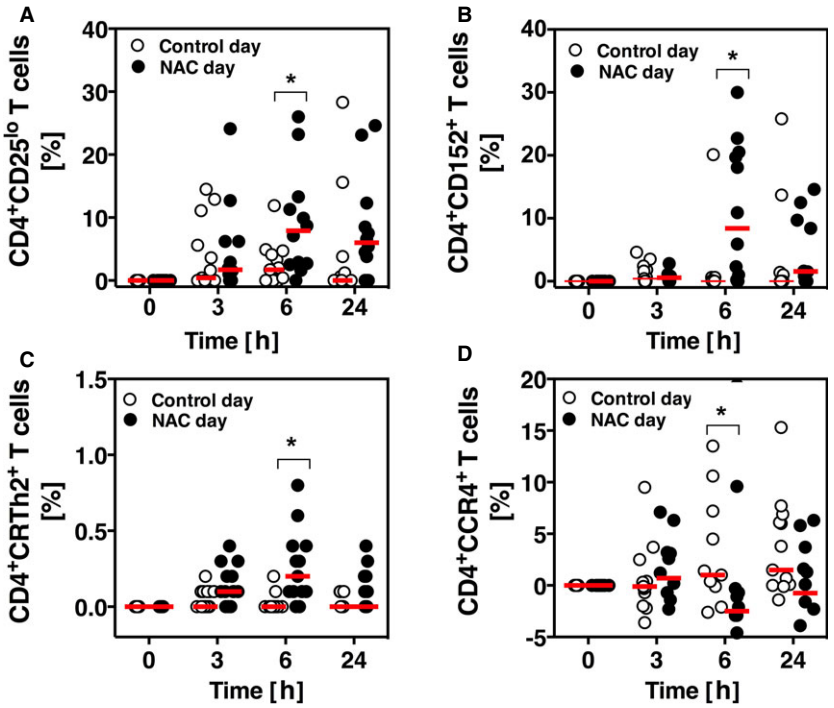

Figure 4 Activation of Th2 cells in the periphery following nasal allergen challenge. Time course of (A) $CD4^+CD25^{lo}$, (B) $CD4^+CD152^+$, (C) $CD4^+CRTh2^+$, and (D) $CD4^+CCR4^+$ T cells on nasal allergen challenge (NAC) and control day. Bars represent medians. $*P < 0.05$.

In this study, the NAC model was used, essentially because it is a well-established model with the benefit of reproducing a direct allergen exposure in a controlled and standardized setting. The increases in TNSS, VAS, and nasal obstruction and the reduction in PNIF are in agreement with previously reported findings (20–22). Unlike the clear-cut biphasic clinical manifestations observed during allergen-induced late responses in the skin and lung, the late responses in the nose are modest and biphasic individual responses are infrequent, resulting in an overall sustained rather than biphasic pattern for the group.

It is not well established whether allergen challenge of the nasal mucosa might result in priming and activation of allergic effector cells in the peripheral blood. In our study, a time course of *ex vivo* allergen-induced basophil responsiveness and $CD123^+CD303^+$ pDC and $CD4^+$ T-cell activation in the peripheral blood was assessed by 8-color multiparametric flow cytometry. The proportions of $CD107a^+$-, $CD63^+$-, and $CD203c^{bright}$-activated basophils were increased, respectively, at 3, 6, and 24 h after NAC. Activated basophils have been reported to produce TSLP, and IL-4 and these Th2 driving mediators may play a role in late allergic responses.

The proportion of $CD123^+CD303^+$ pDCs did not change at any time point. However, $CD123^+CD303^+$ pDCs expressing activation markers such CD80 and CD86 were significantly increased at 6 h on the NAC but not on the control day. These findings suggest that following allergen exposure in the nasal mucosa, pDCs mature, migrate to the periphery, and are primed to activate T cells. pDCs have been shown to efficiently drive allergen-dependent Th2 memory responses, suggesting that they play an active role in the allergic reaction (14). In support of this concept, we observed a parallel increase in the proportion of activated $CD4^+CD25^{lo}$ T cells (memory T cells) at 6 h postallergen challenge. Interestingly, $CD152^+CD4^+$ T cells were also increased post-NAC. These findings suggest that CD152 either is a transient marker of T-cell activation or possibly could play a role in counterbalancing TCR-mediated activation of T cells. It is important to note that these increases in CD25 and CD152 expression were significant but modest and reflect an *in vivo* activation of Th2 cells. Moreover, $CD4^+CRTh2^+$ T cells were increased at 6 h. Interestingly, $CCR4^+CD4^+$ T cells were actually decreased at 6 h on the NAC but not on the control day. These findings could possibly be explained by $CCR4^+$ T cells being recruited into the periphery, possibly to the nasal mucosa, while $CRTh2^+CD4^+$ T cells increase and remain elevated in the periphery. Consistent with this suggestion, we previously showed a selective local increase in $CD3^+$ T cells expressing CCR4 at both messenger RNA and protein levels in the nasal mucosa in parallel with increased local expression of Th2 cytokines at 6–8 h during the late nasal response (21). It is important to note that a limitation of these finding is that we do not know whether these changes in phenotypic characteristics of T cells occurred in antigen-specific cells or whether they reflect an overall upregulation in T-cell activation as a consequence of non-antigen-specific effects of systemic cytokine release. This would be an important question to be addressed in future studies.

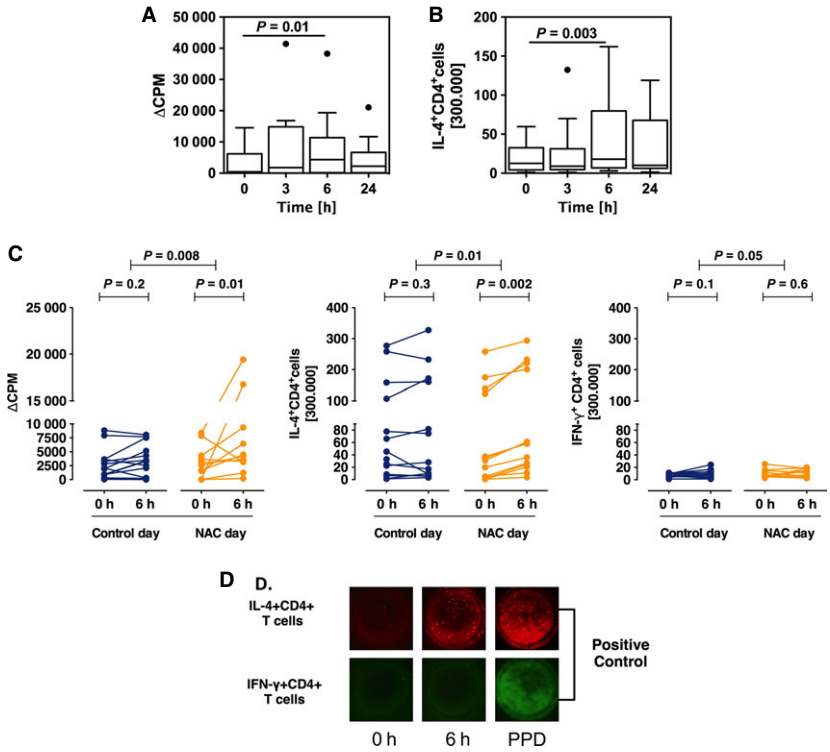

Figure 5 Grass pollen-driven T-cell responses and frequency of Phl p-specific IL-4$^+$CD4$^+$ T cells in the peripheral blood are increased on nasal allergen challenge (NAC) but not on a control day. (A) Time course of grass pollen-driven T-cell proliferative responses (n = 12) and (B) IL-4$^+$CD4$^+$ T cells following NAC. (C) Grass pollen-induced T-cell proliferative responses and the frequencies of IL-4$^+$CD4$^+$ and IFN-γ^+CD4$^+$ T cells at 6 h after challenge. (D) Representative fluorescently labeled spots illustrating IL-4$^+$CD4$^+$ T cells (PE-labeled) and IFN-γ^+CD4$^+$ T cells (FITC-labeled). Purified protein derivative (PPD) was used as positive control. The dots over the box and whiskers represent outliers.

NAC was also associated with a time-dependent increase in the frequency of allergen-specific IL-4$^+$CD4$^+$ T cells and antigen-driven CD4$^+$ T-cell proliferative responses, which peaked at 6 h and returned to baseline at 24 h. To exclude a possible diurnal variation including daily rhythm as responsible for these changes, we repeated the study with measurements on a control day following diluent challenge, and blood samples were collected at the same time during the control and NAC day. In this second cohort, we replicated the antigen-driven increase in CD4$^+$ T-cell proliferative response and an increased frequency of IL-4$^+$CD4$^+$ T cells at 6 h after nasal allergen exposure, but not on the control day. In contrast, no change in IFN-γ^+CD4$^+$T cells was observed after allergen compared to the control day, confirming an allergen-driven selective Th2 signal detectable in the periphery and independent of effects of diurnal variation. It is likely that these cells are CRTh2$^+$CD4$^+$ T cells that predominantly produce IL-4. We chose to use FluoroSpot assays to measure grass pollen-specific IL-4$^+$ and IFN-γ^+ CD4$^+$ cells mainly because the assay represents a superior functional readout compared to the detection of phenotypic antigen-specific T cells by tetramer analysis for which HLA typing is required and only a proportion of individuals could be targeted due to HLA restriction. Furthermore, large volumes of blood would have been necessary for functional studies of tetramer-positive cells, which would not have been feasible in this time course study without eliminating other assays, due to the limiting volume of blood drawing permitted.

Nasal allergen provocation test has a clinical utility in differentiating/confirming sensitization from allergy and has been used to evaluate treatment efficacy (23–25). It has mostly been utilized as a research tool to investigate on the pathophysiological mechanisms of local nasal response to the sensitized allergen (7, 16). Studies of T-cell phenotype after nasal allergen provocation have focused on the changes in the local mucosa by the use of nasal biopsy. Whereas nasal biopsy has been informative for exploring T-cell-associated mechanisms, the technique is complex, more invasive, and cannot easily be repeated in the context of clinical trials. Nouri-Aria and colleagues showed that patients with grass pollen-induced allergic rhinitis have increased eosinophils (EG21$^+$ cells) but not T cells (CD3$^+$ cells) in the nasal mucosa at 6 h postallergen challenge whereas in a separate study, nasal mucosal CD4$^+$ T cells were elevated at 24 h (26). The number of cells expressing IL-4 mRNA and IL-5 mRNA was also increased at 6 h (27). Other studies have investigated mediators and cytokines in nasal fluid during the

early and late allergic responses and have assessed their relationship with disease severity (15, 28). Scadding and colleagues validated the grass pollen allergen provocation model and demonstrated a detailed time course analysis of inflammatory mediators and cytokines in nasal fluid obtained from grass pollen allergic rhinitis. Tryptase levels were increased within 5 min and corresponded to the peak of the EPR. IL-5, IL-13, and ECP reflected Th2-cell and eosinophil activation during LPR, and IL-5 correlated with visual analogue scale (15). In another study, Bauman and colleagues showed that IL-31 and IL-13 were increased in nasal secretions at 5 h following allergen but not after control challenge. They reported a correlation between IL-31 levels in the nasal fluids and IL-13. IL-31 also correlated with symptom scores at 5 h after allergen provocation (28). None of these studies investigated the effects of allergen provocation on peripheral immunologic responses. Nasal fluid sampling is useful for measuring mediators, cytokines, and epithelial transmigrating leukocytes (29–31). However, it is not helpful for assessing T cells that largely compartmentalize to the tissue (16, 27) or assessing peripheral mechanisms of nasal allergen provocation. In our study, we sought to investigate the effect of nasal allergen provocation on cellular responses during early and late allergic response.

Based on these observations, it is reasonable to consider NAC, in conjunction with the assays described in this report, a possible tool to monitor subjective and objective immunological changes following either allergen immunotherapy or novel biologics in patients with severe allergic rhinitis. Reports, by both ourselves and other groups, suggest that incorporating measurement of early- and late-phase biomarkers such as tryptase, eosinophilic cationic protein (ECP), and Th2 cytokines in nasal secretions is a further useful addition to a clinical study, allowing the effect of the treatment in the target organ to be monitored.

In conclusion, following nasal allergen provocation, we have detected and delineated the time course of basophil, T-cell, and dendritic cell activation in peripheral blood. We have further shown increases in *ex vivo* allergen-driven T-cell proliferation and Th2 cytokine expression that occur independent of diurnal variation. Allergy is a systemic disease, and these data provide strong evidence for multiple immune/effector cell activation in the periphery, the first solid evidence for this concept following a local nasal allergen provocation. We believe that a combination of local and peripheral assays may provide a powerful toolbox to generate insights into the mechanism of allergic rhinitis and ultimately yield potential biomarkers to assess disease severity and response to treatment.

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Author contributions

SRD, DP, and MHS conceptualized and delineated research hypotheses. MHS, JAL, and DKMC performed the experimental work. MHS analyzed data, interpreted results, performed statistical analyses, and wrote the first draft of the manuscript. VB, GWS, and MAC recruited all patients, collected clinical data and blood samples, and participated in the discussions. SRD, DP, AT, NT, LAT, AA, and ZG participated in the discussions of data analysis and interpretation and contributed to manuscript preparation. The manuscript was finalized by SRD with the assistance of all authors.

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Conflicts of interest

SRD has received via Imperial College grants lecture fees and consulting fees from ALK and has been consultant for Merck, GSK, Boehringer Ingelheim, Stallergenes, and Circassia; DP, AT, NT, JAL, DKMC, GWS, VB, AA, and ZG have no conflict of interests. LAT has a spouse employed by, and owns equity in, Novartis. MAZ has no conflict of interest. MHS has received via Imperial College research grants from ALK, BioTech Tools and Regeneron.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Methods and material.

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