Structure-function analysis of CCL28 in the development of post-viral asthma

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*Running title: Role of CCL28 structure in asthma
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Background: CCL28 is associated with the pathogenesis of acute post-viral asthma.

Results: In the absence of viral infection, natively folded CCL28 can induce asthma pathology while unfolded CCL28 cannot.

Conclusion: The structure of CCL28 is critical for its role in asthma pathogenesis.

Significance: Inhibition of CCL28 presents a novel therapeutic option for the prevention of post-viral asthma.

ABSTRACT

CCL28 is a human chemokine constitutively expressed by epithelial cells in diverse mucosal tissues and is known to attract a variety of immune cell types including T-cell subsets and eosinophils. Elevated levels of CCL28 have been found in the airways of individuals with asthma, and previous studies have indicated that CCL28 plays a vital role in the acute development of post-viral asthma. Our study builds on this demonstrating that CCL28 is also important in the chronic post-viral asthma phenotype. In the absence of a viral infection, we also demonstrate that CCL28 is both necessary and sufficient for induction of asthma pathology. Additionally, we present the first effort aimed at elucidating the structural features of CCL28. Chemokines are defined by a conserved tertiary structure comprised of a three-stranded β-sheet and a C-terminal α-helix constrained by two disulfide bonds. In addition to the four disulfide bond-forming cysteine residues that define the traditional chemokine fold, CCL28 possesses two additional cysteine residues that form a third disulfide bond. If all disulfide bonds are disrupted, recombinant human CCL28 is no longer able to drive mouse CD4+ T-cell chemotaxis or in vivo airway hyper-reactivity indicating that the conserved chemokine fold is necessary for its biologic activity. Due to the intimate relationship between CCL28 and asthma pathology, it is clear that CCL28 presents a novel target for the development of alternative asthma therapeutics.

Asthma is a chronic inflammatory disease of the airways that affects over 300 million people worldwide and whose prevalence has been increasing in many modernized countries (1). It is a complex disease characterized by airway hyper-responsiveness, inflammation, and tissue remodeling resulting in episodic airflow...
obstruction and clinical symptoms of wheezing, coughing, and shortness of breath (2). Asthma is a major public health issue in the United States as it is the number one illness leading to school absences in children (3) and in 2010 alone, asthma accounted for 3,404 deaths and 1.8 million emergency department visits (4). Currently, there is no cure for asthma. All available therapies focus on providing symptomatic relief and reducing the number and severity of attacks. Understanding the molecular mechanisms by which asthma develops and establishes chronicity is key to elucidating alternative and potentially curative therapies. As of now, the pathways involved in the pathogenesis of asthma have not been conclusively identified.

Respiratory viral infections have been implicated in both the development and exacerbation of asthma, especially infection with the paramyxovirus, Respiratory Syncytial Virus (RSV) (5). Using the mouse paramyxovirus, Sendai Virus (SeV; murine parainfluenza virus type 1), we have documented a mechanistic pathway linking the respiratory infection with development of post-viral asthma. This pathway depends upon recruitment of a specific neutrophil subset to the airways, where they induce expression of the high-affinity receptor for IgE on lung conventional dendritic cells (cDCs). At the same time, IgE is produced against SeV, and subsequent cross-linking of IgE on the cDCs leads to release of the chemokine CCL28, which then recruits Th2 cells to the airway. These Th2 cells produce IL-13 and drive the development of airway hyper-reactivity and mucous cell metaplasia—two hallmarks of asthma pathology (6). The SeV-induced disease includes both an acute form (develops by day 21 post-inoculation (PI) SeV) and a chronic form (develops by day 49 PI SeV) (7). The acute disease is dependent upon Th2 cells, however, the chronic form depends upon iNKT cells producing IL-13 and driving alternatively activated macrophage development. The alternatively activated macrophages produce IL-13, which maintains the disease and may drive even higher levels of mucous cell metaplasia (6, 7). The mechanism connecting the acute to chronic disease is not known. We previously identified CCL28 as a critical player in the acute form of post-SeV asthma (6), but it was unclear whether or not CCL28 played a role in the chronic disease state. In this study, we show that CCL28 plays an important role in the chronic form of post-SeV asthma, as well.

There are several lines of evidence that point toward CCL28’s critical role in asthma pathogenesis. First, high levels of CCL28 have been identified in the sputum and airways of humans with asthma (8, 9). Second, the receptors for CCL28, CCR3 and CCR10, are expressed on eosinophils, Th2 cells, iNKT cells, and regulatory T cells—all cell types that have been associated with asthma and atopic disease (9-14). Taken together, these findings suggest the importance of CCL28 in asthma pathology and identify CCL28 as a potential target for novel asthma therapeutics.

CCL28 is a small ~12 kDa protein in the chemokine family. Chemokines are secreted proteins that are intimately involved in host inflammatory responses and are defined by the presence of four conserved disulfide bond-forming cysteine residues and a common tertiary structure composed of a three-stranded β-sheet and a C-terminal α-helix (15). CCL28 also contains an extended C-terminus and an additional pair of cysteine residues. In this study, we show that these additional cysteine residues form an additional disulfide bond, similar to what has been observed for CCL1, CCL15, CCL21, and CCL23 (16-19), chemokines known to possess six cysteine residues.

The goal of the present study was three fold: to assess the functional role of CCL28 in chronic post-SeV asthma, to produce recombinant human CCL28 (hCCL28) and characterize its basic structural elements, and to establish a simple, robust model for in vivo testing of CCL28 inhibitors with the potential to become future asthma therapeutics. Using an interdisciplinary approach of NMR, mass spectrometry, and both in vitro and in vivo biologic assays, we have identified CCL28 as a chemokine critical in the development of chronic post-viral asthma.

EXPERIMENTAL PROCEDURES:

Animals - Male C57BL6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and used for these experiments when they were between 6 and 12 weeks of age. All animal experiments were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.
CCL28 at Day 49 post inoculation of Sendai virus - Mice were inoculated with $2 \times 10^5$ pfu of SeV or ultraviolet light inactivated SeV (UV-SeV) (6, 7). On day 49 PI, mice were euthanized and one lung removed for mRNA isolation with Trizol (Sigma-Aldrich). cDNA was generated with the QuantiTect reverse transcription kit (Qiagen) as per manufacturer's instructions, and qPCR performed using TaqMan fast master mix and the StepOnePlus PCR system (Applied Biosystems) to compare CCL28 (assay ID Mm00445039_m1) to GAPDH (assay ID 4352339E) copy number.

Anti-CCL28 treatment of Sendai virus induced airway disease – Mice were inoculated with SeV as above and injected s.c. with 200 µg of either anti-CCL28 (clone 134306; R&D Systems) or control rat IgG2b (clone 141945; R&D Systems) weekly from day 14 to 49 PI SeV. Airway hyper-reactivity and mucous cell metaplasia were then determined as outlined below.

Chemotaxis assay - CD4$^+$ T-cells were purified from the spleens of mice using positive immunomagnetic selection with anti-CD4 microbeads (MACS system; Miltenyi Biotec). 7.5 x 10$^5$ CD4$^+$ T-cells in 100ul of RPMI 1640 supplemented with 0.25% BSA (RPMI-0.25) were loaded in the upper chamber of a Transwell system with a 5 um membrane between the upper and lower chambers (Corning Costar Transwell Permeable Supports w/ Polycarbonate membrane). In the upper (for checkerboard analysis) or lower chamber, 1, 3, 10, or 30 µg of recombinant human CCL28 (hCCL28) or unfolded CCL28 (uCCL28) were loaded in 600uL of RPMI-0.25. All samples were tested in duplicate. After incubation for 3 hours at 37 °C, the number of cells transmigrated to the lower chamber was determined by flow cytometry.

CCL28 administration - Mice were anesthetized with i.n. ketamine/xylazine prior to administration of PBS, hCCL28, or uCCL28 in 15 µL PBS on days 0, 1, and 2. On day 3, airway hyper-responsiveness was determined and each mouse sacrificed.

Airway hyper-responsiveness - A dose response curve to aerosolized methacholine (0, 10, 20, 40, 80, and 160 mg/mL in PBS) using two-chamber plethysmography (Buxco) in conscious mice was performed on the days indicated (20). Airway hyper-responsiveness was determined as specific airways resistance (sRaw) and compliance (sGaw) measured with each dose of methacholine and reported as percent change from baseline.

Histologic evaluation - The right lung was removed and fixed in 10% buffered formalin, dehydrated in ethanol and embedded in paraffin before being cut into 5 µm thick sections and stained with PAS (21). The number of PAS positive cells per mm BM of the respiratory epithelium was determined by a blinded observer using Image J with 3 random sections per mouse evaluated (21).

Protein Production and Purification – Recombinant hCCL28 was expressed as an N-terminal His$_6$-SUMO-TEV fusion protein using a modified pET28 vector in BL21 (DE3) E. coli cells, refolded, and purified using established protocols (22). Purity, identity, and molecular weight of the cleaved and refolded CCL28 protein were verified by HPLC and matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The protein produced corresponded to mature, secreted human CCL28, a 108 amino acid protein with the sequence shown in Fig. 3B.

Production of unfolded CCL28 – Purified recombinant hCCL28 was diluted to a concentration of 2mg/mL in 200 mM NH$_4$HCO$_3$ at pH 7.8. All disulfide bonds were reduced by addition of tris(2-chloroethyl)phosphine (TCEP) to a final concentration of 5 mM and incubated for 30 minutes at room temperature with end-over-end rotation. All cysteine residues were alkylated by addition of iodoacetamide to a final concentration of 10 mM and incubated again for 30 minutes at room temperature with end-over-end rotation. Molecular weight of the soluble alkylated CCL28 product was confirmed by MALDI mass spectrometry. The alkylated product was then dialyzed into H$_2$O and repurified by HPLC before use.

NMR Spectroscopy – NMR experiments were performed on a Bruker DRX 600 equipped with a $^1$H/$^15$N/$^13$C Cryoprobe. NMR samples contained 90% H$_2$O, 10% D$_2$O, and 0.02% NaN$_3$, with 20 mM sodium phosphate at pH 6.2. $^1$H, $^15$N, and $^{13}$C resonance assignments for the backbone of CCL28 were obtained using the following experiments: $^{15}$N-$^1$H HSQC (23), 3D HNCO (24), 3D HN(CO)CA (25), 3D HN(CO)CACB, 3D HNCA (26), 3D HNCACB, 3D HNCACO,
3D C(CO)NH (27). NMR data were processed with NMRPipe (28), and XEASY (29) was used for resonance assignments and analysis of NOE spectra. Amino acid sequence and NMR chemical shift information were combined to predict the secondary structure of CCL28 using PECAN, an automated tool for probabilistic secondary structure determination of proteins (30).

**Mass Spectrometry** - Samples (12 µg) were dissolved in 20% acetonitrile with 0.2% AALS-II (Proteabiosciences) then denatured by heating at 90°C for 5 minutes and cooled to room temperature. Samples either proceeded to tryptic digestion directly, or were alkylated with 925 µM iodoacetamide for 20 min at 37°C. Trypsin (Promega) was added to achieve an enzyme:substrate ratio of 1:50 (w/w) and samples were incubated overnight at 37°C with mixing. Digests were desalted using C18 Zip-Tips (Millipore) then reconstituted in Mobile Phase A (0.1% formic acid in water). Peptides were separated by reverse phase nanoflow chromatography at 300 nL/minute using a column (10 cm × 75 µm) packed in-house with Magic C18 AQ 3µm (Michrom) stationary phase, and a gradient of 2-95% Mobile Phase B (0.1% formic acid in acetonitrile) over 75 minutes. The LTQ Orbitrap Velos was set to perform an MS1 scan at 30,000 resolution, an MS2 scan using electron transfer dissociation (ETD) fragmentation with detection in the Orbitrap, and MS3 scans of the 4 most abundant ions formed in the MS2 scan with fragmentation in the high energy collision (HCD) cell and detection in the Orbitrap. The MS2 reagent ion AGC target value was set to 300,000 with 200 ms reaction time for ETD. Three microscans were collected for MS3 scans with a normalized HCD collision energy of 40 and the AGC target value for detection set to 100,000. All isolation widths were 4, MS2 and MS3 scans were collected at a resolution of 7500, and dynamic exclusion was enabled. Data were interpreted manually for disulfide-linked peptides.

**Statistical Analyses** - For parametric data, Student's t-test was used. For comparison of methacholine dose response curves (sRaw and sGaw) a 2 way ANOVA was used. In all cases, significance was set at p ≤ 0.05.

**RESULTS:**

The role of CCL28 in chronic post-viral asthma – In previous studies we have shown the importance of CCL28 in the development of acute asthma pathology following infection with Sendai Virus (SeV) in the mouse (6). However, it was not known whether CCL28 played any role in the chronic disease that develops by day 49 post-inoculation (PI) SeV. Interestingly, we have found that by day 49 PI SeV CCL28 message in the airways is increased above that seen even at day 21 PI SeV (Fig. 1A). This suggests that CCL28 might play a role in the chronic disease phenotype. To further investigate the importance of CCL28 in the development of chronic post-viral asthma pathology, we treated mice with an anti-CCL28 mAb and found that it prevented the development of both mucous cell metaplasia (Fig. 1B) and airway hyper-reactivity (Fig. 1C) at day 49 PI SeV. Together, these findings indicate the critical importance of CCL28 in chronic post-viral asthma pathology and make CCL28 a potential target for the development of novel asthma therapeutics.

NMR structural analysis of CCL28 – To further investigate CCL28, we began the first study aimed at investigating the structure and structure-function properties of this unique chemokine. Recombinant human CCL28 (hCCL28) protein was expressed in E. coli, refolded, and purified using established methods for production of recombinant chemokines (22). Purity and identity of [U-13C,15N] recombinant hCCL28 for NMR analysis were confirmed by reverse-phase high-performance liquid chromatography (HPLC) and mass spectrometry, respectively. The secreted form of hCCL28 is a 108 residue protein with 3 proline, 1 tryptophan, 6 asparagine, and 4 glutamine residues, which could give rise to a total of 126 signals in the 2D 1H-15N HSQC spectrum of hCCL28. The HSQC of recombinant hCCL28 (Fig. 2) contained 125 well-dispersed peaks of uniform intensity, consistent with the presence of a single folded conformation. Standard triple-resonance 3D NMR experiments were conducted and the resulting spectra were used to assign sequence-specific chemical shifts to the backbone of CCL28, as indicated in the HSQC of figure 2. Chemical shift analysis was used to predict the secondary structural elements of CCL28 and it revealed the locations of the three β-
strands and α-helix that comprise the conserved chemokine fold (Fig. 3A).

Compared with most chemokines, the CCL28 sequence is unique in that it contains an extended C-terminal tail, a portion of which resembles the C-terminus of CCL27, a paralog with ~40% sequence identity to CCL28 (Fig. 3B). However, in contrast to CCL27 which contains the traditional four disulfide bond-forming cysteines, CCL28 possesses two additional cysteine residues: Cys30 predicted to be in the β1 strand and Cys80 located in the C-terminal extension. We measured $^{1}H$-$^{15}N$ heteronuclear NOE values to assess the relative mobility of the polypeptide backbone of CCL28 (Fig. 3C), specifically aimed at investigating the C-terminal region encompassing Cys80. Residues 11-70 exhibited NOE values $>0.6$, consistent with the extent of the chemokine fold, with flexible residues (NOE <0.5) at the N-terminus and residues 74-77. In addition, a short segment encompassing Cys80 also displayed NOEs $>0.5$, suggesting that this region of the C-terminal tail is ordered. Additionally, evaluation of the $^{13}C\beta$ chemical shifts from the HN(CO)CACB and HNCACB triple-resonance experiments indicate that all cysteine residues are in the oxidized state ($^{13}C\beta > 35$ ppm), consistent with the presence of three disulfide bonds in CCL28 (Table 1).

Disulfide bond mapping of CCL28 by mass spectrometry - NMR analysis indicated that the CCL28 C-terminal extension might be linked to the first β-strand by a non-conserved Cys30-Cys80 disulfide bond. To confirm the disulfide pairing of all six cysteines, we subjected recombinant CCL28 to a mass spectrometric analysis of non-reduced samples using ETD fragmentation at the MS2 stage to yield liberated peptides that could then be identified in MS3 stage by HCD fragmentation. To ensure that disulfide linked peptides were not a technical artifact of sample processing or scrambling, samples were treated with iodoacetamide to alkylate any available cysteines. The 435.53 $m/z$ ion, which could be observed in samples digested with and without pre-treatment with iodoacetamide, is consistent with the expected $m/z$ of a triply charged di-peptide formed between tryptic peptides VNMCR and GNVCHR. ETD fragmentation of the 435.53 $m/z$ ion generated two dominant ions, 622.28 and 684.31, consistent with singly charged masses for each peptide. In MS3 scans, HCD fragmentation of 622.28 and 684.31 from the MS2 scan are consistent with those expected for VNMCR and GNVCHR, respectively. The presence of the di-peptide in the sample treated with iodoacetamide supports the existence of the disulfide between Cys30 and Cys80 prior to tryptic digestion (Fig. 4). In addition to these data for this previously unreported disulfide bridge, mass spectrometry analysis also confirmed the existence of previously reported disulfides between Cys11 and Cys39 and between Cys12 and Cys54 (Fig. 4).

Structure-function activity analysis of CCL28 – To determine if the recombinant hCCL28 used for structural analysis was biologically active, we performed a chemotaxis assay with mouse CD4$^+$ T cells. We tested both properly folded hCCL28 and an unfolded variant produced by alkylation all cysteine residues with iodoacetamide (uCCL28) to probe the functional requirement for native hCCL28 structure (Fig. 5A,B). Significant chemotaxis was seen only with the properly folded hCCL28, and this was maximal at 3 $\mu$g/ml (Fig. 5C,D). To see whether the recombinant human chemokine could exert any asthma-related in vivo effects in a mouse, we administered 0.1 or 3 $\mu$g hCCL28 or 3 $\mu$g uCCL28 intra-nasally to C57BL6 mice daily for 3 days and then measured airway hyper-reactivity. While the 0.1 $\mu$g dose of hCCL28 had no effect (data not shown), the 3 $\mu$g dose led to significantly increased airway hyper-responsiveness (as evidenced by increased specific airways resistance, sRaw, and decreased airways conductance, sGaw); however, 3 $\mu$g of uCCL28 failed to induce airway hyper-responsiveness (Fig. 6A). In addition, the hCCL28 also drove mucous cell metaplasia (Fig. 6B). These data demonstrate that our recombinant hCCL28 is biologically active and can induce an asthma-like disease in mice with only a short period of administration.

DISCUSSION:

CCL28 is a human chemokine that has been shown to be an important player in the development of allergic airway inflammation and asthma (21, 31, 32). In this study, we provide additional evidence linking CCL28 to the development of chronic asthma pathology. In our
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In a mouse model, we show that CCL28 is elevated at day 49 PI to a level above what is seen at day 21 PI. This provides support for the idea that CCL28 may be playing a role in the establishment of asthma chronicity. We have also shown that CCL28 is necessary for chronic post-viral asthma pathogenesis, as an anti-CCL28 monoclonal antibody significantly decreased airway hyper-responsiveness and mucous cell metaplasia, key markers of asthma. Further, we have shown that recombinant human CCL28 is sufficient to drive this same asthma pathology in the mouse, even in the absence of viral infection. A schematic of how CCL28 activity may drive this disease is shown in Figure 7. As can be seen in the figure, CCL28 binds to CCR3 and/or CCR10 on an IL-13 producing cell, which is recruited to the airways. Once in the airways the IL-13 produced by this cell leads to both mucous cell metaplasia, as well as smooth muscle hypertrophy and airway hyper-reactivity. Taken together, these findings not only suggest the importance of CCL28 in the development of chronic asthma pathology, but also demonstrate that our mouse model is a simple and robust way to further probe the role of CCL28 in asthma pathogenesis.

Nearly all chemokines are stabilized by two disulfide bonds linking the N-terminal region to the core structure (16). In addition to these two conserved disulfide bonds, we have shown that CCL28 possesses a disulfide linking its C-terminal region to its conserved chemokine core. There are five chemokines known to possess the non-traditional six cysteines seen in CCL28 (33), all of which are of the CC subclass (CCL1, CCL15, CCL21, CCL23, and CCL28) (Fig 8). The NMR solution structures of CCL1, CCL15, CCL21, and CCL23 have all shown that the additional cysteines form disulfide bonds (16-19). Interestingly, the way in which the extra disulfide affects the overall chemokine structure varies substantially. For human CCL15 and CCL23, chemokines that share ~64% identity, the extra disulfide seems to have a negligible effect on structure. The extra disulfide in these two chemokines links a $\beta_10$-helical turn just before the first $\beta$-strand to the C-terminal $\alpha$-helix and does not disrupt the conserved chemokine fold (17, 19). In addition, mutational studies with CCL15 have shown that disruption of the extra disulfide has a minimal effect on protein structure (17). However, structural studies on the chemokine CCL1 have shown that its extra disulfide bond significantly affects the conserved chemokine fold, resulting in disruption of the C-terminal $\alpha$-helix and the formation of a short extended C-terminal strand not seen in other chemokines (16). We hypothesize that the extra disulfide bond present in CCL28 links the C-terminal tail distal to the $\alpha$-helix to the first strand of the $\beta$-sheet, creating a novel structured domain not seen in other chemokines. This idea is supported by both the predicted secondary structure and the elevated $^1$H-$^1^5$N heteronuclear NOE values observed in the segment encompassing the C-terminal cysteine involved in the extra disulfide bond (Cys80). We have shown that the structure of CCL28 is critical for its activity in vitro and in vivo, thus further investigations of the unique structural features of CCL28 are needed. Ongoing analysis in our laboratory will confirm the structural and functional significance of the novel disulfide-constrained C-terminal domain in CCL28.

Multiple studies including this one have indicated the importance of CCL28 in asthma pathogenesis. This study comprises the first effort to establish a role for CCL28 in the chronic lung disease that develops by day 49 post-SeV infection in a mouse model. The data we have presented here indicates that CCL28 may be a key factor in the establishment of asthma chronicity, even in the absence of a viral insult. It can be imagined that any stimulus that increases the levels of CCL28 in the lung would have the potential to drive development of asthma. Although these stimuli have not yet been fully elucidated, we postulate that given its association with asthma development, RSV is likely a key stimulus leading to CCL28 production and subsequent asthma pathogenesis.

CCL28 presents an interesting target for the development of novel asthma therapeutics. We propose that by exploiting the unique structural features of CCL28, potent and specific CCL28 inhibitors may be developed. This work is the first step in generating a novel type of asthma therapy that may have the power to prevent development of post-viral asthma in young children.
ACKNOWLEDGEMENTS

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REFERENCES


FOOTNOTES
FIGURE LEGENDS

FIGURE 1: **CCL28 is a key mediator in post-viral asthma.** (A) CCL28 is significantly elevated on day 21 post-Sendai virus infection, with a continued significant increase to day 49 post-Sendai virus infection. Blocking CCL28 with anti-CCL28 monoclonal antibody (mAb) inhibits the development of post-viral (B) mucous cell metaplasia (MCM) and (C) specific airways reactivity (sRaw, measure of airway hyper-responsiveness) at day 49 post-virus. Values represent mean ± SEM *p ≤ 0.05; **p ≤ 0.01; ****p ≤ 0.0001; n= 6 mice/group.

FIGURE 2: **Recombinant human CCL28 is suitable for NMR structural analysis.** A 2D $^1$H-$^1$5N HSQC spectrum with sequence-specific assignments for the backbone of CCL28.

FIGURE 3: **CCL28 adopts the conserved chemokine fold and may possess a unique structural domain.** (A) A graph generated by PECAN indicating the probability of secondary structural elements in CCL28 based on sequence and chemical shift information. Red bars are indicative of alpha helix formation and blue bars are indicative of beta sheets. (B) CCL28 sequence alignment and predicted secondary structure. Amino acid sequences of CCL28, CCL27 (36% identity) and CCL5 (18% identity) were aligned using Clustal Omega. CCL27 secondary structure elements are indicated below the alignments. Locations of CCL28 $\beta$-sheets and $\alpha$-helices predicted by PECAN are indicated above the alignments. (C) $^{15}$N-$^1$H heteronuclear NOE plotted as a function of the CCL28 sequence. Red bars emphasize the elevated NOE values seen for amino acids around Cys80 that are indicative of a more ordered structure.

FIGURE 4: **A novel disulfide bond links the extended C-terminus to the chemokine domain.** (A) Graphical depiction of recombinant CCL28 with cysteines highlighted. Image generated in Protter (34). (B) MS1 and MS2 spectra of the di-peptide resulting from a disulfide bridge between Cys30 and Cys80. (C) MS3 spectra of peptides liberated from ETD fragmentation of the di-peptide using HCD fragmentation to confirm sequence. (D) MS1 spectrum of tri-peptide resulting from bridges between Cys11 and Cys39 and between Cys12 and Cys54. MS2 spectrum using ETD fragmentation of the tri-peptide results in singly charged ions consistent with masses of the liberated peptides ICVSPHNHTVK and ADGDCDLAAVILHKV.

FIGURE 5: **Effect of CCL28 tertiary structure on CD4$^+$ T-cell migration in vitro.** (A) MALDI comparing molecular weights of native (red; hCCL28) and alkylated (blue; uCCL28) CCL28. (B) 1D $^1$H NMR spectra overlay of both native (red; hCCL28) and alkylated (blue; uCCL28). The solid lines highlight the spectral changes between native and alkylated CCL28, which indicate that alkylated CCL28 is unfolded. Migration of purified mouse CD4$^+$ T-cells in response to (C) native or (D) unfolded CCL28. Cells migrated with chemokine in lower chamber are represented by black bars. Cells migrated with chemokine in upper chamber are represented by white bars. Values represent mean ± SEM *p ≤ 0.05; **p ≤ 0.01; samples run in duplicate with data from n=2 separate experiments for hCCL28 and uCCL28.

FIGURE 6: **Effect of CCL28 tertiary structure on mouse asthma pathology in vivo.** Mice received intranasal hCCL28 or uCCL28 daily for 3 days. 3$\mu$g of hCCL28 but not uCCL28 drives (A) airway hyper-responsiveness (as measured by increased sRaw and decreased specific airway conductance, sGaw). In addition, hCCL28 also drove development of (B) mucous cell metaplasia (MCM). Values represent mean ± SEM; 2 Way ANOVA for (A) with * p ≤ 0.001 and NS = not significant. For (B) Student’s t-test with p value as indicated; n≥3 mice/group.

FIGURE 7: **CCL28 is a key player in the pathogenesis of post-viral asthma.** Schematic of how CCL28 activity alone can drive airway hyper-responsiveness (AHR) and mucous cell metaplasia (MCM),
primarily through airway recruitment of a variety of IL-13 producing CCR3 and/or CCR10 expressing cells.

**FIGURE 8: Sequence alignment of all six-cysteine chemokines.** The extra disulfide of CCL28 is shown in blue, CCL1 in green, CCL21 in pink, and CCL15 and CCL23 are shown in red due to their similarity. All alignments generated by Clustal Omega.
Table 1. CCL28 cysteine $^{13}$C chemical shifts

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$^1$Average cysteine and cystine $^{13}$C chemical shifts for 132 proteins in the BioMagResBank (35)
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Figure 1

A

B

C

SeV
UV-SeV

0 21 49
Days PI

PAS+ cells per mm BM

IgG
anti-
CCL28
Ab

p=0.01

S Raw
(% of baseline)

0 20 40
Mch (mg/ml)

SeV + control IgG
SeV + anti-CCL28
UV-SeV

*
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Figure 2
Figure 3

A

Probability

B

CCL28: SEAIPASSCTEVS-HHISSRILLERVWNCRIORADGCDILAVIL-IVHYRIRICVPHNTKDOHMYDOOQAAXXGKNVCHIRKXHHGKXNRAQHHQDHEYGHKTPY
CCL27: FLLPPSTACCTQLYRKPSDKILKIIQLQVDELQEADGCHIQAFVL-MLQAQRSICJHPNQP5LSQWEHQRKHLGTLPKLNFQMLRKG-
CCL5: SPYSDTTPCFAYJARPLRA---HIKEYFYTSGC5PNPAVFVTRNKRQVCANPEKXWREYINSLEMS

C

1H-N H-NOE

Residue Number
Role of CCL28 structure in asthma

Figure 4

A
- Cysteine involved in known disulfide
- Cysteine not previously reported in disulfide
- Trypsin cleavage site (K/R)

B
- 435.53 [M+3H]^3
- VNMC
- GNVCHR
- MS1

- 684.31 [M+H]^+
- MS2
- ETD of 435.53

- 622.28 [M+H]^+

C
- MS3
- HCD of 622.28

D
- MS1
- ETD of 716.79

- 716.79 [M+7H]^4
- ICVSPHNHTVK
- SEAIPASSCCTEVSHHSR
- ADGDCDLAVALHKV

- ICVSPHNHTVK 1639.75382
- ADGDCDLAVALHKV 1234.63562
Role of CCL28 structure in asthma

Figure 5

A

![Mass m/z](mass mz graph)

B

![1H Chemical Shift (PPM)](chemical shift graph)

C

![Chemokine chamber:](chemokine chamber graph)

D

![Chemokine chamber:](chemokine chamber graph)
Role of CCL28 structure in asthma

Figure 6

A

B

hCCL28
uCCL28
PBS

Mch (mg/ml)

Mch (mg/ml)

PAS cells/mm BM

* NS

p = 0.031
Role of CCL28 structure in asthma

Figure 7
Role of CCL28 structure in asthma

hCCL28
SEAILPIASSSCCTEVSH-HI5RRALLERVWKLRIQRADGC3LAAVILHVRK-RRICVSPHNHT VKQHKVQAAQKN--GKGVIVHRQKHQHRK-SVRHAGQKGHETYGKTTPY--

hCCL1
KSMQVFSIRCCFSFAEQEIPKRAI--LVRH--TSSICSNELIFKLKRG--KEAQADTVGAVQIRKRMLRHCPSKRK--

hCCL21
--SDGADQAECLYQQQRKIPAVV--RSYRRQEPESLGESEIFPLAFLPPRSSQAELOCDOPKELVQQLHQLDKTSPQRPQGPZRTGQASTGQKFGKSKGKQKERSQQTKPQGF

hCCL23
LDRFHATSASKFCSXTPRSPECSL--ESYFE--TN5SCSKGPFILTHQG--RRFSANPS8GKQINVMLKLDT3IKTR89N--

hCCL13
--HEAQECSCS1SPHCSMH--RSYFYE--CS5SCSKGPFILTHQG--RQCNPS8QODQBCWNLKYPY8I--