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Allergen-specific CD8+ T cells and atopic disease

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Considerable evidence suggests that IL-10 may have a role in the manifestation of atopic disease. We sought to test the hypothesis that at the single cell level, allergen-specific T cells have diminished IL-10 production capacity in severely affected atopics compared with asymptomatic atopics. We defined three A*0201-restricted Der p 1 CD8+ T cell epitopes. Using human leukocyte antigen-A*0201–peptide (HLA-A*0201–peptide) tetrameric complexes and enzyme-linked immunospot assays to analyze peripheral blood mononuclear cells from A*0201-positive severely symptomatic atopics, asymptomatic atopics, and nonatopic controls, we observed a significant association between the frequency of the Der p 1–specific CD8+ T cells and disease activity. The specific T cells expressed an antigen-experienced cell surface phenotype, and 45.7% were positive for cutaneous lymphocyte-associated antigen. The specific T cells were able to produce IFN-γ efficiently, but their IL-10 production was significantly reduced in severely affected atopics. In contrast, viral-specific CD8+ T cells were able to produce equivalent amounts of IL-10 in the severely affected atopics compared with asymptomatic atopics and nonatopics. Through defining the first human atopic allergen HLA class I epitopes, we have provided a possible cellular mechanism to link the previous association of low IL-10 levels and severe atopic disease. These data are consistent with a role for CD8+ T cells in atopic disease pathogenesis and may provide a basis for future T cell immunotherapy strategies.

Introduction

The atopic diseases include atopic dermatitis (AD), asthma, and allergic rhinitis and now carry a combined prevalence of up to 30% in developed countries and a high overall morbidity (1). The United Kingdom refinements of the Hanifin and Rajka Diagnostic Criteria for AD have now been validated in many populations and facilitate the easy and reproducible identification of affected individuals using a relatively narrow disease definition (2, 3). The six area six sign atopic dermatitis (SASSAD) disease severity score has been found to be simple, rapid, reproducible, and an effective means to monitor AD disease activity (4).

Individuals with AD have an increased tendency to react to common environmental antigens. For example, up to 75% of individuals with AD have skin prick test reactivity and/or specific IgE to house dust mites, cat and dog dander, and grass (5). Patch test challenge with house dust mite extract leads to the development of a clinical and histological eczematous response in 34% of individuals with AD (6). Furthermore, careful house dust mite avoidance measures can be associated with improvement in disease severity scores (7).

Increased frequencies of both CD4+ and CD8+ T cells producing type 2 cytokines (including IL-4, IL-13, and IL-5) have been observed in the peripheral blood of individuals with AD (8, 9), particularly within the putative skin-homing, cutaneous lymphocyte-associated antigen-positive (CLA-positive) subset (10). In addition, CLA+CD8+ and CLA+CD4+ T cells derived from the peripheral blood of AD patients are equipotent in inducing IgE production by B cells and enhancing eosinophil survival (11). Dust mite–specific CD4+ T cell responses have been identified and the HLA class II–restricting elements defined for several Der P antigens (12–25). The responses have been found in blood and lesional skin of affected individuals and following dust mite patch test challenge (21, 26). A significant proportion of the allergen-specific T cell clones isolated from lesional skin are of a CD8+ phenotype (26). Further evidence for a role of CD8+ T cells comes from the demonstration of IFN-γ production by CD8+ T cells from the peripheral blood of atopics, which is then reduced following dust mite immunotherapy (27). Animal model data support such observations; for example, a murine Db-restricted Der p 1 epitope has been identified and used to prime mice (28); in a separate system, ovalbumin-specific CD8+ T cells can inhibit IgE production (29, 30).

Circumstantial evidence exists that IL-10 may have a role in atopic disease pathogenesis. IL-10 levels in bronchoalveolar lavage fluid from severely affected atopics are significantly lower than levels in mildly affected individuals (31). PBMCs from asthmatics produce less IL-10 mRNA in response to LPS stimulation than those isolated from nonatopics (31), and low IL-10–producing promoter polymorphisms have been associated with severe asthma (32). Alveolar macrophages from asthmatics produce less IL-10 than those from nonatopic controls (33), and in Gabon there is an inverse association between levels of IL-10 produced by PBMCs incubated with schistosomal antigens and skin test reactivity to house dust mites (34). Animal model data support such observations; IL-10 suppresses murine airway inflammation and cytokine production, and IL-10 knockout mice have enhanced Langerhans cell migration to regional lymph nodes (35–38).

We sought to test the hypothesis that severely affected atopics have diminished production of IL-10 by individual allergen epitope-specific T cells compared with asymptomatic atopics. To proceed, we first needed to define precisely T cell epitopes within a common atopic allergen. We therefore mapped three HLA-A*0201–restricted CD8+ T cell epitopes within Der p 1 and generated HLA-A*0201 tetrameric complexes based on these peptides. We compared the frequency and phenotype of the epitope-specific T cells and observed that severely affected atopics have high frequencies of Der p 1–specific CD8+ T cells in the peripheral blood but that the T cells produce relatively less IL-10. Using tetrameric complexes, enzyme-linked immunospot (ELISpot) assay, and intracellular cytokine analysis, nonatopics had no identifiable Der p 1–specific CD8+ T cells within the peripheral blood. These data identify a functional difference in the T cells of severe atopics and potentially provide a mechanism for the existing associations of low IL-10 levels and severe atopic disease. Furthermore, the characterization of the first human Der p 1 CD8+ T cell epitopes will provide a tool to objectively measure the T cell response to Der p 1 in future studies.

Methods
Subjects. Nine moderate to severely symptomatic, eight currently asymptomatic atopic HLA-A*0201–positive (based on sequence-specific PCR) individuals with a history of AD, and eight HLA-A*0201–positive nonatopic controls were recruited through the department of dermatology, Churchill Hospital, Oxford, United Kingdom, under ethical approval from the Oxfordshire Clinical Research Committee. In total, 60 individuals were screened to obtain the A*0201-positive groups detailed above. AD was defined according to the United Kingdom AD diagnostic criteria, which are specific and sensitive for AD (2, 3). The criteria are as follows: an itchy skin condition plus three or more of the following: onset below 2 years of age; history of skin crease involvement; history of generally dry skin; visible flexural der-

matitis; personal history of another atopic disease (or history of atopic disease in a first-degree relative). All severely symptomatic atopic patients had, by definition, a disease severity SASSAD score of greater than 54 (of possible 108 total) (4). The SASSAD score is graded 0–3 (absent to severe) for each of the six clinical features (erythema, exudate, excoriation, lichenification, dryness, and cracking) at six sites (head/neck, thorax/proximal limbs, mid-upper limbs, mid-lower limbs, hands, and feet). All of the atopics had positive skin prick tests and IgE radioallergosorbent test (RAST) tests to house dust mites. The mean house dust mite IgE RAST test (assay range 0–6) for each group was: symptomatic atopic, 5.2 (SD 1.26); asymptomatic atopic, 3.5 (SD 0.5); nonatopic, 0 (SD 0). The mean total IgE level for each group was: symptomatic atopic, 3215 kilounit (kU)/l (SD 2187); asymptomatic atopic, 424 kU/l (SD 190); nonatopic, 20 kU/l (SD 13). The age, sex, and racial origin distribution of all three groups was not statistically different. The mean ages of the three groups were: symptomatic atopic, 24.8 years; asymptomatic atopic, 27.9 years; and nonatopic, 28.3 years. The male/female ratios for the three groups were: symptomatic atopic, 6:3; asymptomatic atopic, 6:2; nonatopic, 5:3. All individuals were Caucasian; HLA-A*0201 is one of the most common class I alleles within that group. None of the patients or controls had received systemic immunosuppressive treatments for the preceding 6 months. All individuals are likely to have continued exposure to the ubiquitous Der p 1 because none were practicing house dust mite avoidance measures, and previous reports have shown that both atopics and nonatopics may have allergen-specific CD4+ T cells (39). Furthermore, all individuals had been exposed to the antigen through skin prick testing.

ELISpot. ELISpot analyses were undertaken as described previously (40). Briefly, ELISpot plates (Millipore Corp., Bedford, Massachusetts, USA) were coated with anti-human IFN-γ or IL-10 Ab overnight (Mabtech AB, Nacka, Sweden). The plates were washed six times and incubated for 1 hour with RPMI-1640 and 5% human serum. Titrating numbers of PBMCs were added in duplicate wells to a final volume of 200 µl. Peptide was added at a final concentration of 20 µM. The plates were incubated either overnight (IFN-γ) or for 10 hours (IL-10) at 37°C and 5% CO_2. The cells were removed and the plates developed with a second biotinylated Ab to human IFN-γ or IL-10 and washed a further six times. The plates were developed with streptavidin-alkaline phosphatase (Mabtech AB) and colorimetric substrate, and the spots enumerated using an automated ELISpot reader. Peptide-specific reactivity was calculated by subtracting the counts from an irrelevant peptide control, and the results expressed as spot-forming cells per total number of PBMCs plated. Although background counts varied between individuals, these were all below 50 spots per million PBMCs.

HLA-peptide tetrameric complexes. Complexes were synthesized as described previously (41, 42). Purified HLA

heavy chain and β2 microglobulin were synthesized by means of a prokaryotic expression system (pET; Novagen, Madison, Wisconsin, USA). The heavy chain was modified by deletion of the transmembrane/cytosolic domain and C-terminal addition of a sequence containing the biotin-protein ligase BirA enzymatic biotinylation site. Heavy chain, β2 microglobulin, and peptide were refolded by dilution. The 45-kDa refolded product was isolated by fast protein liquid chromatography and then biotinylated by BirA (Avidity, Denver, Colorado, USA) in the presence of biotin, ATP, and Mg^{2+} (all from Sigma-Aldrich, St. Louis, Missouri, USA). Streptavidin-PE conjugate (Sigma-Aldrich) was added in a 1:4 molar ratio, and the tetrameric product was concentrated to 1 mg/ml.

Flow cytometry. Four-color flow cytometric analysis was performed using a FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) with CellQuest software (Becton Dickinson). PBMCs (10^6) were centrifuged at 300 g for 5 minutes and resuspended in a volume of 50 μl. Tetrameric complex was added and incubated at 37°C for 20 minutes. Directly-conjugated Ab's, including anti-CD8 PerCP, anti-CD45RO-APC (Becton Dickinson), anti–CLA-FITC, anti-CD45RA FITC, anti-CD28 FITC (BD Biosciences Pharmingen, San Diego, California, USA), were added according to the manufacturers' instructions, and the samples were incubated for 60 minutes at 4°C. After two washes in cold PBS, the samples were fixed in 2% formaldehyde. Specific controls used were PBMCs from all patient groups stained with an HLA-A*0201-tetramer based on an irrelevant peptide (hepatitis B core 18–27) and PBMCs from HLA-A*0201–negative individuals from all patient groups stained with the A*0201-Der p 1 tetramers. In line with previous reports (43), the mean plus 3 SDs of tetramer-binding cells in the PBMCs from all control individuals was less than 0.02% of CD8+ T cells.

Intracellular cytokine staining. Simultaneous flow cytometric assessment of T cell phenotype and cytokine synthesis capability was performed as described previously (44). The PBMCs were stimulated using recombinant Der p 1 (optimal dose 10 μg/ml; Indoor Biotechnologies, Cardiff, United Kingdom) for 8 hours, with brefeldin A being added during the last 6 hours. This time profile was selected after preliminary experiments showed that 8 hours was the optimal time of stimulation. Following stimulation, the cells were washed twice with PBS containing 0.1% BSA, incubated in 0.5 ml permeabilizing solution (Becton Dickinson) at room temperature for 10 minutes, washed with PBS/BSA, and then incubated for 25 minutes with Ab-fluorochrome conjugates (IL-4, IL-5, IL-10, IL-13, IFN-γ, and TNF-α). After staining, the cells were washed and resuspended in 0.5 ml of 1% paraformaldehyde in PBS before flow cytometric analysis. Isotype control reagents were used to verify the staining specificity of experimental Ab's and as guides for setting markers to delineate positive and negative populations.

Statistics. Statistical analyses were performed using non-parametric t tests and Pearson correlation coefficient.

Results

*Predicting A*0201-restricted Der p 1 CD8+ T cell epitopes.* Using the published Der p 1 sequences and known polymorphisms (45), we identified six potential HLA-A*0201–binding peptides that satisfied A*0201-binding preferences, namely leucine or methionine at position 2 and valine or leucine at position 9 (46). Three (P3, P5, and P6) of the peptides were able to refold with HLA-A*0201 in the presence of β2 microglobulin, consistent with potential HLA-A*0201–binding capacity (Table 1). Having established the presence of A*0201-binding peptides within Der p 1, we then proceeded to investigate whether these were immunogenic in our cohort.

HLA-peptide tetrameric complexes. HLA-A*0201–based tetrameric complexes were generated using the three A2-binding peptides (P3, P5, and P6) and used to stain PBMCs of individuals from the three groups of study subjects, symptomatic atopic, asymptomatic atopic, and nonatopic. High frequencies of Der p 1–specific CD8+ T cells for all three epitopes were observed within the PBMCs of symptomatic atopic (S) subjects (Figure 1). The symptomatic atopic mean (SD) number of tetramer-binding cells per million CD8+ T cells for the three tetramers was as follows: P3 863 (SD 182), P5 693 (SD 104), P6 1078 (SD 268). Der p 1–specific CD8+ T cells were also present in the asymptomatic atopic group of subjects, but at significantly lower frequencies ($P < 0.01$). The asymptomatic atopic mean (SD) number of tetramer-binding cells per million CD8+ T cells for the three tetramers was as follows: P3 573 (SD 75), P5 499 (SD 67), and P6 670 (SD 69). Using the tetramers, we were not able to identify Der p 1–specific CD8+ T cells in the nonatopic controls above negative control levels of staining: P3 12.5 (SD 35.4), P5 0 (SD 0), and P6 50 (SD 53.5). In line with previous reports (43), the mean plus 3 SD of tetramer-binding cells in the PBMCs from control stains was less than 0.02% of CD8+ T cells. The negative control stains comprised PBMCs of HLA-A*0201–negative patients from all three clinical groups stained with the A2 tetramers and PBMCs of HLA-A*0201–positive patients from all three groups stained with tetramers based on an irrelevant peptide or HLA allele (Figure 1). Figure 1 also shows an example of tetramer staining of atopic symptomatic PBMCs with tetramer-phycoerythrin (tetramer-PE; A*0201/P3) along the x axis and anti-CD8 Tricolor along the y axis.

Table 1
Peptide sequences of defined HLA-A*0201 epitopes

	Sequence	Precursor position
P3	MMIEEYPYV	309–317
P5	SLDLAEQEL	152–160
P6	YLAYRNQSL	145–153

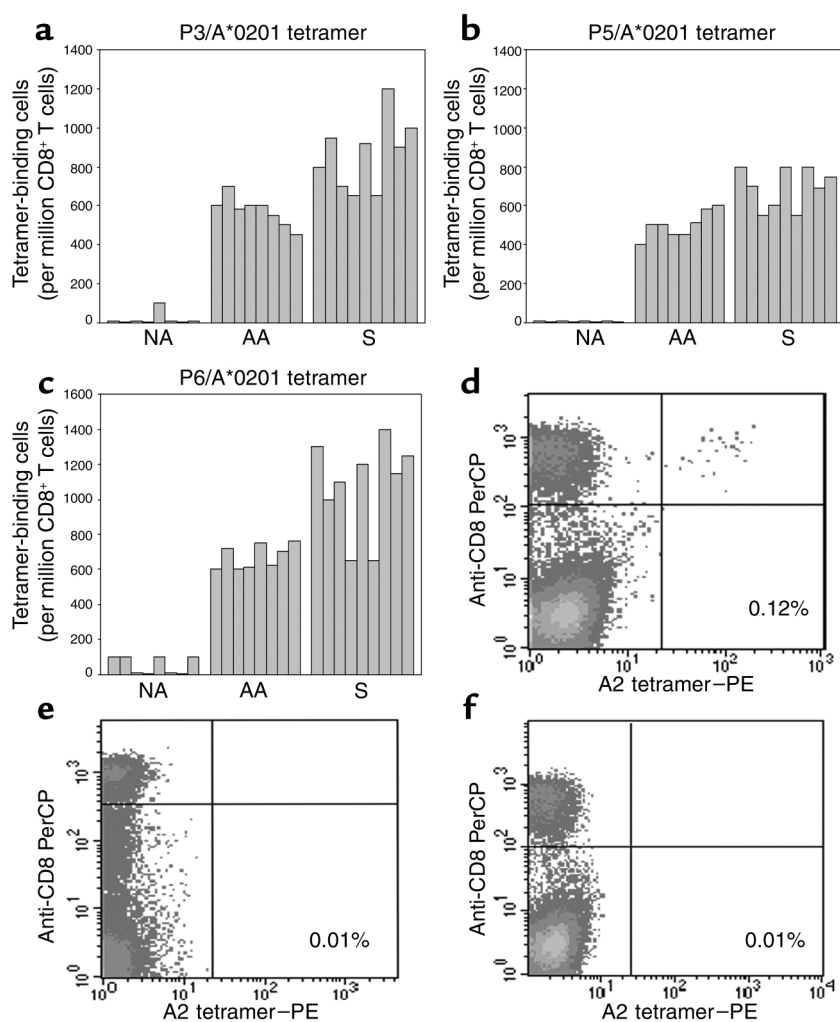Amino acid sequences of the defined HLA-A*0201 epitopes.

Figure 1

(**a**–**c**) Illustration of the number of CD8^{+} T cells staining with each tetramer (P3 A, P5 B, P6 C) per million CD8^{+} T cells for each of the subject groups. NA, nonatopic; AA, atopic asymptomatic; S, atopic symptomatic. (**d**) An example of tetramer staining of PBMCs from an A*0201–positive atopic symptomatic donor with the P6-based tetramer: *x* axis represents staining with tetramer and *y* axis represents staining with anti-CD8. In this example, 0.12% of CD8^{+} T cells stained with the tetramer. (**e** and **f**) Examples of negative control staining with **e** showing PBMCs from an A*0201–positive nonatopic donor and **f** showing PBMCs from an A*0201–positive atopic stained with an A*0201-tetramer containing a peptide derived from hepatitis B core. The horizontal gates have been applied to include the CD8-high population.

We also observed significant correlations ($P < 0.01$) between the frequency of the mean (P3, P5, and P6 tetramer mean) Der p 1–specific T cells and the total IgE ($r = 0.872$, $P < 0.01$) and house dust mite–specific IgE ($r = 0.873$, $P < 0.01$). This was confirmed on subgroup analyses that showed significant correlations ($P < 0.01$) for the symptomatic atopic group between total IgE ($r = 0.90$) and house dust mite–specific IgE ($r = 0.90$) and for the asymptomatic atopic group between total IgE ($r = 0.93$) and house dust mite–specific IgE ($r = 0.95$). For the individual peptides, correlation r values were 0.82, 0.71, and 0.25 for P6, P5, and P3, respectively, between Der p 1–specific CD8^{+} T cells and house dust mite–specific IgE. This suggests that by analyzing the combined responses to all three peptides, the overall responses may be more representative.

Overall, these data demonstrate that Der p 1–specific CD8^{+} T cells exist in the peripheral blood of atopic individuals and that increased frequencies are associated with severe disease, high total IgE, and high house dust mite–specific IgE. Having mapped the precise CD8^{+} T cell epitopes and quantified the overall frequency of the Der p 1 epitope-specific T cells, we were able to proceed to the testing of our primary hypothesis.

Cytokine production by Der p 1 epitope-specific CD8^{+} T cells. A previous study suggested that IFN-γ is a dominant cytokine produced by Der p 1–specific CD8^{+} T cells in house dust mite–sensitive individuals (27). Therefore, using both IFN-γ and IL-10 ELISpot assays, the cytokine responses to the three Der p 1 epitopes were measured within the PBMCs of the three groups of subjects (symptomatic atopic, asymptomatic atopic, and nonatopic). The Der p 1 epitope–specific CD8^{+} T cells were able to produce IFN-γ and the mean frequency within the symptomatic atopic subjects was significantly higher for each peptide ($P < 0.01$) than in the asymptomatic atopic and nonatopic groups (Figure 2). Furthermore, the frequency of the IFN-γ–secreting Der p 1 epitope–specific CD8^{+} T cells was significantly higher in the asymptomatic atopic group than in the nonatopic group of subjects ($P < 0.01$). These data demonstrate that the many of the cells identified with the tetramers were functional and able to produce IFN-γ, a putative type 1 T$_c$ cytokine (29, 47, 48). Cytokine production was uniformly present using CD4^{+} T cell depletion prior to ELISpot analysis, suggesting that the responses were CD8^{+} T cell–derived. Consistent with previous reports, the proportion of
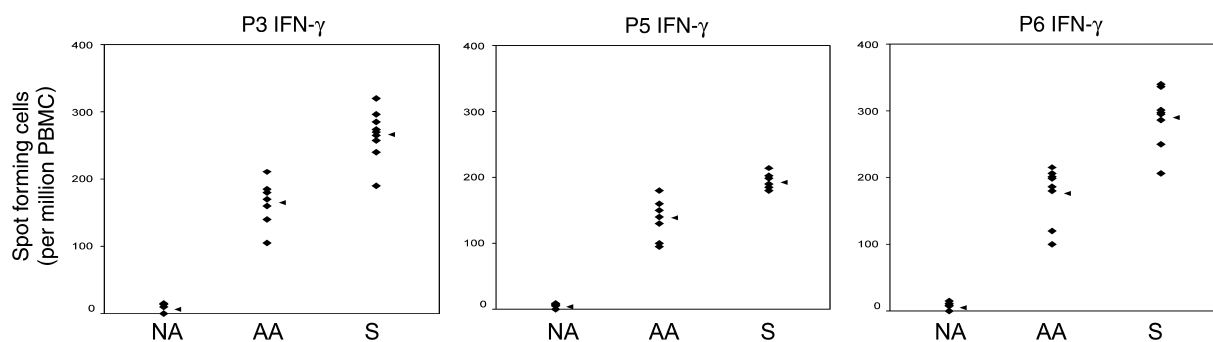
Figure 2
The figure shows the IFN-γ ELISpot data for the three peptides (P3, P5, P6) represented as spot-forming cells per million PBMCs for each group of subjects.

tetramer-binding cells able to produce sufficient IFN-γ to be detectable in an overnight ELISpot assay was approximately 25% (49, 50).

Figure 3 shows the data for the IL-10 ELISpot and demonstrates that the frequency of Der p 1 epitope–specific CD8+ T cells producing IL-10 in the symptomatic atopic group was significantly less than in the asymptomatic atopic group ($P < 0.01$). In contrast, the frequency of the Der p 1 epitope–specific CD8+ T cells producing IL-10 was significantly higher in the asymptomatic atopic group than in the nonatopic controls. As expected, the three non-A*0201–binding peptides (P1, P2, and P4) were not able to induce cytokine production by PBMCs from the A*0201–positive individuals. Overall, these data confirm that the Der p 1–specific CD8+ T cells were able to produce IL-10, a cytokine produced by both type 1 and type 2 Tc /Th cells (29, 47, 48), but that the T cells produced relatively less IL-10 in the symptomatic atopic group than in the asymptomatic atopic group.

We proceeded to further characterize the functional capacity of other nonallergen-reactive CD8+ T cells, namely T cells specific for the persistent virus, Epstein Barr virus (EBV) BMLF1 protein (280-8 GLCTLVAML) (51). Using A*0201-BMLF1 tetramers, IFN-γ, and IL-10 ELISpots, we observed that the viral-specific CD8+ T cells were able to efficiently produce IL-10 in all three groups of subjects, nonatopic, asymptomatic atopic,

and symptomatic atopic (Figure 4). The frequency of IFN-γ– and IL-10–producing BMLF1-specific CD8+ T cells showed a significant positive correlation ($P < 0.01$, $r = 0.987$). These data demonstrate that the relative defect in IL-10 expression by Der p 1–specific CD8+ T cells in the symptomatic atopic group of subjects was not present in a viral-specific CD8+ T cell population.

Cutaneous lymphocyte-associated antigen (CLA) is a carbohydrate-modified form of P-selectin glycoprotein ligand-1 expressed by a number of cell types, including T lymphocytes, and is believed to bind E-selectin on endothelial cells at sites of cutaneous inflammation (52). We examined the cell surface phenotype of the BMLF1- and Der p 1–specific CD8+ T cells and showed (Table 2 and Figure 5) that the Der p 1–specific CD8+ T cells express markers consistent with an activated, antigen-experienced phenotype and contain a large proportion that are positive for CLA. There were no differences between the phenotypic analyses of Der p 1–specific CD8+ T cells from symptomatic or asymptomatic atopics. In contrast, only 1.79% (SD 0.77, Table 2) of the BMLF-1–specific CD8+ T cells expressed CLA. These data suggest that many of the Der p 1–specific CD8+ T cells are antigen experienced and are able to home to sites of cutaneous inflammation.

Cytokine production in response to recombinant Der p 1. To more closely define the cytokine production by
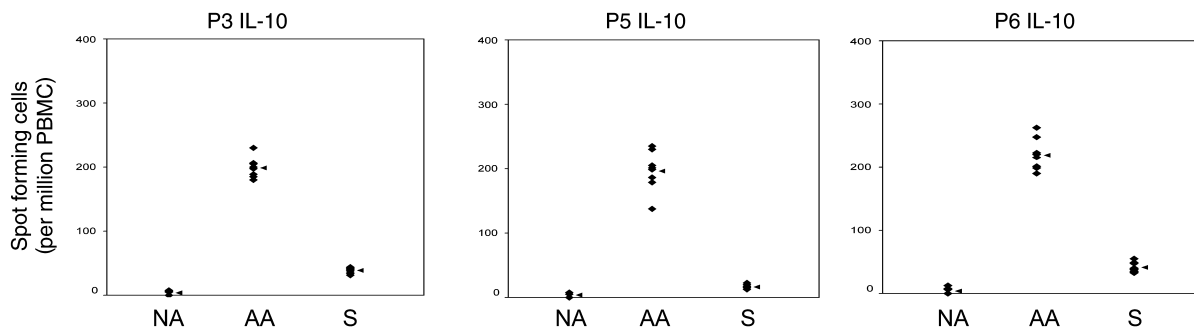

Figure 3
The figure shows the IL-10 ELISpot data for the three peptides (P3, P5, P6) represented as spot-forming cells per million PBMCs for each group of subjects.
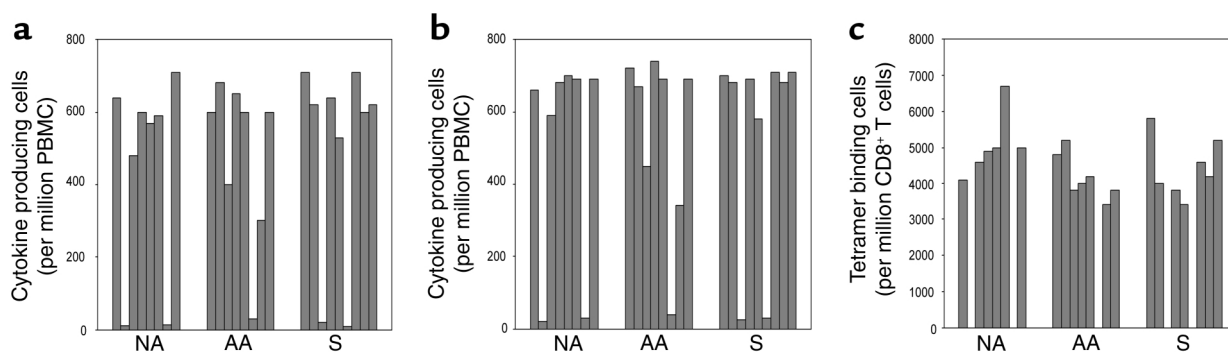
Figure 4
The figure illustrates the IL-10 (**a**), IFN-γ (**b**), and tetramer staining (**c**) for the BMLF1-A*0201 peptide (280-8 GLCTLVAML; ref 51) in the three groups of individuals, nonatopic, atopic asymptomatic, atopic symptomatic.

Der p 1–specific CD8^{+} T cells, we investigated the intracellular cytokine phenotype of CD8^{+} T cells reactive to exogenous recombinant Der p 1, which may be the dominant antigenic form encountered in vivo. Following prolonged PBMC incubation with recombinant Der p 1, a broad CD8^{+} T cell cytokine production profile, including both putative Tc1 and Tc2 cytokines IL-4, IL-5, IL-10, IL-13, IFN-γ, and TNF-α, was induced (Figure 6) from PBMCs derived from the atopics but not from the nonatopics. The symptomatic atopics had significantly higher ($P < 0.01$) frequencies of Der p 1–reactive cells producing IL-4, IL-5, IL-13, IFN-γ, and TNF-α than the asymptomatic atopics. However, IL-10 production in response to whole Der p 1 was significantly greater in the asymptomatic atopic patients than the symptomatic atopics ($P < 0.01$). The CLA[+] subset produced significantly more (>90%, $P < 0.01$) of the cytokines in response to whole recombinant Der p 1 than the CLA^{−} subset, consistent with dominance in the skin-homing subset of CD8^{+} T cells. The maximal frequencies of reactive cells were 0.12% of CD8^{+} T cells, which were comparable to the responses determined by the tetramer analyses (maximum 0.15% of CD8^{+} T cells). This suggests that the A*0201-epitope–specific responses are highly dominant and make significant contributions to the overall Der p 1 response within these individuals.

Discussion

Existing studies have suggested that atopic-derived PBMCs produce less IL-10 than PBMCs derived from normal subjects and that low levels of IL-10 within broncho-alveolar lavage fluid are associated with severe atopic disease (31, 53). We tested the hypothesis that at the single cell level, Der p 1–specific T cells produced less IL-10. By mapping the first human Der p 1 CD8^{+} T cell epitopes and quantifying the frequency of reactive T cells in the peripheral blood of severely symptomatic atopics, asymptomatic atopics, and nonatopic controls, we have shown in a cross-sectional analysis that the overall frequency of the Der p 1–specific CD8^{+} T cells is highest in those with severe disease, but the Der p 1–specific cells produce relatively less IL-10.

Considerable circumstantial evidence exists that CD8^{+} T cells may have a role in the pathogenesis of atopic disease. Increased frequencies of CLA^{+} CD8^{+} T cells producing type 2 cytokines (including IL-4, IL-13, and IL-5) are present in the peripheral blood of individuals with AD (8–10). CLA^{+}CD8^{+} and CLA^{+}CD4^{+} T cells derived from the peripheral blood of AD patients are both able to induce IgE production by B cells and enhance eosinophil survival (11). A significant proportion of the allergen-specific T cell clones isolated from AD lesional skin are of a CD8^{+} phenotype (26). Perforin and granzyme B are strongly expressed in AD lesional skin and show significant correlation with the degree of epidermal edema (54). Perforin-positive T cells are also elevated in the peripheral blood of asthmatics (55), and CD8^{+} T cells are sequestered in the airways during episodes of acute asthma (56). Indeed, activated CD8^{+} T cell infiltration into peribronchial tissue has been associated with asthma death (57). Our data illustrate that Der p 1–specific CD8^{+} T cells exist in humans and that it is possible to map the precise class I epitopes and characterize patterns of cytokine production. The demonstration that individuals with severe atopic disease have high frequencies of Der p 1–specific CD8^{+} T cells is consistent with a role for such cells in disease pathogenesis.

IL-10 is produced by numerous cell types including monocytes, macrophages, dendritic cells (DCs), B cells, T cells, mast cells, keratinocytes, and eosinophils. It is

Table 2
Percentage of tetramer-binding cells staining for specific cell surface markers

	Mean P3/P5/P6 a*0201-tetramer-binding cells	EBV BMLF1 a*0201-tetramer-binding cells
CLA	45.7% (12.4)	1.79% (0.77)
CD45RA	0.80% (0.77)	29.6% (16.2)
CD28	82.4% (8.4)	67.4% (19.9)
CD45RO	90.6% (5.02)	ND
CD27	73.4% (8.98)	93.0% (5.4)

The figures show the percentage of the tetramer-binding cells that express each phenotype, with the SD in parentheses. The samples were from all individuals of the symptomatic atopic and asymptomatic atopic groups. There were no significant differences between the two subgroups. ND, not determined.
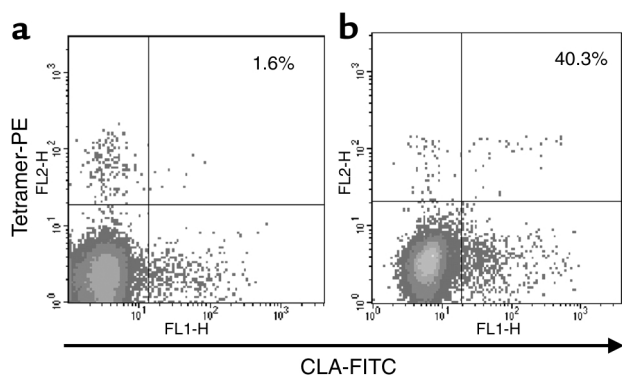
Figure 5
The FACS images show an example of staining of asymptomatic atopic (AA) PBMCs with anti-CLA (FITC, *x* axis) and tetramer (PE, *y* axis). (**a**) Data for the EBV BMLF1 A*0201 tetramer. (**b**) Data for the P6 A*0201 tetramer with the percentage of tetramer-binding cells that are CLA shown in the top right-hand corner of each image.

pleiotropic in function with known regulatory effects on diverse cell types including T cells, NK cells, B cells, DCs, keratinocytes, mast cells, granulocytes, and endothelial cells (58). IL-10 treatment of DCs can induce or contribute to a state of anergy in allo-antigen– or peptide-antigen–activated T cells (59–64). While both macrophages and DCs have been reported to cross-present exogenous antigens to CD8+ T cells, it is the DCs that are believed to be more effective in inducing naive CD8+ T cells to respond through cross-priming (65–67). Such a pathway may be particularly susceptible to inhibition by IL-10 in atopics. Our data are consistent with these studies and potentially provide a cellular mechanism to link the low levels of IL-10 and severe atopic disease. We observed no change in the IL-10 production capacity of the viral-specific CD8+ T cells in the severe atopics, demonstrating that there is not a complete absence of IL-10. Instead, there is a relative difference in cells specific for different classes of antigens, perhaps reflecting the cytokine pattern present at the time of T cell priming or activation. Genetically, susceptible atopic individuals may have a relative epithelial defect, facilitating uptake of exogenous Der p 1 by epidermal Langerhans cells or dermal dendritic cells, perhaps via keratinocyte apoptosis (68, 69). Subsequent cross-presentation to IFN-γ–producing Der p 1–specific T cells may contribute to a local inflammatory response, which is particularly severe in the context of diminished IL-10. It is possible that in the asymptomatic atopics, the IL-10–producing Der p 1–specific T cells are functioning in an analogous manner to CD4+ regulatory T cells, for which IL-10 may be an important mediator of active suppression (70, 71). Interestingly, IL-10–producing CD8+ T cells have been shown recently to inhibit the allospecific proliferation of naive CD8+ T cells to monocytes and DCs (72). The pattern of IL-10 production by Der p 1–specific CD4+ T cells is currently under investigation, as are the longitudinal changes in specific CD8+ T cells. Allergen-spe-

cific CD4+ T cells have been identified in both atopics and nonatopics, but in response to allergen stimulation there is a Th2 bias in the former (39). The current data demonstrate that like allergen-specific IgE, allergen-specific CD8+ T cells are restricted to the atopic group. Therefore, both atopics and nonatopics are sensitized to the common allergens, but atopics mount both IgE and CD8+ T cell responses, which may contribute to the development of clinical disease.

CD8+ T cells from atopics were also able to respond to whole recombinant Der p 1 through production of IL-4, IL-5, IL-10, IL-13, IFN-γ, and TNF-α. The symptomatic atopics produced significantly less IL-10 in response to whole Der p 1 than the asymptomatic atopics, consistent with the findings for the peptide-based ELISpot responses. The maximal frequencies of the responses were comparable to the responses determined by the A*0201-tetramer analyses. These data suggest that the A*0201 responses measured using the tetramers are highly dominant and make a significant contribution to the overall Der p 1–specific responses.
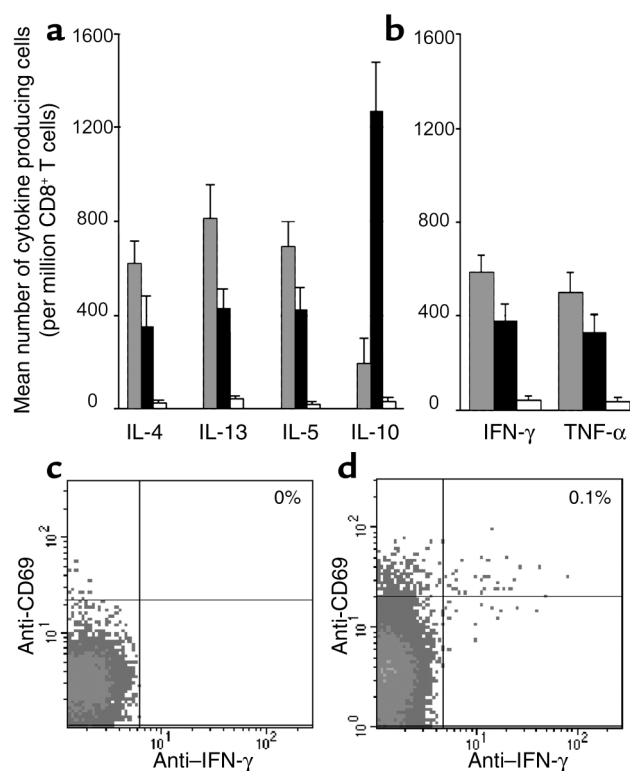

Figure 6
The number of cytokine-producing cells per million PBMCs in response to whole recombinant Der p 1. The gray bars represent atopic symptomatic patients (S), the black bars represent the atopic asymptomatic patients (AA), and the white bars represent the nonatopic individuals (NA). (**a**) Data for cytokines IL-4, IL-5, IL-13, and IL-10. (**b**) Data for cytokines IFN-γ and TNF-α. (**c** and **d**) Unstimulated PBMCs and recombinant Der p 1–stimulated PBMCs, respectively (gated on CD8+ T cells). The *y* axis shows staining with anti-CD69, and the *x* axis shows staining with anti–IFN-γ. (**d**) 0.1% of CD8+ T cells produced IFN-γ in response to whole recombinant Der p 1 staining.

However, the cytokine responses were largely restricted to the CLA+ subset, raising the possibility that in addition to identifying tissue-specific homing capacity, the presence of CLA may also be associated with increased functional activity. The ability of whole exogenous Der p 1 and Der p 1–derived peptides to induce cytokine production by Der p 1–specific CD8+ T cells would be consistent with cross-presentation of the class I–binding peptides by antigen-presenting cells within the PBMC population. Overall, these data show that Der p 1–specific CD8+ T cells derived from atopics can produce both Tc1 and Tc2 cytokines and that responses are likely to be mediated, at least in part, through APC cross presentation.

A large proportion (mean 45.7%) of the Der p 1–specific CD8+ T cells expressed CLA and therefore potentially had the capacity to home to the skin and mediate tissue damage. In contrast, few of the EBV-specific CD8+ T cells expressed CLA. Since the symptomatic atopic group had high frequencies of Der p 1–specific CD8+ T cells detectable with tetramers and IFN-γ–ELISpot, but only a small fraction (mean 15%) were able to produce IL-10, the presence of CLA was not an absolute correlate of low IL-10 production.

By defining the first human Der p 1–specific CD8+ T cell epitopes, we have been able to generate insight into a number of questions relating to atopic disease pathogenesis. We have provided a possible cellular mechanism to link low levels of IL-10 and severe atopic disease. The frequency of the Der p 1–specific CD8+ T cells correlates with disease activity, and the cells are able to produce IFN-γ with capacity similar to viral-specific CD8+ T cells. However, severely affected atopics have relatively lower production of IL-10 by the Der p 1–specific CD8+ T cells. Immunotherapeutic manipulation of allergen-specific immunity using minimal T cell epitopes is becoming an increasingly accessible goal (73, 74), and we hope that our data will ultimately be of value for such an approach.

Acknowledgments

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