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Enhanced Antigen-presenting and T-cell Functions During Late-phase Allergic Responses in the Lung

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Abstract

Background—Allergic inflammation is a common feature of asthma and may contribute to both development and perpetuation of disease. The interaction of antigen presenting cells (APC) with sensitized helper T lymphocytes (TC) producing Th2 cytokines may determine the inflammatory response. Recruitment of APC and TC to the lung during allergic responses has been demonstrated, but functional studies in humans have been limited.

Objective—This study examined the function of APC and TC accumulating at sites of inflammation after segmental allergen challenge (SAC).

Methods—15 allergic patients underwent SAC, and cells from bronchoalveolar lavage (BAL) were collected after 24 hours. APC and TC from the blood and BAL were purified based on expression of the monocyte marker, CD14; the plasmacytoid dendritic cell (pDC) marker, BDCA4, identifying neuropilin 1 (NRP1); and the helper T cell marker, CD4. Functional activity was assessed using allergen-induced T cell proliferation. Flow-cytometry identified cells expressing CD14 and NRP1.

Results—SAC resulted in a 12-fold increase of mononuclear cells having the morphologic appearance of blood monocytes. Most of these cells co-expressed CD14 and NRP1. After saline challenge, BAL mononuclear cells demonstrated little APC function. Following SAC, BAL mononuclear cells showed function equal to pDC from blood and greater than blood monocytes. Purified NRP1⁺ cells from BAL had even greater function than pDC cells from blood ($p = .008$). Using consistent sources of APC, enhanced proliferation of TC from lung compared to blood was also demonstrated ($p = .002$).

Conclusions—The marked increase in APC function for allergen-specific TC proliferation during allergic inflammation is largely due to the recruitment of monocytes and dendritic cells. There is also an enhanced response in the lung TC population, consistent with recruitment of allergen-specific T cells. Interactions between recruited APC and TC may occur as an early event promoting allergic airway inflammation.

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INTRODUCTION

Allergic inflammation is a common feature of asthma, and exposure of the sensitized individual to allergens may result in development or worsening of disease.(1) Allergen challenge has been used as an *in vivo* model to examine the role of allergic inflammation in asthma, and has demonstrated the dominant role of the mast cell in the immediate response to allergen exposure, followed by a late-phase of cellular recruitment that includes cells associated with both the innate (i.e. neutrophils, macrophages and dendritic cells) and adaptive (i.e. Th2 lymphocytes, eosinophils, basophils) immune responses.(2, 3) Molecules involved in the highly selective recruitment of cells characterizing allergic responses (i.e. Th2 lymphocytes, eosinophils, and basophils) include cytokines, chemokines, and adhesion molecules.(4) Recently, recruitment of multiple dendritic cell (DC) subsets during allergic inflammation has been demonstrated suggesting an important mechanism for enhancing immune responses within tissues by enhancing antigen-presenting cell (APC) function.(5–7) DCs initiate primary immune responses, link innate and adaptive immunity, and regulate TC responses. Animal models suggest an obligatory role for DCs in both the initiation and maintenance of allergic inflammatory responses in the lung.(8, 9) Distinct subsets of DC may modulate immune responses to promote or inhibit Th1 vs Th2 responses.(10)

Previous human studies of allergen exposures in the nose and lung have demonstrated the accumulation of multiple DC populations within 6–24 hrs.(6, 7, 11) of allergen exposure. These findings suggest that enhancement of antigen-presenting cell (APC) function within airway tissues may activate sensitized, memory T cells to provide a specific and accelerated response to exposure that does not require recirculation through lymph nodes or lymphoid tissues. These memory T cells may represent effector memory cells or resident memory T cells that populate mucosal surfaces, distinct from central memory cells that recirculate through lymphoid tissues such as lymph nodes and spleen.(12) Specific T cell activation defines distinctive features of the allergic inflammatory response characterized by Th2 lymphocytes and cytokines, eosinophils, and basophils. The recruitment and differentiation of DCs at sites of inflammation may also play a critical role in T cell recruitment and differentiation within inflamed tissues.

In the present study, we have used the model of segmental allergen challenge (SAC) in allergic individuals to examine the phenotypic characteristics of monocytoïd and dendritic cells that accumulate in the lung in response to allergen in the atopic individual. **The plasmacytoid DC marker, BDCA4, was used to purify a DC subset distinct from monocytes with a possible role in promoting Th2 allergic responses.** For the first time, APC function of these lung DCs compared to circulating or resident APC in blood and lung is examined. The results demonstrate the marked recruitment and rapid differentiation of DCs from blood monocyte and DC precursors. This accumulation results in augmentation of APC function for CD4⁺, helper T cell proliferation in response to allergen. Furthermore, the accumulation of DCs is associated with an enhanced capacity of CD4⁺ helper T cells from the lung following SAC to respond to allergen.

METHODS

Subjects

15 allergic rhinitic subjects with or without asthma, ages 18–43 years, were characterized on the basis of symptoms, skin prick testing to a panel of 9 common inhalant allergens, lung function tests, and reactivity to methacholine as previously described.(12) Asthmatics had mild disease managed only with bronchodilator as needed. Study protocols were approved by the Johns Hopkins Medicine Institutional Review Board, and subjects gave written informed consent. Investigational New Drug approval was obtained from the Food and Drug Administration for use of standardized allergens for airway challenge (IND number: BB-IND11311). Bronchoscopy was performed in accordance with National Institutes of Health guidelines and previous allergen challenge studies. (12)

Segmental Allergen Challenge (SAC) and Bronchoalveolar Lavage (BAL)

SAC was performed in all patients to obtain lung cells for the studies shown. The procedure includes an initial bronchoscopy to perform the challenge, followed by a second bronchoscopy to collect cells by BAL at sites of challenge for cellular studies.

Subjects sensitive to ragweed (RW) and/or dust mite (DM) allergen were selected for SAC. Standardized allergens, short ragweed (*Ambrosia elatior*) and dust mite (*Dermatophagoides farinae* or *D. pteronyssinus*) selected for low endotoxin content (<25 endotoxin units/ml in undiluted stock preparation) were purchased from Greer Laboratories (Lenoir, NC). The allergen concentration used for SAC was individualized based on a titrated endpoint response to intradermal skin testing as previously described. (13)

The initial bronchoscopy was performed following premedication with atropine (0.6 mg IV) and local anesthesia achieved by inhalation of nebulized lidocaine 4%, followed by viscous lidocaine 4% intranasally or benzocaine 10% sprayed onto the oropharynx. Mild sedation with fentanyl (0.1 mg IV) was given immediately before bronchoscopy. An Olympus BF10 fiberoptic bronchoscope (Olympus Corporation, Lake Success, NY) was inserted into the lower airways. Local anesthesia was supplemented with lidocaine 2%. A control challenge with normal saline (NS) was performed by instilling 5 ml NS into a wedged segment of the lingula or right middle lobe. Allergen challenge (AG) was then performed in a segment of the opposite lung with 5 ml of ragweed or dust mite. After 20–24 hours, a second bronchoscopy was performed with bronchoalveolar lavage (BAL) at the sites of NS and AG challenges. BAL was performed with 5 × 20 ml aliquots of NS, pre-warmed to 37° C. BAL fluid collected from each site was pooled and processed separately. Blood samples were collected prior to bronchoscopy for cell analysis and functional studies.

Allergen cross-over study

In experiments designed to evaluate the T cell specificity of the allergic response, three allergic subjects (two asthmatic, 1 rhinitic) with sensitivity to both ragweed and dust mite underwent SAC with each allergen separated by 3–7 months. On each occasion, SAC was identical except for the allergen used for challenge. On each occasion, the *in vitro* responses

of T cells to both allergens were measured so that T cell recruitment in response to SAC with different allergens could be compared in the same individual.

Cell Counts

Total fluid recovered from each BAL was measured, filtered through gauze, and aliquots removed for cell counts performed using a hemocytometer. Cell viability was determined by trypan blue dye exclusion. Differential cell counts were performed by enumerating 500 cells on cytocentrifuge preparations (Cytospin, Shandon Southern Instruments, Sewickley, PA) stained with Diff-Quik (American Scientific Products, McGaw Park, IL).

Total cell number was calculated as volume of BAL fluid recovered $\times$ cells/ml by hemocytometer count. The total number of each cell type was calculated as total cells $\times$ percent of each cell type enumerated by differential cell counts.

The macrophage population was further divided by morphologic criteria into an “alveolar” macrophage population characterized by large size and low nuclear:cytoplasmic ratio; and a “monocytoid” macrophage population with the cytologic features of blood monocytes with smaller size and higher nuclear:cytoplasmic ratio than the resident alveolar macrophage population (Figure 1). In a separate count, 300 macrophages were categorized, and total number of each type was calculated as percent of each type $\times$ total macrophage number.

Flow cytometry of monocytoid cell subsets

In 2 subjects included in the functional studies and 3 additional subjects, BAL cells from the SAC protocol were analyzed by flow cytometry for expression of the monocyte and DC markers, CD14 and blood DC antigen 4 (BDCA4), used for isolation of dendritic cells. Flow cytometry was performed using fluorochrome-conjugated monoclonal antibodies to CD14 and BDCA4 (Miltenyi Biotec, Auburn, CA) and isotype-matched control monoclonal antibodies. Cell populations for analysis were selected by light-scatter characteristics of monocytes, and subsets were identified by staining with CD14-Tricolor and BDCA4-PE after comparison with matched, fluorochrome-conjugated, control monoclonal antibodies.

Cell Purification

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood using density gradient centrifugation (Histopaque, specific gravity 1.077, Sigma Diagnostics, St. Louis, MO). BAL mononuclear cells (BAL-MC) were isolated from total BAL cells by the same density gradient centrifugation procedure used for PBMC. Blood monocytes (MNC), helper T lymphocytes (TC), and dendritic cells (DC) were purified from PBMC by positive selection, using immunomagnetic beads against CD14, CD4, and blood DC antigen 4 (BDCA4), respectively, according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Lung DC and TC from sites of allergen challenge were purified from BAL-MC by the same procedures used for blood DC and TC. Non-DC cells from sites of allergen challenge were cells remaining following removal of BDCA4⁺ lung DC.

Cell culture: Allergen-induced proliferative response (APR)

The APC of CD4⁺ TC was assessed by tritiated (³H)-thymidine incorporation. In brief, purified CD4⁺ T cells (1×10^5) were co-cultured with $0.3 - 2.5 \times 10^4$ purified and irradiated APCs (e.g. macrophages, monocytes, DC) at APC:TC ratios from 1:32 to 1:4. Cultures with and without allergen were performed in triplicate in U-bottom, 96-well microtiter plates in RPMI 1640 or IMDM media (Gibco, Rockville, MD) containing penicillin/streptomycin and 5% human AB serum at 37° C and 5% CO₂. In all experiments, APCs were irradiated ($3887 \text{ rads} \times 8 \text{ minutes}$) to block proliferation of the APC population. Dust mite and ragweed allergens were used at concentrations of 100 allergy units (AU) or 100 protein nitrogen units (PNU) per ml, respectively. One PNU of RW equals .005 mcg of ragweed Antigen E or Ambal. After 5 days of co-culture, ³H-thymidine (1 microCi/well, Perkin Elmer, Boston MA) was added. After 16 hours, cells were harvested onto glass fiber filters using distilled water and a cell harvester (PhD, Cambridge Technology, Cambridge, MA). Radioactivity (counts per minute, cpm) was measured using liquid scintillation spectrometry (Bio-Safe II, Research Products International, Mt. Prospect, IL).

Using the mean values of triplicate determinations, proliferative responses were calculated as a stimulation index (SI): (cpm in cultures with allergen) / (cpm in cultures without allergen), or as net cpm: (cpm in cultures with allergen) - (cpm in cultures without allergen). When multiple control cultures without allergen were performed, the mean of all control cultures for an experiment was used to minimize the effect of small variations in control cultures on calculated SI.

Normalized stimulation index

Due to the large range of responses among individuals, proliferation was normalized to one condition within an individual experiment by dividing the SIs from all APC/TC co-cultures by the SI of DC/TC from blood at a single ratio, typically 1:8. Responses of different APC:TC ratios or cell source combinations were compared using the normalized SI.

Statistical Analysis

Data are reported as median (range) unless otherwise indicated. Cell counts were compared using the Wilcoxon signed-rank test. Correlations were assessed using the Spearman rank correlation. Function of APC populations from lung and blood after SAC at multiple APC:TC ratios were compared by 2-way ANOVA and Bonferroni post-tests. Results of cell mixing experiments using BDCA4⁺ DC and CD4⁺ TC purified from blood and lung following SAC were analyzed by one-tailed, paired T-test with significance determined after correction for multiple comparisons by the false discovery rate procedure (14). P-value < .05 was considered significant.

RESULTS

Subject characteristics

Individual subject characteristics with the allergen used for SAC are shown in Table 1. Subject number is used to specify patients participating in each study protocol.

Cellular responses to SAC

In the primary group undergoing the SAC protocol for cellular studies, the recovery of BAL fluid was not different between NS- and AG-challenged sites (mean % + SD: NS 46+11 vs AG 45+7). As expected, SAC induced a vigorous inflammatory cell response with marked increases in total cells, lymphocytes, monocytoïd macrophages, neutrophils and eosinophils (Table 2). Total cells increased by almost 3-fold, largely due to the increase in eosinophils. Recovery of total resident alveolar macrophages was not significantly changed by allergen challenge, but the monocytoïd macrophage population increased nearly 12-fold. This monocytoïd population is likely to contain both recruited monocytes and dendritic cells.

Characterization of the monocytoïd macrophage population

The cytologic appearance of alveolar and monocytoïd macrophages from BAL are illustrated in Figure 1A. Alveolar macrophages (AM) were characterized by large size and low nuclear:cytoplasmic ratio, while monocytoïd macrophages (MNC) had the appearance of monocytes with smaller size and higher nuclear:cytoplasmic ratio.

Examination by flow cytometry of the monocytoïd macrophage population using CD14 as a monocyte marker and BDCA4 as a DC marker revealed large numbers of cells expressing both CD14 and BDCA4 as illustrated in Figure 1B. At both NS and AG sites (n = 5), the percentage of BDCA4⁺ cells expressing CD14 (dual) was consistently greater than the percentage of cells expressing BDCA4 only (single) (NS: dual 3.4–57.6%, single 2.1–4.3% with 62.7–95.1 % co-expression, median 89.1%; AG: dual 11.4–65.5%, single 2.6–8.4% with 67.8–93.1% co-expression, median 81.7%). There were no significant differences between NS and AG.

APC function in the lung is markedly enhanced following SAC

In subjects 1–6, APR to the allergen used for SAC (RW: n=5, DM: n=1) was assayed using APC populations from blood or BAL co-cultured with CD4⁺TC from the blood. Stimulation indexes within each experiment were normalized to the response of blood DC at the APC:TC ratio of 1:8. APC populations included blood monocytes; blood DC; lung mononuclear cells from BAL following normal saline challenge; lung mononuclear cells from BAL following allergen challenge; lung DC purified from BAL following allergen challenge; and lung, non-DC remaining after DC purification.

The reference SI of DC/TC from blood used for normalization ranged from 6.6 – 14.8, median 8.4. These reference SI were calculated from ³H-thymidine incorporation proliferation data ranging from 365–597 cpm in control cultures to 3202–5983 cpm in allergen-stimulated cultures using DC/TC from blood at a 1:8 ratio. As shown in Figure 2, both the cell type and the APC:TC ratio had highly significant effects (p < .0001) on the APR with 62% of total variance due to the cell type and 26% due to the ratio. All APC populations from blood and from BAL following SAC, demonstrated better APC function than the resident BAL cell population following control challenge with normal saline, comprised mainly of alveolar macrophages. APR was directly related to the number of APC with the greatest effect observed with lung DC. From blood, the BDCA4⁺ plasmacytoid DC (pDC) had better APC function than CD14⁺ monocytes. The mixed mononuclear cell

population in BAL following SAC with allergen demonstrated APC function identical to the pDC population from the blood. However, when this mixed population was further separated into DC and non-DC cell populations based on expression of the pDC marker, BDCA4, the lung DC population revealed greater APC function compared to all others, including pDC from blood.

The remaining, non-DC population had APC function identical to that of blood monocytes.

Both DC and TC functions in the lung are enhanced following SAC

Subjects 7–12 were studied in these experiments. To assess the reproducibility of the APR measured in individual subjects, assays were performed using DC and TC purified from blood prior to each bronchoscopy, i.e. before SAC and 20–24 hours after SAC (n=6). Individual APR responses demonstrated wide variation, with SI ranging from 3.25 – 100.95 (median 6.3) before SAC, to 4.71 – 85.63 (median 9.2) after SAC. APR between assays was not different and highly correlated by linear regression analysis, $r^2 = .99$, $p < .0001$ (data not shown). The mean % difference between assays was $45 \pm 16\%$ (SE). These data demonstrate that despite the marked variability among individual responses, the assay of APR from blood cells for each individual was very reproducible and did not appear to be affected by SAC.

Results of mixing DC and TC from blood or lung following SAC are shown in Figure 3. In each experiment, blood DC/TC showed the lowest response of all the combinations tested with proliferation data ranging from 331–1062 cpm in control cultures to 3210–50,929 cpm in allergen-stimulated cultures. This corresponded to SI ranging from 4.71–85.63. These SI values were used as the reference responses for the normalized stimulation index data shown in Figure 3. Similar to results shown in Figure 2, the lung DC/blood TC combination demonstrated 20–168% enhanced APR compared to reference blood DC/TC ($p = .008$) confirming enhanced function of lung DC. Similarly, the blood DC/lung TC combination demonstrated 4–49% enhanced APR compared to reference blood DC/TC ($p = .002$). Hence, enhanced TC function in lung TC compared to blood TC was demonstrated under conditions using blood DC to provide a consistent source of APC. These TC responses between blood and lung were correlated ($p = .0167$, Spearman rank correlation).

This finding of enhanced lung TC function was further supported by a similarly enhanced response of lung TC compared to blood TC, when both were cultured with DC from the lung. Under these conditions, the overall level of APR was significantly higher due to the effect of using lung DC rather than blood DC, but the enhanced response of lung TC was apparent. Mixing both DC/TC from the lung, resulted in the highest TC proliferative response of 31–731% enhancement over reference ($p = .013$), since both the DC and TC components of enhanced APR were present. The lung DC/TC response was significantly greater than either of the enhanced responses using lung DC or lung TC ($.021 < p < .039$). Taken together, these results confirm the previous findings of enhanced APC function of DC in the lung. In addition, the results demonstrate an enhanced TC response suggesting that enhanced immune responses at sites of allergic inflammation also involved recruitment of allergen-specific TC.

Allergen challenge results in sensitized TC recruitment with biased recruitment of TC specific to the allergen used for challenge: Further evidence for allergen specific TC recruitment

Subjects 13–15 participated in the allergen-crossover study. Each subject sensitive to both RW and DM allergens was challenged with each allergen during SAC separated by 3–7 months. The cellular responses to SAC with each allergen were similar. With RW challenge, total cells recovered was $74 \pm 42 \times 10^6$ (mean $\pm$ SE) with 26 $\pm$ 16% macrophages, 14 $\pm$ 6% lymphocytes, 13 $\pm$ 7% neutrophils, and 46 $\pm$ 19% eosinophils. With DM challenge, corresponding cell numbers were $107 \pm 41 \times 10^6$ total cells with 13 $\pm$ 4% macrophages, 9 $\pm$ 3% lymphocytes, 25 $\pm$ 13% neutrophils, and 53 $\pm$ 13% eosinophils.

The APR to both RW and DM allergens was assayed on both occasions, using irradiated PBMC (1×10^5) to provide equal APC function, and CD4⁺ TC (5×10^4) purified from blood and BAL following SAC. Using the proliferative response assessed by the net cpm, an allergen-specific response ratio was calculated by the formula: (net cpm to the allergen used for SAC in vivo) / (net cpm to the alternate allergen). The response to the alternate, i.e. non-specific allergen, was used as a control to represent non-specific TC recruitment related to inflammation. A ratio greater than 1 represents a greater proliferation to the allergen used for SAC than to the non-specific allergen, and supports an allergen-specific response.

Results from each experiment (subjects 13–15) are shown in Table 3. Significant APR to both allergens were measured using both blood and lung TC following SAC. In the lung, T-cell responses following SAC were consistently greater to the allergen used for challenge compared to the non-specific allergen. This enhancement was observed when either RW or DM was used for SAC in the same individual. In contrast to the lung, a response ratio less than 1 in blood was consistent with SAC using DM, suggesting depletion of allergen-specific TC from the blood; however SAC using RW gave mixed results. Overall, the results from this small number of experiments support an allergen-specific component to the TC recruitment observed during the allergic response in the lung. TC recruitment to the lung may be accompanied by depletion of allergen-specific TC from the blood.

DISCUSSION

We report some of the first assessments of APC and TC function of immune cells accumulating at sites of allergen challenge in the lung. The results highlight the rapid recruitment and differentiation of APC from circulating monocyte and dendritic cell precursors that accompanies the influx of other cells that characterize allergic inflammation. This recruitment results in conversion of the immune status of the lung from one of limited immune responsiveness due to minimal APC function of the predominant alveolar macrophage population, to markedly augmented APC function due to recruited monocyte and DC populations. These recruited populations demonstrate APC function that is even greater than that of their circulating counterparts. The potential for interactions within the lung resulting in rapid mobilization of specific immune responses and establishing tissue-specific memory is clear.

Based on cytologic appearance, SAC results in a marked change in the macrophage population from one that is almost exclusively alveolar macrophages to one that includes a small, but significant proportion of cells (about 5%) resembling blood monocytes. The co-expression of CD14 and blood DC antigen 4 (BDCA4) on these cells suggests that circulating monocytes rapidly expressed this dendritic cell marker, identifying plasmacytoid DC. Our findings and those of others(11) demonstrate that in this inflammatory state, BDCA4 is not specific for the plasmacytoid DC subset found in blood. Nevertheless, BDCA4 expression identifies a subset of monocytoïd cells in the lung with enhanced APC function that likely includes both circulating pDC and monocytes transitioning into monocyte-derived or myeloid DC. The selected inflammatory BDCA4⁺ population demonstrates greater APC function than blood pDC that only express BDCA4. The role of BDCA in these responses has not been determined.

The BDCA4 expressing cells likely represent both recruitment of circulating pDC and monocytes in transition to DCs. This transition is likely driven by exposure to cytokines involved in DC differentiation such as IL-4, GM-CSF, and TGF- α known to be present in this model.(3) IL-15 from airway epithelial cells may also cause differentiation of monocytes to DC.(15) Using a similar SAC model and four-color flow cytometry, Bratke et al clearly demonstrated increases in both mDC and pDC (up to medians of .56% and 1.82%, respectively) in total BAL cells at 24 hours after SAC(11). They also noted that BDCA4 was not specific for pDC at sites of allergen challenge, while BDCA2 remained specific for pDC. In the present study, the large majority of BDCA4⁺ cells co-expressed CD14⁺ suggesting that the enhanced APC function lay within the recruited monocyte population.

BDCA4 has been identified as neuropilin 1 (NRP1) that was initially described as a neuronal receptor involved in nerve growth guidance. (16) An important role for NRP1 in immune responses is now recognized. (17) Tordjman et al have demonstrated that human DC and TC can both express NRP1 and that NRP1 appears to be an essential molecule involved in cell clustering and TC proliferation since a blocking antibody against NRP1 inhibited both functions. (18) In the present study, induced expression of NRP1 on recruited monocytes appears to identify cells demonstrating enhanced APC function. This supports the speculation that NRP1 is playing a role in the heightened APC function, however, such a role has not been established. Many changes associated with DC maturation including heightened expression of major histocompatibility antigens (MHC), costimulatory molecules and cytokine production are likely to play important roles but were not examined in the present study.

Previous studies have also demonstrated the diversity of mononuclear phagocyte populations in the lung, changes provoked by inflammatory stimuli, and functional changes. A recent flow cytometric analysis of mononuclear phagocytes isolated from human lung tissue identified 5 distinct populations resident in the non-vascular compartment: alveolar macrophages, 3 monocyte-derived groups and pulmonary dendritic cells. CD1c⁺ population that contained pulmonary DC and monocyte-derived cells were the most potent allostimulators in the mixed leukocyte response.(19) In a study of endotoxin effects on responses to segmental allergen challenge, endotoxin enhanced allergic inflammation that included monocyte and myeloid DC recruitment. Only the pulmonary myeloid DC

stimulated T cell proliferation in an autologous mixed lymphocyte culture assay. (20) Increased monocyte numbers were also found in the lung as part of the inflammatory response within 6 hours of segmental challenge with endotoxin.(21) Lee et al also demonstrated a new population of recruited alveolar macrophages derived from monocytes after SAC associated with increased levels of macrophage chemotactic proteins (MCP 1 and 3), supporting the role for epithelial-derived MCP1 in monocyte recruitment.(22)

Our study also demonstrates allergen-specific T cell recruitment to sites of allergic inflammation. When the proliferative responses of T-helper cells recruited to the lung were compared to those from blood using identical APC populations, the recruited lung T-cell population consistently demonstrated greater proliferation, suggesting a selective process for recruitment of allergen-specific cells from the circulation. This suggestion was further supported by the limited results from the allergen-crossover study that not only demonstrated both allergen-specific and non-specific TC responses within the TC population recruited to the lung, but also confirmed a recruitment bias favoring the allergen used for challenge. These results support a previous study by Till et al comparing TC from BAL and blood following segmental allergen-challenge that demonstrated increased IL-5 production from BAL TC compared to blood, but showed no difference in proliferation.(23) Considering their rapid appearance, the allergen-specific TC are certainly memory TC derived from prior allergen sensitization. Previous studies have shown that the large majority of TC recruited following allergen challenge bear a CD4⁺CD45RO⁺ (helper, memory) phenotype, despite memory cells being the minority phenotype in the circulation.(3) Central memory TC recirculating through lymph nodes, effector memory TC recirculating from blood to tissues, and resident memory TC with prolonged residence in the lung, may all contribute to the allergen-driven response.

We assume that the changes in proliferation of helper TC observed in the present studies represent changes in the number of responding cells, but the lack of direct enumeration of responding TC number is a limitation. However, prior studies have demonstrated a relationship between TC number and proliferative responses. In a study of plasmacytoid DC activation of T-helper cells responding to grass pollen under conditions similar to the present study, Farkas *et al* demonstrated that proliferation measured by thymidine incorporation corresponded to numbers of proliferating TC enumerated by carboxyfluorescein diacetate succinimidyl ester (CFSC) staining and flow cytometry.(24) This association suggests that enhanced APR assessed by thymidine incorporation in the present study represents increases in the number of allergen-specific helper TC recruited to sites of allergen challenge. However, the absolute numbers of these cells could not be estimated.

The function of TC during the allergic response will certainly be diverse, but certainly includes the production of Th2 cytokines, including IL-4, IL-5 and IL-13, that have been previously demonstrated, as well as IL-2.(3) Afshar et al demonstrated the recruitment of CD4⁺CD25⁺Foxp3⁺ regulatory T cells within the recruited lymphocyte population following SAC. These Tregs inhibited autologous CD4⁺ lymphocyte proliferation to CD3 stimulation. (25)

The characteristic features of allergic inflammation may be dependent on the enhance accessory cell function and specific TC recruitment demonstrated here. Such as response does not require immune cell stimulation in lymph nodes since the necessary components are already present within the lung tissue or can be directly recruited from the circulation. TC activation in lymphoid tissues occurs as a later event to amplify the allergen-specific response.

In conclusion, we have formally demonstrated that monocyte and DC recruitment are early components of the cellular response to allergen exposure and markedly enhance antigen presentation and allergen-specific TC responses in the lung. This interaction is associated with accumulation of allergen-specific TC that may determine the characteristic features of allergic inflammation.

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Figure 1.

Cytology of alveolar and monocytoïd macrophages. A. Photomicrograph of cytocentrifuge preparation of BAL cells following SAC (500 $\times$). AM: alveolar macrophages, MNC: monocytoïd macrophages. B. Flow cytometry for CD14 and BDCA4 of the monocytoïd macrophage populations in BAL following normal saline and allergen challenge.

Figure 2.

Enhanced APC function in the lung following SAC. Results from subjects 1–6 are shown. The effect of accessory cells from the lung and blood on blood CD4⁺TC proliferation was assayed. Stimulation indexes (SI) within each experiment were normalized to the SI obtained with blood DC at the APC:TC ratio of 1:8. APC populations: blood monocytes (Blood/MNC), blood DC (Blood/DC), lung mononuclear cells from BAL following normal saline challenge (NS/BAL-MC), lung mononuclear cells from BAL following allergen challenge (AG/BAL-MC), lung DC (AG/BAL-DC) purified from AG/BAL-MC, and lung non-DC (AG/BAL-non DC) remaining after DC purification. Data presented as mean $\pm$ SE, $n = 4 - 6$, except for Blood/MNC, $n = 2$. Differences related to cell type and ratio were significant, $p < .0001$.

Figure 3.

Enhanced DC and TC functions in the lung following SAC. Results from subjects 7–12 are shown. Purified BDCA4⁺ DC and CD4⁺ TC populations from blood and BAL at sites of allergen challenge were cultured at a DC:TC ratio of 1:8 with or without the allergen used for SAC (RW: n=5, DM: n=1). The stimulation index (SI) for each DC:TC combination was calculated, and the results from individual experiments were normalized to the SI for the blood DC:TC response. Symbols and line represents results from one individual, referenced by subject number in Table 1: subject number 7 (), 8(+), 9(), 10(x), 11(□), 12(o). Solid bars are medians. * indicates .002 < p < .014 versus blood DC+blood TC; # indicates p < .05 versus lung DC+lung TC.

Table 1

Subject Characteristics

Subject Number	Age	Gender	Race	Diagnosis	Allergen	conc/ml
1	42	F	C	R	DP	1 BAU
2	25	M	H	A	RW	1 mcg
3	37	M	C	R	RW	0.1 mcg
4	41	F	AA	A	RW	0.1 mcg
5	35	F	AA	R	RW	1 mcg
6	33	M	AA	A	RW	10 mcg
7	41	F	AA	R	RW	1 mcg
8	25	M	C	R	RW	100 mcg
9	43	F	C	R	DP	10 BAU
10	43	F	AA	A	RW	10 mcg
11	18	M	AA	A	RW	10 mcg
12	42	M	AA	R	RW	0.01mcg
13	36	M	C	A	RW	100 PNU
14	28	M	AA	A	DF	10 PNU
					RW	20 PNU
15	28	F	AA	R	DP	100 PNU
					RW	500 PNU
					DF	100 PNU

AA: African American, C: Caucasian, H: Hispanic, R: Allergic Rhinitis, A: Allergic Asthma, RW: Ragweed, DP: Dermatophagoides pteronyssinus, DF: Dermatophagoides farinae, BAU: Biologic allergy units, mcg: micrograms, PNU: Protein nitrogen units

Table 2

Cellular Responses to SAC

Cells ($\times 10^6$)	Normal Saline (NS)	Allergen (AG)	P value [*]
Total	16.10 (4.90–42.00)	44.30 (13.50–270.00)	.0005
Macrophages	12.07 (3.68–39.73)	17.84 (10.26–66.96)	.0122
Alveolar	11.52 (3.59–39.60)	15.65 (9.64–54.91)	NS
Monocytoid	.22 (.08–1.18)	2.60 (.62–12.05)	.0005
Lymphocytes	.99 (.30–7.21)	4.73 (1.21–22.18)	.0005
Neutrophils	.87 (.03–10.73)	5.60 (1.30–49.10)	.0005
Eosinophils	.08 (0–1.80)	9.08 (.38–141.5)	.0005

median (range), n=12

*
NS versus AG

Table 3

Allergen-specific T cell responses in the allergen crossover protocol

Source of TC:	Allergen-specific Response Ratio (ARR)			
	BAL		Blood	
Allergen used for SAC:	RW	DM	RW	DM
<u>Subject:</u>				
13	1.92	1.12	.49	.45
14	6.39	2.54	9.40	.06
15	1.49	1.71	.45	.47

ARR= (net cpm to the allergen used for SAC) / (net cpm to the alternate allergen)

TC: T cells, BAL: bronchoalveolar lavage, SAC: segmental allergen challenge, RW: ragweed, DM: dust mite.