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CCL5-independent Helper T Lymphocyte Responses to Immuno-dominant Pneumococcal Surface Protein A Epitopes

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Abstract

Understanding the requirements for protection against pneumococcal carriage and pneumonia will greatly benefit efforts in controlling these diseases. Several antigens, in addition to the polysaccharide capsule, have been implicated in both the virulence and protective immunity against *Streptococcus pneumoniae*; one of the best-studied *S. pneumoniae* antigens is pneumococcal surface protein A (PspA). Recently, it was shown that genetic polymorphisms could diminish CCL5 expression, which results in increased susceptibility to and progression of infectious diseases. We previously showed CCL5 blockade reduced PspA-specific humoral and cellular pneumococcal immunity, during *S. pneumoniae* strain EF3030-induced carriage, by diminishing IFN-γ and enhancing IL-10 secretion by effector T cells. We also identified immuno-dominant helper T lymphocyte (HTL) epitopes in PspA peptide 19-23 ($PspA_{199-246}$), which caused comparatively more cytokine secretion and proliferation responses by splenic and cervical lymph node (CLN) $CD4^+$ T cells from mice previously challenged with *S. pneumoniae* strain EF3030. In this study, we sought to determine if $PspA_{199-246}$–specific $CD4^+$ T cells responses were resistant to the effect of CCL5 deficiency. In short, T cell responses against these HTL epitopes were resistant to CCL5 inhibition, than compared to cells from control or naïve mice, and unaffected by reduced co-stimulatory molecule expression caused by CCL5 blockade. CCL5 deficiency also corresponded with a higher number of IL-10^+ $CD11b^+$ $CD11c^{Lo}$ and $CD11b^+$ $CD11c^{Hi}$ cells and lower IFN-γ expression by similar cells, than compared to controls. These data confirm CCL5 is an essential factor for optimal pneumococcal adaptive immunity and show $CD4^+$ T cell responses to $PspA_{199-246}$ are largely resistant to CCL5 deficiency.

Keywords

T helper cytokine; Streptococcus pneumoniae; HTL epitope

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Introduction

Pneumonia caused by *S. pneumoniae*, is the most common cause of childhood deaths in the developing world and among the top ten causes of death in aged populations. Recently, antibiotic-resistant *S. pneumoniae* strains have emerged worldwide [1-3]. Pneumococci in nasopharyngeal carriage are thought to be the main human reservoir for these potentially lethal bacteria. Moreover, nasopharyngeal carriage is thought to be an intermediate stage that precedes invasive disease [4]. Vaccination against pneumococcal infections is greatly needed. However, the host factors that determine pneumococcal immunity are imprecisely known. This study addresses the contribution of an essential host factor and dominant HTL epitopes in pneumococcal immunity.

Chemokines have emerged as important factors and possible mucosal adjuvants that function in lymphocyte activation and recruitment [5-7]. Indeed, a qualitative relationship exists between the class of chemokines secreted following infection, the type of immune response (cellular or humoral immunity) elicited, and the fate of the host following infection [8-11]. The profile of chemokine expression serves as an indicator of immune response type (i.e., Th1 vs. Th2). In this respect, the CCL5-CCR5 axis has been demonstrated to be involved in the activation and function of Thl cells [6, 12, 13]. CCL5 is secreted by epithelial cells, macrophages, fibroblasts, platelets, and activated T cells [14]. This CC chemokine is known to regulate T cell differentiation and polarize Th1 ⩾ Th2 subtypes as well as numerous physiological functions of leukocytes including migration [6, 9, 14, 15]. Genetic variations in *ccl5* contribute to differences in infectious disease progression. Indeed, polymorphisms in *ccr5* and *ccl5* genes play critical roles in susceptibility to and progression of infectious diseases, namely HIV/AIDS and *Chlamydia* [16-18]. CCL5 acts as an adjuvant for antigen-specific humoral and cellular immune responses in both mucosal and systemic compartments [6]. However, it is not certain what effect these variations have on *S. pneumoniae* disease susceptibility, progression, and/or protective T cell immunity.

Recently, we showed that *S. pneumoniae* strain EF3030 induced bronchial epithelium to express CCL5, which was required for optimal pneumococcal humoral and cellular immunity [19]. In fact, CCL5 inhibition resulted in fewer local and systemic antigen-specific $CD4^+$ T cells that produced IL-4 and IFN-γ, while increasing T helper cells that secreted IL-10. Recently, we revealed a region in PspA, spanning residues 199 to 246 ($PspA_{199-246}$), with dominant HTL epitopes that theoretically bind a broad range of HLA-DR, -DQ, and –DP alleles as well as I-A and I-E. Overlapping peptides in this region, i.e., PspA peptides 19, 20, 21, 22, and 23, induced significant IFN-γ and IL-10 secretion and proliferative responses after *ex vivo* stimulation of T helper cells from previously pneumococcal-challenged mice [20].

Our study specifically addresses an important question “are dominant HTL epitopes resistant to CCL5 deficiency?” This is an important question to design better vaccines against *S. pneumoniae*, especially when one considers the health disparities associated with CCL5 expression caused by the In 1.1 T/C mutation [17]. We used a novel human isolate of capsular group 19 pneumococci that was passed in mice to yield *S. pneumoniae* strain EF3030, which has a greater propensity to cause nasal or pulmonary infections than to cause sepsis and death when given intranasally [21]. Through antibody-mediated inhibition, we show that dominant PspA HTL epitopes are largely resistant to CCL5 deficiency, despite the significant contribution this chemokine has on pneumococcal immunity.

Materials and Methods

Mice

Female F1 (C57BL/6 × BALB/c) mice, aged 8 to 12 weeks, were procured from Jackson Laboratories (Bar Harbor, MA). All mice were housed in horizontal laminar flow cabinets free of microbial pathogens. Routine Ab screening for a large panel of pathogens and histological analysis of organs and tissues were performed to insure that mice were pathogen - free. The Morehouse School of Medicine Institutional Review Board approved all procedures using mice.

Anti-CCL5 antibody generation and treatment

CCL5 and Freund's or incomplete Freund's adjuvants (Sigma, St. Louis, MO) were used to generate anti-CCL5 antibody titers of ~ $1{:}10^6$ such that 10 μl of rabbit anti-CCL5 antiserum neutralized 20 ng of CCL5. This antiserum was titrated by direct ELISA and no cross-reactivity was detected, when tested against other CCR5 ligands, chemokines (CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, XCL1, CCL1, CCL2, CCL4, CCL7, CCL8, and CCL11), and cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, and TNF-α). Subsequently, normal or anti-CCL5 sera were heat-inactivated and purified using an IgG isotype-specific protein A column (Pierce Biotechnology, Rockford, IL). Anti-CCL5 antibody titers or non-immunized sera were adjusted to $1{:}4{\times}10^5$ (i.e., 50 × dilution) in PBS (CCL5 antibody solution), which were administered to mice two days before bacterial challenge and every third day thereafter.

S. pneumoniae strain EF3030 growth and challenge

S. pneumoniae capsular strain EF3030 was among the human isolates of capsular group 19 that were previously examined and found to be relatively non-invasive in mice [22]. Pneumococci were grown in Todd Hewitt broth and stored frozen in aliquots at −70 °C, in 20% glycerol, in sterile lactated Ringer's solution (Ringer's) (Abbott Labs) [23, 24]. To establish nasal carriage, groups of F1 (C57BL/6 × BALB/c) mice were nasally administered 10^7 colony-forming units (CFU) of EF3030 in 15 μL of Ringer's solution [25]. Experimental groups consisted of 10 mice and studies were repeated 3 times. The guidelines proposed by the committee for the Care of Laboratory Animal Resources Commission of Life Sciences - National Research Council were followed to minimize animal pain and distress. The Morehouse School of Medicine review board approved all procedures involving mice.

Pneumococcal antigens

Previously, we revealed immuno-dominant PspA helper T lymphocyte (HTL) epitopes recognized by *S. pneumonia* strain EF3030-challenged $CD4^+$ T cells [20]. These epitopes were identified by "peptide walking" using PspA peptides 15 amino acids in size, which overlapped by four amino acids. This region spanned residues 199-246 of the PspA protein sequence (NCBI Acession no. NP_359087). For this study, we synthesized peptides 19-23, DAEEVAPQAKIAELE, AELENQVHRLEQELK, QELKEIDESESEDYA, EDYAKEGFRAPLQSK, and LQSKLDAKKAKLSKL, respectively, by the multipin synthesis method by Chiron Mimotopes Peptide Systems [26] (Table 1). All peptides were acetylated at the N-terminus and ended with a COOH-terminal. Purity of these peptides was approximately 95% and free of endotoxin contamination. The peptides were dissolved in a mixture (v/v) of 75 % dimethyl sulfoxide and 25 % water, to a concentration of 70 mM, divided into small aliquots and stored frozen at −80 °C.

Tissue collection and cell isolation

Mice were sacrificed by CO_2 inhalation to collect the spleen and CLNs for single cell isolation of lymphocytes. Single cell suspensions of spleen and CLNs were collected 0, 7, 14 and 28 days following *S. pneumoniae* strain EF3030 challenge, prepared by aseptically removing tissues and passage through a sterile wire screen. $CD4^+$ T cells were further separated by OctoMACS™ (Miltenyi Biotec) using negative selection. Remaining (non-$CD4^+$ T cells), were used as accessory feeder cells for peptide-specific stimulation assays after mitomycin C (Sigma) treatment.

Cytokine quantitation by Luminex™ analysis

Purified $CD4^+$ T cells and mitomycin C-treated feeder cells were cultured at a density of 5×10^6 and 10^6 cells per ml, respectively, in complete medium containing 1 μM of each PspA peptide at 37°C in 5% CO_2. For the assessment of cytokine production, 100 μL of culture supernatants from 96-well flat bottom plates (Corning Glass Works) were harvested 3 days after *ex vivo* PspA peptide stimulation to determine the levels of IL-10 and IFN-γ secreted by $CD4^+$ T cells. Phorbol-12-myristate-13-acetate (PMA) 1 μg/mL was used as a positive control, ovalbumin (1 μg/mL) and medium only were used as negative controls. Supernatant cytokine levels were determined by the Beadlyte™ mouse multi-cytokine detection (Bio-Rad). Briefly, filter bottom ELISA plates were rinsed with 100 μL of Bio-plex assay buffer and liquid was removed using a Millipore™ Multiscreen Separation Vacuum Manifold System set at 5 mm Hg. Analyte beads in assay buffer were added to the wells followed by 50 μL of serum or standard solution. The plates were incubated for 30 minutes at room temperature with continuous shaking (at setting #3) using a Lab-Line™ Instrument Titer Plate Shaker. The filter bottom plates were washed, as before, and centrifuged at 300 × g for 30 seconds. Subsequently, 50 μL of anti-mouse IL-10 or IFN-γ antibody-biotin reporter solution was added in each well, after which the plates were incubated with continuous shaking for 30 min followed by centrifugation and washing. Next, 50 μL streptavidin-phycoerythrin (PE) solution was added, and the plates were incubated with continuous shaking for 10 min at room temperature (25°C). 125 μL of Bio-plex assay buffer was added, and Beadlyte™ readings were measured using a Luminex™ System and calculated using Bio-plex™ software (Bio-Rad). The cytokine Beadlyte™ assays were capable of detecting > 5 pg/mL for each analyte.

Cell proliferation

Lymphocyte proliferation was measured by a 5-bromo-2′-deoxy uridine (BrdU) absorption and detection (Roche Diagnostics). In brief, purified $CD4^+$ T cells were cultured at a density of 5×10^6 cells/mL, with 10^6 mitomycin C-treated feeder cells/mL in complete medium containing 1 μM of PspA peptide at 37°C in 5% CO_2. After 2 days of *ex vivo* antigen stimulation, cells were transferred to polystyrene 96 well plates (Corning Glass Work). 10 μL of BrdU labeling solution (10 μM final concentration per well) were added and incubated for 18 hours at 37°C with 5% CO_2. The cells were then fixed and incubated with 100 μL of nuclease in each well for 30 minute at 37°C. The cells were washed with complete media and incubated with BrdU-POD solution for 30 minute at 37°C. BrdU incorporation was developed with a 2,2′–azino-bis 3-ethylbenzthia-zoline-6-sulfonic acid (ABTS) solution and optical density (OD) was read at 450 nm. The proliferation index (PI) was calculated as follows. Antigen-specific $CD4^+$ T cell proliferation was obtained by measuring 5-Bromo-2′-deoxy uridine (BrdU) incorporation, according to manufacturer's instructions (Roche Diagnostics). BrdU absorption or optical density at 450nm (OD_{450}) was detected using a scanning multi-well SpectraMax 250 spectrophotometer (Molecular Devices). PI = BrdU OD_{450} of peptide -stimulated cells / BrdU OD_{450} in un-stimulated cells × 100 %. The results were expressed as mean ± the standard error mean (SEM) of the response of three replicate

determinations from three independent experiments. Statistical significance was assessed by student's *t* test.

Flow cytometry

After single cell isolation of lymphocytes, fluorescently tagged monoclonal antibodies (BD-Pharmingen) were added to characterize $CD4^+$ $CD11b^+$ and $CD11c^+$ lymphocytes. Cells were washed 3 times in PBS (supplemented with 0.5% BSA) and treated with 1 μg of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, allophycocyanin (APC)-, or Cy5-conjugated IgG control isotype or rat anti-mouse CD4, CD11b, CD11c, IL-10 and/or IFN-γ antibodies per 10^5 cells at 4°C for 30 minutes. Subsequently, wells were washed with PBS to remove unbound antibodies. Labeled cells were fixed in 500 μL of 2% paraformaldehyde and 10^4 CD4− or CD11b-gated cells were analyzed using a FACS Caliber™ flow cytometer (BD) and Flowjo software (Tree Star).

Statistics

Data were expressed as mean ± SEM and compared using a two-tailed student's *t*-test or an unpaired Mann Whitney U test. The results were analyzed using Microsoft Excel and considered statistically significant if $p < 0.01$. When expression levels were below the detection limit (BD), then values were recorded as one-half the lower detection limit for statistical analysis. Kolmogorov-Smirnov (K-S) two sample tests were used to compute the statistical significance between histograms; results were considered statistically significant if $p < 0.01$.

Results

Proliferation and cytokine responses of $PspA_{199\text{-}246}$-specific systemic and local $CD4^+$ T cells following pneumococcal carriage

$PspA_{199\text{-}246}$-specific $CD4^+$ T cell responses were characterized 28 days after *S. pneumoniae* strain EF3030 challenge along with anti-CCL5 or control antibody treatment. In general, pneumococcal carriage led to substantial increases in PspA-specific proliferative responses (Figure 1). In confirmation with our previous finding, CCL5 inhibition significantly lowered PspA (i.e., whole protein) proliferative *ex vivo* antigen restimulation responses [19, 27]. Surprisingly, CCL5 blockade during pneumococcal carriage resulted in slightly increased splenic as well as CLN $CD4^+$ T cell proliferative recall responses to PspA peptides 19-23, than compared to naïve mice or control antibody-treated mice. Of the peptides tested, PspA peptide #21 induced the highest recall proliferation response by both systemic as well as local $CD4^+$ T cells, which were not significantly effected by CCL5 deficiency.

In confirmation with our prior studies [19, 27], splenic and CLN derived $CD4^+$ T cells, from pneumococcal -challenged mice that received anti-CCL5 antibody, secreted significantly more IL-10 in response to *ex vivo* restimulation with whole PspA protein, than compared to cells from naïve mice or similar cells from infected mice treated with control antibody. In general, CLN $CD4^+$ T cells from *S. pneumoniae* strain EF3030-challenged mice secreted significantly higher levels of IL-10 after PspA peptide 19-23 *ex vivo* restimulation, than compared to similar splenic T helper cells from control mice. However, PspA peptides 22 and 23 induced significantly more IL-10 secretion by *ex vivo* restimulated CLN $CD4^+$ T cells from pneumococcal-challenged, anti-CCL5 antibody-treated mice than compared to naïve T helper cells or similar cells from mice treated with control antibody that were *ex vivo* restimulated with PspA peptides 19, 20, or 21. Splenic $CD4^+$ T cells from *S. pneumoniae* strain EF3030-challenged mice secreted similar levels of IL-10 after *ex vivo* restimulation with PspA peptides 19-23. However, splenic T helper cells from infected mice that were *ex vivo* restimulated with PspA peptides 19 and 20 secreted comparatively more

IL-10 than similar cells *ex vivo* restimulated with PspA peptides 21-23. Taken together, local and systemic $CD4^+$ T cells from pneumococcal-challenged mice, treated with either control antibody or anti-CCL5 antibody during carriage, secreted similar levels of IL-10 in response to PspA peptides 19 and 20, but not PspA peptides 21-23.

We previously showed splenic and CLN $CD4^+$ T cells from pneumococcal-challenged mice receiving anti-CCL5 antibody produced significantly less IFN-γ in response to whole PspA protein *ex vivo* restimulation, than compared to similar cells from infected mice treated with control antibody during infection [19, 27]. In contrast, T helper cells from *S. pneumoniae* strain EF3030-challenged mice secreted similar amounts of IFN-γ when *ex vivo* restimulated with PspA peptides 19-23, regardless of whether these mice received control antibody or anti-CCL5 antibody during infection. As seen with CLN T helper cell IL-10 recall responses, PspA peptide 23 induced CLN-derived $CD4^+$ T cells, from anti-CCL5 antibody-treated, pneumococcal-challenged mice to secrete significantly more IFN-γ than compared to similar cells from infected mice treated with control antibody during infection. Hence, CLN- and spleen-derived T helper cells from mice receiving either control antibody or anti-CCL5 antibody during pneucoccal carriage secrete comparable amounts of IFN-γ when ex vivo restimulated with PspA peptides 19-22, but not PspA peptide 23.

Effect of CCL5 blockade on co-stimulatory molecule expression

Co-stimulatory molecule expression by leukocytes from pneumococcal-infected mice was evaluated to determine whether CCL5 blockade modulated CD40L, CD28, CD80 and CD86 expression during carriage. Anti-CCL5 antibody treatment during carriage lead to an decrease in CD40L and CD28 expression by splenic as well CLN-derived $CD4^+$ T cells 7 and 14 days after pneumococcal challenge (Figure 2). There were also significant decreases in CD80 and CD86 expression by spleen and CLN-derived $CD11b^+$ and $B220^+$ cells from anti-CCL5 antibody-treated mice, than compared to control mice 7 and 14 days after pneumococcal challenge (Figure 3). Our findings show anti-CCL5 antibody treatment during carriage lead to a reduction in CD28, CD40L, CD80 and CD86 expression by both systemic and local leukocytes up to 2 weeks after pneumococcal challenge.

Effect of CCL5 blockade on IL-10 and IFN-γ expression by T helper cells and monocytes

Previously, we showed CCL5 inhibition increased IL-10 secretion by PspA-specific splenic $CD4^+$ T cells, but decreased IFN-γ production by similar cells isolated from CLNs, 28 days post pneumococcal challenge [19]. To better elucidate changes in the expression of these cytokines caused by CCL5 deficiency during carriage, the effect of anti-CCL5 antibody treatment on the frequency of IL-10- and IFN-γ-expressing spleen- and CLN-derived $CD4^+$ T cells were examined 7 and 14 days post pneumococcal challenge. CCL5 blockade during pneumococcal carriage resulted in dramatically higher IL-10 expression by both spleen- and CLN-derived T helper cells isolated 7 and 14 days after challenge (Figure 4A), than compared to similar cells from the control antibody-treated mice or isotype control antibody staining of pooled lymphocytes from both groups. In contrast to our previous findings observed 28 days post bacterial challenge, anti-CCL5 antibody treatment during carriage lead to a modest, yet measurable, increase in IFN-γ expression, than compared to similar cells from infected mice receiving control antibody (Figure 4B).

Seven days after challenge, 0.75% of splenic $CD11b^+$ $CD11c^{Hi}$ cells from control antibody-treated mice were $IL10^+$, compared to 1.55% of similar cells isolated from anti-CCL5 antibody-treated mice (Figure 5). This two-fold increase was also observed 14 days post challenge. Interestingly, CCL5 inhibition led to a two fold decrease in the percentage of $IL\text{-}10^+$ $CD11b^+$ $CD11c^{Lo}$ spleen cells 7 days (i.e. 4.37% to 2.13%) and more modest decreases (3.48% to 2.72%) 14 days after pneumococcal challenge. A similar trend was

observed in the percentage of IL-10^{+} CD11b^{+} CD11c^{Hi} cells isolated from the CLN. Unlike splenic IL-10^{+}CD11b^{+}CD11c^{Lo} cells, the percentage of these cells in the CLN were significantly higher and 14 days post pneumococcal carriage following CCL5 blockade, than cells from mice treated with control antibody (Tables 1 and 2, respectively). Remarkably, the establishment of pneumococcal carriage lead to a significant increase in the percentage of IFN-γ^{+} CD11b^{+} CD11c^{-} and IL-10^{+} CD11b^{+} CD11c^{-} cells 14 days post challenge, than compared to cells 7 days post challenge. However, these B220^{+} cells (data not shown) did not appear to be significantly affected by CCL5 deficiency, because there were no major changes in the percentage of IFN-γ^{+} CD11b^{+} CD11c^{-} or IL-10^{+} CD11b^{+} CD11c^{-} cells from mice treated with either control antibody or anti-CCL5 antibody.

In contrast to IL-10^{+} monocytes and dendritic cells in the spleen, CCL5 deficiency resulted in nearly two fold decreases in the percentage of IFN-γ^{+} CD11b^{+} CD11c^{Hi} splenocytes 7 and 14 days post pneumococcal challenge, than compared to similar cells from experimental control mice. Similar decreases occurred in CLN –derived IFN-γ^{+} CD11b^{+} CD11c^{Hi} cells 7 and 14 days after *S. pneumoniae* strain EF3030-induced carriage from anti-CCL5 antibody - treated mice compared to control antibody-treated mice. The percentage of CLN and splenic IFN-γ^{+} CD11b^{+} CD11c^{Lo} cells from pneumococcal-challenged mice receiving anti-CCL5 antibody was approximately two-fold lower than similar cells from mice administered control antibody. These changes largely corresponded with trends in CLN- and spleen-derived IFN-γ^{+} CD11b^{+} CD11c^{Hi} and IFN-γ^{+} CD11b^{+} CD11c^{Lo} cells. Taken together, the data show that increases in splenic IL-10^{+} DCs following CCL5 blockade coincided with increases in the number of IL-10^{+} CD4^{+} T cells and decreases in splenic CCR5^{+} DCs, 7 and 14 days following challenge (Tables 1 and 2).

Discussion

PspA is a highly conserved, cell wall-associated surface protein that plays a major role in pneumococcal virulence by inhibiting both bactericidal effect of human apolactoferrin and complement deposition on the bacterial surface [28]. Our previous studies showed CCL3, CCL4, and CCL5 enhance adaptive immunity through cytokine and co-stimulatory molecule modulation [6, 7]. We also demonstrated that pneumoccal carriage induces PspA-specific cellular response [27], which were regulated in part by CCL5 [19]. Indeed, lack of CCL5 resulted in potential lethal effects approximately 5 days after pneumococcal challenge, during the end of the innate response and beginning of the recognition phase of the adaptive immune response. CCL5 blockade resulted in a dramatic (~10^{4} fold) increase in *S. pneumonia* strain EF3030 colony forming units (CFUs) from nasal tract wash and lung lavage samples. These heightened bacterial loads, relative to infected controls, continued 28 days after challenge. For these reasons and to better determine the effects of CCL5 deficiency on immuno-dominant pneumococcal T cell epitopes after the recognition phase, the current study used F1 (C57BL/6 × BALB/c) mice that were less susceptible to the lethality of CCL5 blockade. I-A^{d} and I-E^{d} peptide binding and associated responses have been used to identify sequence motifs for immunogenic peptide regions and correlate with DR-binding [29, 30, Alexander, 1994 #19402, Sidney, 1994 #19403]; hence, studying PspA$_{199\text{-}246}$-specific CD4^{+} T cell responses in mice might give insights into similar responses in man. Using these approaches, this study sought to ascertained whether PspA$_{199\text{-}246}$-specific CD4^{+} T cells responses were resistant to the previously described effects of CCL5 deficiency.

IL-10 leads to macrophage/monocyte deactivation as well as suppresses the release of reactive -oxygen species and -nitrogen intermediates, which are involved in the pathophysiology of pneumococcal meningitis [31]. This Th2 cytokine reduces pulmonary vascular leakage and the appearance of red blood cells in the alveoli during pneumococcal

pneumoniae [32]. IL-10 has also been shown to enhance susceptibility to pneumococcal infections [33]. Interestingly, this selective increase in IL-10 secretion was representative of unique Th2 cell subsets that are often prevalent at early stages of Th2 differentiation that diminish over time [34]. IL-10^+ $CD4^+$ T cells also exist as T regulatory (Tr1) cells. While additional studies will be necessary to characterize the potential of CCL5 deficiency on $CD4^+$ $CD25^+$ Tr1 cells or merely the lack of appropriate T helper cell development, our data suggest that the development of antigen-specific IL-10-secreting $CD4^+$ T cells, in the presence of diminished Th1 (e.g. IFN-γ and TNF-α), correlated with a higher number of IL-10^+ $CD11b^+$ $CD11c^+$ cells, 7 days post infection.

Marginal zone (MZ) B cells interact with B1 B cells to generate a massive wave of IgM antibodies in the initial 3 days of a primary response to pneumococcal infection [35]. B1 B cell anti-phosphorylcholine (PC) responses confer protection against *S. pneumoniae* [36], although homing to lung and/or nasal tract is not required. While the precise role of MZ, B1, B2, and follicular B cells in thymus-dependent or -independent pneumococcal responses remains uncertain, B cell apoptosis controls the level of humoral *S. pneumoniae* responses [37]. Interestingly, high CCL5 expression during an immune response is associated with expanded conventional B2 cells, but not MZ or B1 cells proliferation [38]. In contrast, innate-like B1 B cells play housekeeping roles, including spontaneous production of IgM, anti-PC antibody, and IL-10 [39]; the latter has been shown to regulate macrophage polarization during inflammation and infection [40]. Our data show that $CD11b^+$ $CD11c^-$ leukocytes were a major source of IL-10 (Figure 5), at least 14 days after pneumococcal carriage was established in mice receiving control or anti-CCL5 antibody. The precise function of these $B220^+$ B cells (data not shown) in the context of CCL5 and *S. pneumoniae* carriage remains to be determined. Additional studies will be required to determine the role of CCL5 in MZ, follicular, and B1 B cell IL-10 responsiveness and their collective role in pneumococcal immunity.

IFN-γ is required for protective host immunity against pneumococcal disease(s) [41]. PspA peptide-specific IFN-γ $CD4^+$ T cell secretion were dramatically reduced in the absence of CCL5. In contrast to Th2 cells, Th1 cells preferentially express CCR5 [12]. Hence, expression of CCR5 ligands (e.g. CCL5, CCL4 and CCL3) often precedes recruitment of $CCR5^+$ cells for Th1 responses. To this end, we show the number of $CCR5^+$ T cells and antigen-presenting cells significantly increased following pneumococcal challenge. As expected, leukocyte subpopulations in the spleen and CLNs of in anti-CCL5 antibody-treated mice contained less IFN-γ^+ $CD4^+$ T cells relative to control antibody-treated mice. $PspA_{199\text{-}246}$-specific $CD4^+$ T cells were able to mount both IL-10 and IFN-γ responses in either control or anti-CCL5 antibody treated mice. IL-10 and IFN-γ double-producers are found in a subpopulation of Tr1 and Th1 cells, which suppress DCs ability to activate $CD4^+$ T cells [42]. It is tempting to speculate that the previously reported IFN-γ^+ IL-10^+ $CD4^+$ T cells might play some function in the T cell responsiveness attributed by CCL5. However, additional studies will be required to elucidate the precise role of IL-10 and IFN-γ double-producer T cell populations in pneumococcal immunity.

The biological determinants that influence the probability of *S. pneumoniae* transmission and progression can include, but are not limited to, the characteristics of the infecting strain (carriage *versus* invasive), susceptibility of uninfected hosts as well as infected individuals. Polymorphisms in CCL5 that negatively affect its expression are associated with enhanced susceptibility and progression to HIV-1/AIDS [17, 18, 43]. This accounts for an important health disparity; up to 67% of African Americans carry at least one of these polymorphisms. It is tempting to speculate that some of the health disparities associated with pneumococcal immunity may be partially mediated by this *ccl5* genetic variance, which would result in diminished expression of CCL5 protein. We show CCL5 deficiency increased IL-10

expression by DCs as well as $CD4^+$ T cells to support the generation of $CD4^+$ T cells that secrete IL-10 in response to *ex vivo* $PspA_{199\text{-}246}$ restimulation. However, the contribution of IL-10 from these accessory cells did not prevent the generation of $PspA_{199\text{-}246}$-specific, IFN-γ secreting T helper cells.

T cell epitopes used in a future pneumococcal vaccine should mount optimal responses, even in individuals with CCL5 deficiencies. To explain, African Americans have a higher mortality rate associated with pneumococcal infection, compared to their Caucasian counterparts [45]. The rate of pneumococcal pneumonia is higher in blacks than in whites [46]. Indeed, rates of infection are three-times higher in African Americans than in Caucasian Americans. Moreover, African Americans are known to have a higher incidence of invasive pneumococcal infection than Caucasian Americans [47]. Interestingly, 67% of African Americans, compared to only 3% in Caucasians, possess genetic variants in the *ccl5* gene that results in lower CCL5 protein expression. This has been shown to be a critical factor in HIV/AIDS incidence and disease progression, respectively. Unfortunately, there are no animal models that precisely mimic these genetic variations, which is why we used antibodies to limit CCL5 availability. Previously, we showed CCL5 differentially supports CD28, CD40L, CD30, and/or 4-1-BB expression during T cell activation [6]. We also showed that CCL5, IFN and IL-10 expression by $CD4^+$ T cells, $CD8^+$ T cells, $CD11b^+$ monocytes, and $CD11b^+$ $CD11c^{Hi}$ DCs were significantly increased four days after pneumococcal carriage challenge [19]. Hence, this is the first study to directly show how CCL5 deficiency during pneumococcal infection affects the ability of T cells as well as $CD11b^+$ $CD11c^-$, $CD11b^+$ $CD11c^{Lo}$ $CD11b^+$ $CD11c^{Hi}$ leukocytes to produce IL-10 and IFN-γ as well as mount optimal responses to pneumococcal HTL epitopes.

In conclusion, PspA is a highly immunogenic surface protein of *S. pneumoniae* and considered to be a promising vaccine candidate [47-48]. $PspA_{199\text{-}246}$ is the HTL immunodominant region and likely encompasses MHC II binding epitopes to support pneumococcal immunity. While the precise role of CCL5 interactions in adaptive pneumococcal immunity remains uncertain, this study addresses important questions that are relevant to many individuals that display *ccl5* polymorphisms, which diminish CCL5 protein expression. The consequences of the genetic variants might affect infectious disease outcomes and optimal responses to vaccines. In short, the $PspA_{199\text{-}246}$ region is largely resistant to CCL5 deficiency and might prove useful to include in peptide-based pneumococcal vaccines.

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References

[1]. Dagan R. Impact of pneumococcal conjugate vaccine on infections caused by antibiotic-resistant *Streptococcus pneumoniae*. Clin Microbiol Infect. 2009; 3:16–20. [PubMed: 19366365]

[2]. Dagan R, Givon-Lavi N, Leibovitz E, Greenberg D, Porat N. Introduction and proliferation of multidrug-resistant *Streptococcus pneumoniae* serotype 19A clones that cause acute otitis media in an unvaccinated population. J Infect Dis. 2009; 199:776–85. [PubMed: 19434927]

[3]. Reinert RR. The public health ramifications of pneumococcal resistance. Clin Microbiol Infect. 2009; 3:1–3. [PubMed: 19366361]

[4]. Katsarolis I, Poulakou G, Analitis A, Matthaiopoulou I, Roilides E, Antachopoulos C, et al. Risk factors for nasopharyngeal carriage of drug-resistant *Streptococcus pneumoniae*: data from a nation-wide surveillance study in Greece. BMC Infect Dis. 2009; 9:120. [PubMed: 19640285]

[5]. Lillard JW Jr. Boyaka PN, Hedrick JA, Zlotnik A, McGhee JR. Lymphotactin acts as an innate mucosal adjuvant. J Immunol. 1999; 162:1959–65. [PubMed: 9973465]

[6]. Lillard JW Jr. Boyaka PN, Taub DD, McGhee JR. RANTES potentiates antigen-specific mucosal immune responses. J Immunol. 2001; 166:162–9. [PubMed: 11123289]

[7]. Lillard JW Jr. Singh UP, Boyaka PN, Singh S, Taub DD, McGhee JR. MIP-1alpha and MIP-1beta differentially mediate mucosal and systemic adaptive immunity. Blood. 2003; 101:807–14. [PubMed: 12393512]

[8]. Sallusto F, Lanzavecchia A, Mackay CR. Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. Immunology Today. 1998; 19(12):568–74. [PubMed: 9864948]

[9]. Luther SA, Cyster JG. Chemokines as regulators of T cell differentiation. Nature Immunol. 2001; 2:102–7. [PubMed: 11175801]

[10]. Darville T, Andrews CW Jr. Sikes JD, Fraley PL, Braswell L, Rank RG. Mouse strain-dependent chemokine regulation of the genital tract T helper cell type 1 immune response. Infect Immun. 2001; 69:7419–24. [PubMed: 11705916]

[11]. Schrum S, Probst P, Fleischer B, Zipfel PF. Synthesis of the CC-chemokines MIP-1alpha, MIP-1beta, and RANTES is associated with a type 1 immune response. J Immunol. 1996; 157:3598–604. [PubMed: 8871660]

[12]. Bonecchi R, Bianchi G, Bordignon PP, D'ambrosio D, Lang R, Borsatti A, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med. 1998; 187:129–34. [PubMed: 9419219]

[13]. Sallusto F, Mackay CR, Lanzavecchia A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. Science. 1997; 277:2005–7. [PubMed: 9302298]

[14]. Appay V, Rowland-Jones SL. RANTES: a versatile and controversial chemokine. Trends Immunol. 2001; 22:83–7. [PubMed: 11286708]

[15]. Makino Y, Cook DN, Smithies O, Hwang OY, Neilson EG, Turka LA, et al. Impaired T cell function in RANTES-deficient mice. Clin Immunol. 2002; 102:302–9. [PubMed: 11890717]

[16]. Ifere GO, He Q, Igietseme JU, Ananaba GA, Lyn D, Lubitz W, et al. Immunogenicity and protection against genital Chlamydia infection and its complications by a multisubunit candidate vaccine. J Microbiol Immunol Infect. 2007; 40:188–200. [PubMed: 17639158]

[17]. Liu H, Chao D, Nakayama EE, Taguchi H, Goto M, Xin X, et al. Polymorphism in RANTES chemokine promoter affects HIV-1 disease progression. Proc Natl Acad Sci U S A. 1999; 96:4581–5. [PubMed: 10200305]

[18]. McDermott DH, Beecroft MJ, Kleeberger CA, Al-Sharif FM, Ollier WE, Zimmerman PA, et al. Chemokine RANTES promoter polymorphism affects risk of both HIV infection and disease progression in the Multicenter AIDS Cohort Study. Aids. 2000; 14:2671–8. [PubMed: 11125885]

[19]. Palaniappan R, Singh S, Singh UP, Singh R, Ades EW, Briles DE, et al. CCL5 modulates pneumococcal immunity and carriage. J Immunol. 2006; 176:2346–56. [PubMed: 16455992]

[20]. Singh R, Singh S, Sharma PK, Singh UP, Briles DE, Hollingshead SK, et al. Helper T cell epitope-mapping reveals MHC-peptide binding affinities that correlate with T helper cell responses to pneumococcal surface protein A. PLoS One. 2010; 5:e9432. [PubMed: 20195541]

[21]. Briles DE, Hollingshead SK, Paton JC, Ades EW, Novak L, Van Ginkel FW, et al. Immunizations with Pneumococcal Surface Protein A and Pneumolysin Are Protective against Pneumonia in a Murine Model of Pulmonary Infection with *Streptococcus pneumoniae*. Infect Immun. 2003; 188:339–48.

[22]. Briles DE, Crain MJ, Gray BM, Forman C, Yother J. Strong association between capsular type and virulence for mice among human isolates of *Streptococcus pneumoniae*. Infect Immun. 1992; 60:111–6. [PubMed: 1729176]

[23]. Aaberge IS, Eng J, Lermark G, Lovik M. Virulence of *Streptococcus pneumoniae* in mice: a standardized method for preparation and frozen storage of the experimental bacterial inoculum. Microb Pathog. 1995; 18:141–52. [PubMed: 7643743]

[24]. Briles DE, Ades E, Paton JC, Sampson JS, Carlone GM, Huebner RC, et al. Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. Infect Immun. 2000; 68:796–800. [PubMed: 10639448]

[25]. Kadioglu A, Gingles NA, Grattan K, Kerr A, Mitchell TJ, Andrew PW. Host cellular immune response to pneumococcal lung infection in mice. Infect Immun. 2000; 68:492–501. [PubMed: 10639409]

[26]. Valerio RM, Benstead M, Bray AM, Campbell RA, Maeji NJ. Synthesis of peptide analogues using the multipin peptide synthesis method. Anal Biochem. 1991; 197:168–77. [PubMed: 1952061]

[27]. Palaniappan R, Singh S, Singh UP, Sakthivel SK, Ades EW, Briles DE, et al. Differential PsaA-, PspA-, PspC-, and PdB-specific immune responses in a mouse model of pneumococcal carriage. Infect Immun. 2005; 73:1006–13. [PubMed: 15664944]

[28]. Shaper M, Hollingshead SK, Benjamin WH Jr. Briles DE. PspA protects *Streptococcus pneumoniae* from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin. Infect Immun. 2004; 72:5031–40. [PubMed: 15321996]

[29]. Alexander J, Sidney J, Southwood S, Ruppert J, Oseroff C, Maewal A, et al. Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. Immunity. 1994; 1:751–61. [PubMed: 7895164]

[30]. Sette A, Buus S, Appella E, Smith JA, Chesnut R, Miles C, et al. Prediction of major histocompatibility complex binding regions of protein antigens by sequence pattern analysis. Proc Natl Acad Sci U S A. 1989; 86:3296–300. [PubMed: 2717617]

[31]. Koedel U, Bernatowicz A, Frei K, Fontana A, Pfister HW. Systemically (but not intrathecally) administered IL-10 attenuates pathophysiologic alterations in experimental pneumococcal meningitis. J Immunol. 1996; 157:5185–91. [PubMed: 8943431]

[32]. Wang JF, Park IW, Groopman JE. Stromal cell-derived factor-1alpha stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. Blood. 2000; 95:2505–13. [PubMed: 10753828]

[33]. Van der Sluijs KF, van Elden LJR, Nijhuis M, Schuurman R, Pater JM, Florquin S, et al. IL-10 is an important mediator of the enhanced susceptibility to *pneumococcal pneumonia* after influenza infection. J Immunol. 2004; 172:7603–9. [PubMed: 15187140]

[34]. Cousins DJ, Lee TH, Staynov DZ. Cytokine coexpression during human Th1/Th2 cell differentiation: direct evidence for coordinated expression of Th2 cytokines. J Immunol. 2002; 169:2498–506. [PubMed: 12193719]

[35]. Martin I, Shastri VP, Padera RF, Yang J, Mackay AJ, Langer R, et al. Selective differentiation of mammalian bone marrow stromal cells cultured on three-dimensional polymer foams. J Biomed Mater Res. 2001; 55:229–35. [PubMed: 11255174]

[36]. Nixon JC, Ferrell S, Miner C, Oldham AL, Hochgeschwender U, Webb CF. Transgenic mice expressing dominant-negative bright exhibit defects in B1 B cells. J Immunol. 2008; 181:6913–22. [PubMed: 18981111]

[37]. Chattopadhyay G, Khan AQ, Sen G, Colino J, DuBois W, Rubtsov A, et al. Transgenic expression of Bcl-xL or Bcl-2 by murine B cells enhances the in vivo antipolysaccharide, but not antiprotein, response to intact *Streptococcus pneumoniae*. J Immunol. 2007; 179:7523–34. [PubMed: 18025197]

[38]. Zhang S, Cubas R, Li M, Chen C, Yao Q. Virus-like particle vaccine activates conventional B2 cells and promotes B cell differentiation to IgG2a producing plasma cells. Mol Immunol. 2009; 46:1988–2001. [PubMed: 19376580]

[39]. Hansell CA, Schiering C, Kinstrie R, Ford L, Bordon Y, McInnes IB, et al. Universal expression and dual function of the atypical chemokine receptor D6 on innate-like B cells in mice. Blood. 2011; 117:5413–24. [PubMed: 21450903]

[40]. Wong SC, Puaux AL, Chittezhath M, Shalova I, Kajiji TS, Wang X, et al. Macrophage polarization to a unique phenotype driven by B cells. Eur J Immunol. 2010; 40:2296–307. [PubMed: 20468007]

[41]. Pomeroy C, Rubins JB. Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. Infect Immun. 1997; 65:2975–7. [PubMed: 9199475]

[42]. Yanagawa Y, Iwabuchi K, Onoe K. Co-operative action of interleukin-10 and interferon-gamma to regulate dendritic cell functions. Immunol. 2009; 127:345–53.

[43]. Anonymous. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. WHO Technical Report Series. 2000; 894:i–xii. 1–253. [PubMed: 11234459]

[44]. Hausmann LR, Ibrahim SA, Mehrotra A, Nsa W, Bratzler DW, Mor MK, et al. Racial and ethnic disparities in pneumonia treatment and mortality. Med Care. 2009; 47:1009–17. [PubMed: 19648832]

[45]. Marrie TJ. Pneumococcal pneumonia: epidemiology and clinical features. Semin Respir Infect. 1999; 14:227–36. [PubMed: 10501310]

[46]. Harrison LH, Dwyer DM, Billmann L, Kolczak MS, Schuchat A. Invasive pneumococcal infection in Baltimore, Md: implications for immunization policy. Arch Intern Med. 2000; 160:89–94. [PubMed: 10632309]

[47]. McDaniel LS, Yother J, Vijayakumar M, McGarry L, Guild WR, Briles DE. Use of insertional inactivation to facilitate studies of biological properties of pneumococcal surface protein A (PspA). J Exp Med. 1987; 165:381–94. [PubMed: 3546575]

[48]. Berry AM, Yother J, Briles DE, Hansman D, Paton JC. Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae*. Infect Immun. 1989; 57:2037–42. [PubMed: 2731982]

PspA is a candidate vaccine antigen against pneumococcal disease.

Unfortunately, little is known regarding PspA peptide epitopes that induce the greatest helper T lymphocyte (HTL) responses.

In previous studies we used *in silico* analysis tools and mouse models to reveal PspA immuno-dominant peptides of Streptococcus pneumoniae (family 1) PspA.

This region optimally binds with a broad range of HLA-DR, -DQ, and -DP allelles.

In the current study, we show that these immuno-dominant epitopes and possible peptide vaccine candidates are partially resistant to CCL5 deficiencies.

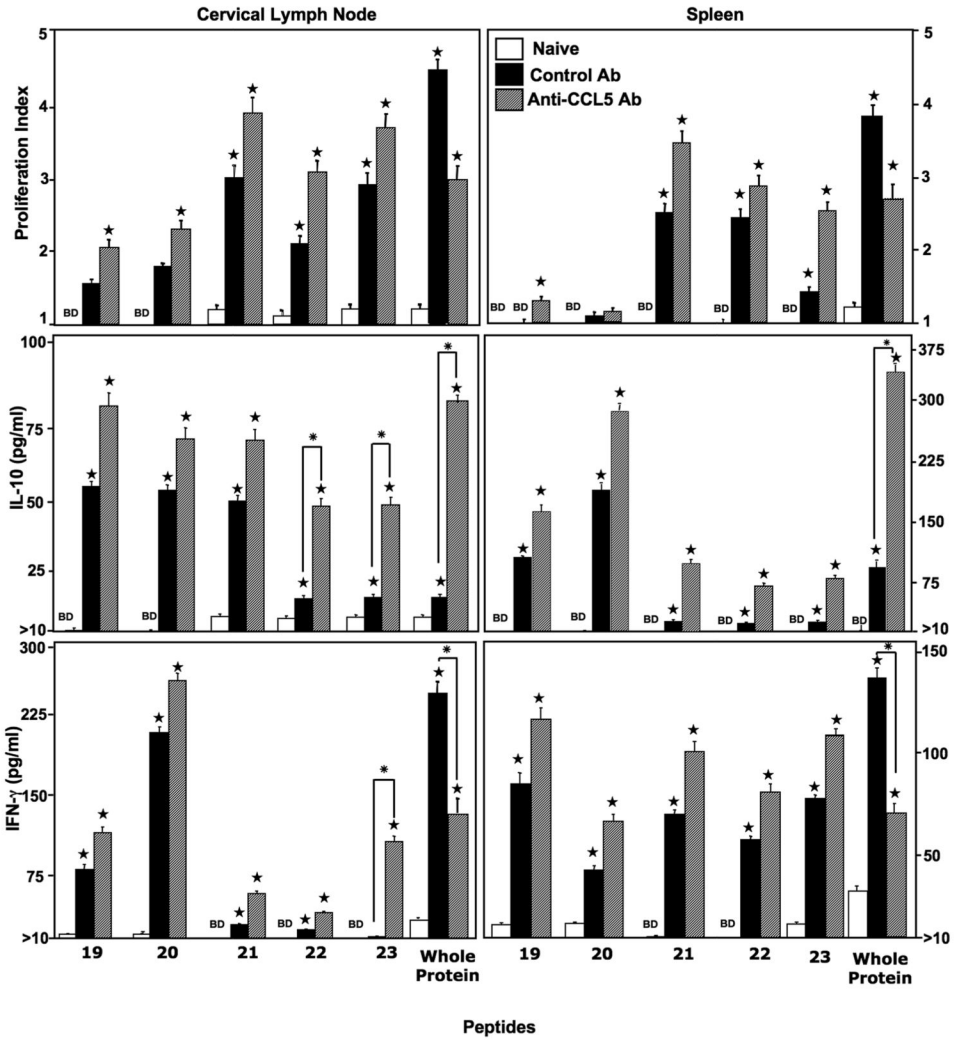

Figure 1. Proliferation and cytokine response of PspA peptide-specific systemic and local CD4$^+$ T cells that express CCL5 during pneumococcal carriage

Cervical lymph node (CLN) and spleen lymphocytes were isolated from female F1 (C57BL/6 × BALB/c) naïve mice (open box) and 28 days after intranasal challenge with *Streptococcus pneumoniae* strain EF3030 and treated with control (solid box) or anti-CCL5 (hashed box) antibody (Ab) solutions. CD4$^+$ T cells were incubated with 1μM of PspA peptides plus mitomycin C-treated naïve syngeneic feeder cells, for 3 days, at a ratio of 5:1 × 10^6 cells. Proliferation was measured by BrdU incorporation, which was measured by ELISA. The data presented are the mean OD_{450}. Stars (★) indicate statistically significant ($p < 0.01$) increases between naïve *versus* control and anti-CCL5 antibody-treated groups. Whereas asterisks (*) indicate statistically significant ($p < 0.01$) increases between control antibody- and anti-CCL5 antibody-treated groups. Experimental groups consisted of 10 mice and experiments were repeated three times. The data presented are the mean ± SEM optical densities of quadruplicate cultures from each group. IL-10 and IFN-γ production of cultured supernatants was determined by luminex capable of detecting > 2 pg/ml.

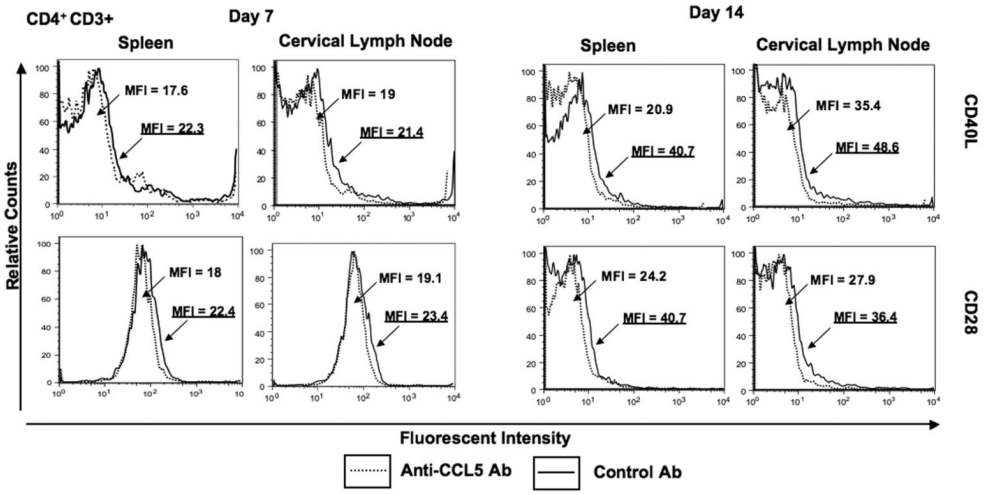

Figure 2. Flow cytometry analysis of CD28 and CD40L expression by CD4$^+$ T cells following pneumococcal challenge

Representative plots from three separate experiments are shown where spleen- and cervical lymph node (CLN)-derived CD4$^+$ T cells from female F1 (C57BL/6 × BALB/c) mice, treated with control antibody (Ab, solid line) or anti-CCL5 Ab (dotted line) solutions, were isolated 7 and 14 days after intranasal challenge with *Streptococcus pneumoniae* strain EF3030. Mean fluorescence intensity (MFI) and fluorescence intensity histograms of CD28 and CD40L expression by CD4$^+$ cells are illustrated and were analyzed using Flow Jo version 8.3 software. Underlined MFI values represent bacterial-challenged, anti-CCL5 antibody-treated groups.

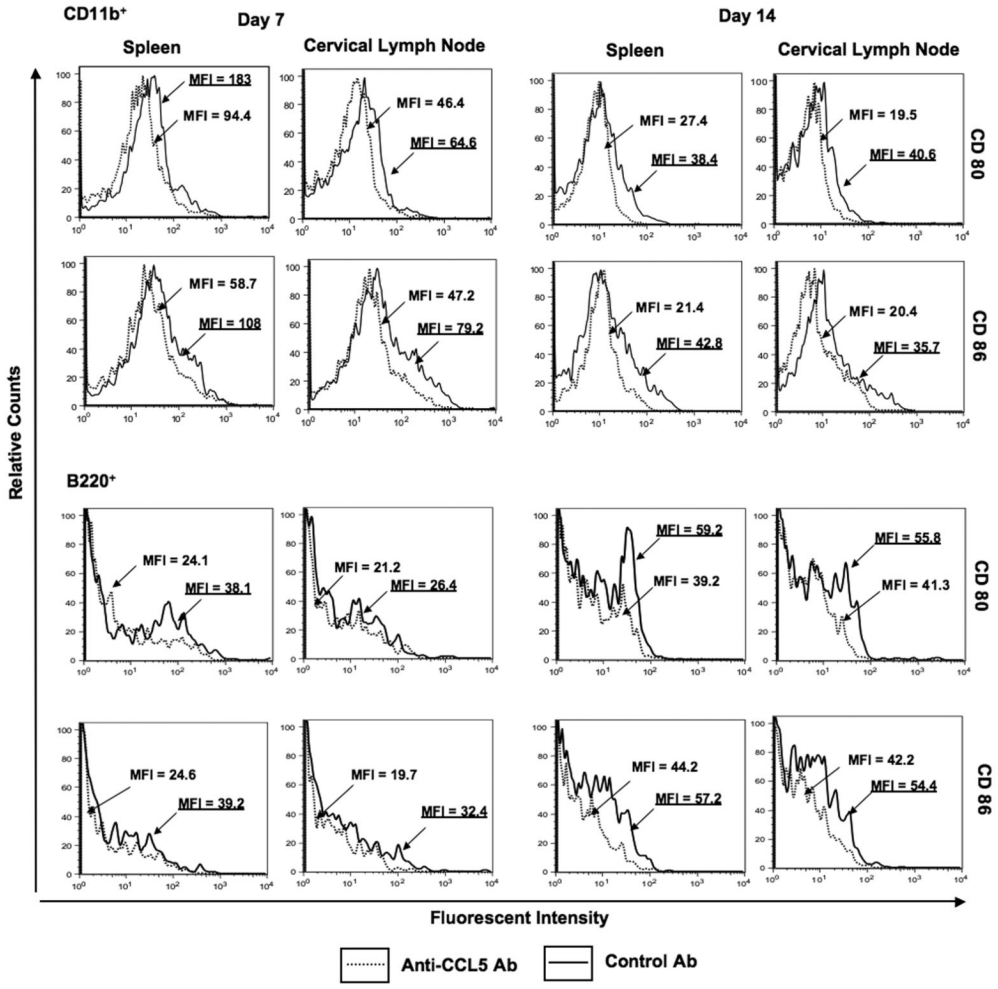

Figure 3. Flow cytometry analysis of CD80 and CD86 expression by CD11b$^+$ and B220$^+$ cells following pneumococcal challenge

Representative plots from three separate experiments are shown where spleen- and cervical lymph node-derived CD4$^+$ T cells from Female F1 (C57BL/6 × BALB/c) mice, treated with control or anti-CCL5 antibody (Ab) solutions, were isolated 7 and 14 days after intranasal challenge with *Streptococcus pneumoniae* strain EF3030. Mean fluorescence intensity (MFI) and fluoroscence intensity histograms of CD80 or CD86 expression by CD11b$^+$ and B220$^+$ cells are illustrated and were analyzed using Flow Jo version 8.3 software. Underlined values represent MFI recorded bacterial-challenged, anti-CCL5 antibody-treated groups.

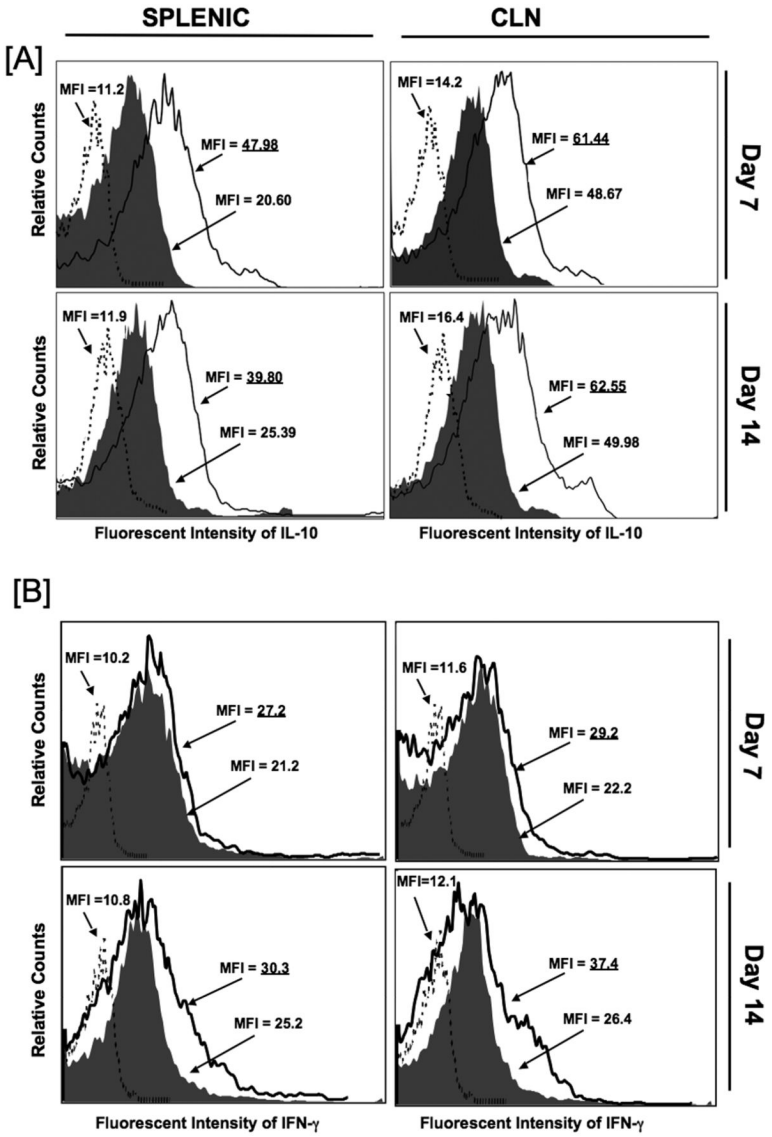

Figure 4. Change in IL-10- and IFN-γ-expressing splenic and cervical lymph node CD4$^+$ T cells following pneumococcal challenge

Female F1 (C57BL/6 × BALB/c) mice were intranasally challenged with 10^7 CFUs of *Streptococcus pneumoniae* strain EF3030 in a 15μl volume of Ringer's solution. Anti-CCL5 antibody (open histogram) or control antibody (solid histogram) antibody were administered by intraperitoneal route every 3 days, starting 2 days before challenge. Anti-CCL5 antibody- and control antibody-treated groups consisted of 10 mice each and studies were repeated 3 times. *Panel A* shows the mean fluorescence intensity (MFI) and fluorescence intensity histograms of IL-10 expression by cervical lymph node (CLN)- and spleen-derived CD4$^+$ T cells from anti-CCL5 and control antibody-treated groups as well as isotype control antibody staining (dotted histogram) of pooled lymphocytes from these groups, which were analyzed using Flow Jo version 8.3 software. Underlined values represent MFI recorded bacterial-challenged, anti-CCL5 antibody-treated groups. *Panel B* shows the mean fluorescence intensity (MFI) and fluorescence intensity histograms of IFN-γ expression by CLN- and spleen-derived CD4$^+$ T cells from anti-CCL5 and control antibody-treated groups as well as isotype control antibody staining (dotted histogram) of pooled lymphocytes from

these groups, which were analyzed using Flow Jo version 8.3 software. Underlined values represent MFI recorded bacterial-challenged, anti-CCL5 antibody-treated groups

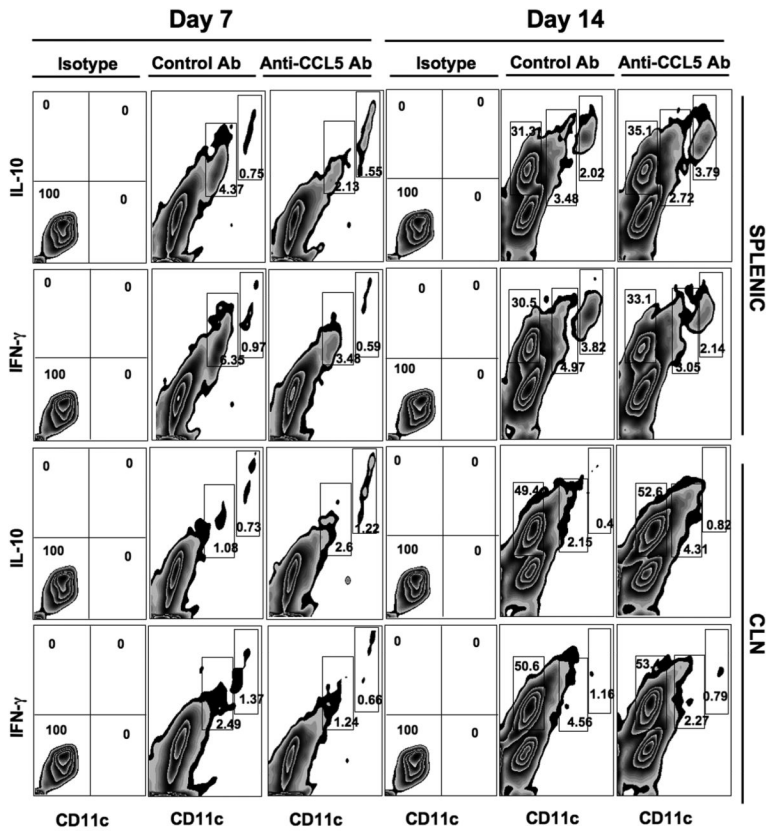

Figure 5. Change in IL-10$^+$ and IFN-γ$^+$ splenic and cervical lymph node CD11c$^+$ leukocytes following pneumococcal challenge

Female F1 (C57BL/6 × BALB/c) mice were intra-nasally challenged with 10^7 CFUs of *S. pneumoniae* strain EF3030 in a 15μl volume of Ringer's solution. Anti-CCL5 antibody or control antibodies were administered by intra peritoneal route every 3 days, starting 2 days before challenge with *Pneumococci*. Splenic and cervical lymph node (CLN) lymphocytes from anti-CCL5 and control antibody-treated groups as well as isotype control antibody groups were stained and analyzed by flow cytometry. Experimental and control groups consisted of 10 mice each and studies were repeated 3 times. Representative density plots along with percentages of IL-10$^+$, IFN-γ$^+$ and CD11b$^+$ CD11c^{Hi} or CD11b$^+$ CD11c^{Lo} populations are shown of CD11b-gated cells isolated 7 or 14 days after bacterial challenge.

Table 1

Overlapping PspA peptides

01	MNKKKMILTSLASVA
02	ASVAILGAGFVASQP
03	ASQPTVVRAEESPVA
04	SPVASQSKAEKDYDA
05	DYDAAKKDAKNAKKA
06	AKKAVEDAQKALDDA
07	LDDAKAAQKKYDEDQ
08	DEDQKKTEEKAALEK
09	ALEKAASEEMDKAVA
10	KAVAAVQQAYLAYQQ
11	AYQQATDKAAKDAAD
12	DAADKMIDEAKKREE
13	KREEEAKTKFNTVRA
14	TVRAMVVPEPEQLAE
15	QLAETKKKSEEAKQK
16	AKQKAPELTKKLEEA
17	LEEAKAKLEEAEKKA
18	EKKATEAKQKVDAEE
19	DAEEVAPQAKIAELE
20	AELENQVHRLEQELK
21	QELKEIDESESEDYA
22	EDYAKEGFRAPLQSK
23	LQSKLDAKKAKLSKL
24	LSKLEELSDKIDELD
25	DELDAEIAKLEDQLK
26	DQLKAAEENNNVEDY
27	VEDYFKEGLEKTIAA
28	TIAAKKAELEKTEAD
29	TEADLKKAVNEPEKP
30	PEKPAPAPETPAPEA
31	APEAPAEQPKPAPAP
32	APAPQPAPAPKPEKP
33	PEKPAEQPKPEKTDD
34	KTDDQQAEEDYARRS
35	ARRSEEEYNRLTQQQ
36	TQQQPPKAEKPAPAP
37	APAPKTGWKQENGMW
38	NGMWYFYNTDGSMAT

39	SMATGWLQNNGSWYY
40	SWYYLNSNGAMATGW
41	ATGWLQYNGSWYYLN
42	YYLNANGAMATGWAK
43	GWAKVNGSWYYLNAN
44	LNANGAMATGWLQYN
45	LQYNGSWYYLNANGA
46	ANGAMATGWAKVNGS
47	VNGSWYYLNANGAMA
48	GAMATGWLQYNGSWY
49	GSWYYLNANGAMATG
50	MATGWAKVNGSWYYL
51	WYYLNANGAMATGWV
52	TGWVKDGDTWYYLEA
53	YLEASGAMKASQWFK
54	QWFKVSDKWYYVNGL

Individual, yet overlapping, *Streptococcus pneumoniae* strain R6 PspA peptides, 15 amino acids in length were used in *ex vivo* and *in silico* assays.

Table 2

Number of $CD11b^{+}CD11c^{Hi}$ and $CD11b^{+}CD11c^{Lo}$ cells 7 days after pneumococcal challenge

		IL-10	IFN-γ	CCR5
Spleen	$CD11b^{+}CD11c^{Hi}$ *Control Ab*	4.40 ± 0.3	7.93 ± 0.2	17.10 ± 0.3
	$CD11b^{+}CD11c^{Hi}$ *Anti-CCL5 Ab*	8.96 ± 0.1*	5.12 ± 0.1*	11.50 ± 0.2*
Cervical Lymph Node	$CD11b^{+}CD11c^{Hi}$ *Control Ab*	0.10 ± 0.1	0.38±0.1	1.70 ± 0.2
	$CD11b^{+}CD11c^{Hi}$ *Anti-CCL5 Ab*	0.35 ± 0.2*	0.18 ± 0.1*	1.60 ± 0.2
Spleen	$CD11b^{+}CD11c^{Lo}$ *Control Ab*	4.76 ± 0.3	4.79 ± 0.1	7.40 ± 0.2
	$CD11b^{+}CD11c^{Lo}$ *Anti-CCL5 Ab*	5.90 ± 0.2	7.09 ± 0.3*	5.43 ± 0.2*
Cervical Lymph Node	$CD11b^{+}CD11c^{Lo}$ *Control Ab*	0.10 ± 0.1	0.17 ± 0.1	0.84 ± 0.1
	$CD11b^{+}CD11c^{Lo}$ *Anti-CCL5 Ab*	0.15 ± 0.1	0.14±0.2	0.40 ± 0.1*

C57BL/6 × BALB/c F1 mice were intranasally challenged with PBS (uninfected) or 10^{7} CFUs of *S. pneumoniae* strain EF3030 in a 15 μL volume of Ringer's solution and treated with either control or anti-CCL5 antibodies. Spleen and cervical lymph node lymphocytes were purified and prepared for cell surface and intracellular flow cytometry analysis 7 days after bacterial challenge. The fold increases ± SEM in the number of ($\times 10^{6}$) of $CD3^{-}CD11b^{+}$ $CD11c^{Hi}$ or $CD3^{-}$ $CD11b^{+}$ $CD11c^{Lo}$ lymphocytes that were CCL5, IL-10, IFN-γ or CCR5 positive are shown. Asterisks (*) indicate statistically significant ($p < 0.01$) increases between infected over infected local cell subpopulations from three separate experiments with two groups containing 10 mice each.

Table 3

Number of $CD11b^{+}CD11c^{Hi}$ and $CD11b^{+}CD11c^{Lo}$ cells 14 days after pneumococcal challenge

		IL-10	IFN-γ	CCR5
Spleen	$CD11b^{+}CD11c^{Hi}$ *Control Ab*	2.32 ± 0.2	3.73 ± 0.2	19.6 ± 0.1
	$CD11b^{+}CD11c^{Hi}$ *Anti-CCL5 Ab*	4.41 ± 0.2*	2.57 ± 0.3*	12.5 ± 0.2*
Cervical Lymph Node	$CD11b^{+}CD11c^{Hi}$ *Control Ab*	0.10 ± 0.1	0.29±0.1	1.66 ± 0.1
	$CD11b^{+}CD11c^{Hi}$ *Anti-CCL5 Ab*	0.21 ± 0.2*	0.11 ± 0.1*	1.58 ± 0.2
Spleen	$CD11b^{+}CD11c^{Lo}$ *Control Ab*	8.85 ± 0.3	7.68 ± 0.3	7.56 ± 0.1
	$CD11b^{+}CD11c^{Lo}$ *Anti-CCL5 Ab*	10.4 ± 0.2	8.62 ± 0.3	6.09 ± 0.1
Cervical Lymph Node	$CD11b^{+}CD11c^{Lo}$ *Control Ab*	0.18 ± 0.1	0.40 ± 0.1	0.99 ± 0.2
	$CD11b^{+}CD11c^{Lo}$ *Anti-CCL5 Ab*	0.22 ± 0.1	0.73±0.2*	0.77 ± 0.1

C57BL/6 × BALB/c F1 mice were intranasally challenged with PBS (uninfected) or 10^{7} CFUs of *S. pneumoniae* strain EF3030 in a 15 μL volume of Ringer's solution and treated with either control or anti-CCL5 antibodies. Spleen and cervical lymph node lymphocytes were purified and prepared for cell surface and intracellular flow cytometry analysis 14 days after bacterial challenge. The fold increases ± SEM in the number of ($\times 10^{6}$) of $CD3^{-}CD11b^{+}$ $CD11c^{Hi}$ or $CD3^{-}$ $CD11b^{+}$ $CD11c^{Lo}$ lymphocytes that were CCL5, IL-10, IFN-γ or CCR5 positive are shown. Asterisks (*) indicate statistically significant ($p < 0.01$) increases between infected over infected local cell subpopulations from three separate experiments with two groups containing 10 mice each.