Role of CCL5 (RANTES) in Viral Lung Disease

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CCL5/RANTES is a key proinflammatory chemokine produced by virus-infected epithelial cells and present in respiratory secretions of asthmatics. To examine the role of CCL5 in viral lung disease, we measured its production during primary respiratory syncytial virus (RSV) infection and during secondary infection after sensitizing vaccination that induces Th2-mediated eosinophilia. A first peak of CCL5 mRNA and protein production was seen at 18 to 24 h of RSV infection, before significant lymphocyte recruitment occurred. Treatment in vivo with Met-RANTES (a competitive chemokine receptor blocker) throughout primary infection decreased CD4+ and CD8+ cell recruitment and increased viral replication. In RSV-infected, sensitized mice with eosinophilic disease, CCL5 production was further augmented; Met-RANTES treatment again reduced inflammatory cell recruitment and local cytokine production. A second wave of CCL5 production occurred on day 7, attributable to newly recruited T cells. Paradoxically, mice treated with Met-RANTES during primary infection demonstrated increased cellular infiltration during reinfecion. We therefore show that RSV induces CCL5 production in the lung and this causes the recruitment of RSV-specific cells, including those making additional CCL5. If this action is blocked with Met-RANTES, inflammation decreases and viral clearance is delayed. However, the exact effects of chemokine modulation depend critically on time of administration, a factor that may potentially complicate the use of chemokine blockers in inflammatory diseases.

Bronchiolitis resulting from respiratory syncytial virus (RSV) infection is the single major cause of infant hospitalization in the developed world (25). It is characterized by excessive cell recruitment to the lung, leading to bronchial obstruction and sometimes ventilatory failure (24). RSV bronchiolitis is associated with the recurrent wheezing and asthma diagnosis in later childhood (33).

CCL5 (RANTES) is a potent chemoattractant cytokine that recruits monocytes, T cells, and eosinophils, acting via the receptors CCR1, CCR3, and CCR5 (30). Infection of respiratory epithelial cells with RSV causes upregulation of CCL5 secretion (21) by NF-kB translocation (39) and by increasing the stability of CCL5 mRNA (16), as does stimulation of epithelial cells with the Th1 cytokine gamma interferon (IFN-γ) (37). Children with RSV infections have increased CCL5 protein levels in both the upper and lower airway secretions, and levels of CCL5 in upper airway secretions correlate positively with disease severity (2, 9, 11, 36). In mice, CCL5 induction by RSV infection contributes to subsequent allergic pulmonary inflammation (14). CCL5 is a key chemokine in recruitment of CD8+ T cells to the lung (6) and has been implicated in classical IFN-γ dominant Th1 responses, and yet it is also involved in eosinophilic disease driven by Th2 cells (7, 8, 17, 27).

In mice, RSV infection can prime for Th1- or Th2-biased T-cell populations that control infection but also enhance inflammation upon subsequent exposure to RSV, allowing us to examine situations in which polarized cytokine responses can be achieved in the context of identical viral challenge (24). In this system, we have recently shown that the pattern of chemokine release is directly affected by priming with individual RSV proteins (4).

To further explore the timing of CCL5 production, its cellular source, and the associated pattern of pathology, we used the inhibitory CCL5 analogue Met-RANTES (28) to block CCL5 activity in a mouse model of acute viral lung disease. To further investigate the role of CCL5 in Th2-biased immunopathology, we primed mice with RSV-G prior to RSV challenge. In the first 2 days of RSV infection, CCL5 protein was abundant in bronchoalveolar lavage fluid but not in the eluted cells by intracellular staining or by enzyme-linked immunosorbent (ELISPOT) assay; at 7 days this situation was reversed. Therefore, CCL5 and its coaggregants have potent time-dependent actions in viral lung disease, both in recruitment of inflammatory cells and in controlling virus infection.

MATERIALS AND METHODS

Virus stocks, mouse infection, and cell recovery. RSV (strain A2) and recombinant vaccinia virus expressing RSV G protein (rVV-G) or β-galactosidase (rVV-βgal) was grown in HEp-2 cells (ATCC). UV inactivation of virus was performed in a UV Stratalinker (Stratagene) for 10 min. Eight-week-old female BALB/c mice (Harlan Ltd., Veryan, United Kingdom) were maintained in pathogen-free conditions according to institutional and United Kingdom Home Office guidelines. All studies were reviewed and approved by the local institutional review committee. In some experiments, mice were primed to individual RSV proteins by scarification on the rump using 3 × 10^7 pfu.
10⁶ PFU of vaccinia virus, 2 weeks prior to RSV challenge. Mice were infected with 2 × 10⁶ PFU RSV intranasally (i.n.) in 100 μl.

Bronchoalveolar lavage (BAL) fluids and lung tissues were harvested as described previously (12). Briefly, the lungs of each mouse were inflated six times with 1 ml of 12 mM lidocaine in Eagle’s minimal essential medium and BAL fluid kept on ice. 100 μl was centrifuged onto glass slides and stained with hematoxylin and eosin. The remainder was centrifuged, the supernatant retained at −80°C, and the pellet resuspended at 10⁶ cells/ml. Lungs were homogenized by passage through 100-μm cell strainers (Falcon), red blood cells lysed in ammonium chloride buffer, and the remaining cells resuspended in RPMI medium with 10% fetal calf serum. Viable cell numbers were determined by trypan blue exclusion.

**Met-RANTES.** Met-RANTES was produced as previously described. It was reconstituted from a lyophilized powder to 80 μg/ml in phosphate-buffered saline (PBS) and 200 μl given intravenously on each day of treatment, from days 0 to 7 during primary RSV infection and days 5 to 6 during RSV challenge following vaccinia sensitization. **Analysis of cell types.** Surface and intracellular staining was carried out as previously described (20). Cells (10⁶/ml) were incubated with 50 ng/ml phorbol myristate acetate, 500 ng/ml ionomycin, and 10 μg/ml Brefeldin A for 4 h at 37°C. Cells were blocked with Fc block prior to being stained with fluorescently labeled antibody against surface markers for 30 min on ice and then fixed for 20 min at room temperature with 2% formaldehyde. Samples were permeabilized with 0.5% saponin in PBS (1% bovine serum albumin–0.1% azide) for 10 min. Anticytokine antibodies (anti-IFN-γ, BD and anti-CCL5:R&D) were added for a further 20 min at room temperature, washed with PBS (1% bovine serum albumin–0.1% azide), and analyzed on a Coulter EPICS Elite flow cytometer collecting data on at least 40,000 lymphocytes. All chemicals were from Sigma (Poole, United Kingdom), and all antibodies were from BD-Pharmingen (Oxford, United Kingdom) except where stated.

**ELISPOT.** Assays were performed as described previously (23), with minor modifications. Briefly, plates were coated with 1 μg/ml of the appropriate anti-cytokine antibody in 0.1 M carbonate/bicarbonate buffer, pH 9.6, overnight at 4°C. One hundred microliters of cell suspension in doubling dilutions (from 10⁵ to 1.25 × 10⁶ cells) was added per well. For live RSV stimulation, 2 PFU RSV per cell was added and incubated overnight at 37°C. Controls of equivalent amounts of UV-inactivated RSV were also performed, as was mock stimulation by the addition of lysate from uninfected HEp-2 cells. Each well was then incubated with 100 μl of the appropriate biotinylated antibody: anti-CCL5 overnight at 4°C or anti-IFN-γ (BD) and anti-CCL5:R&D (Amersham Pharmacia). Assays were carried out using the RNase protection assay III kit (Ambion). Analyses were performed as described previously (23).

**RESULTS**

**CCL5 mRNA and protein quantification.** RNA was extracted from snap frozen whole lung using RNA-Stat-60 (Tel-Test, Inc.). CCL5 mRNA expression was determined by using the probe kit mCK5 (BD Biosciences). Probes were transcribed using T7 RNA polymerase and α-32P-labeled UTP (Amersham Pharmacia). Assays were carried out using the RNase protection assay III kit (Ambion) with analysis by polyacrylamide gel electrophoresis, and band intensity was assessed electronically (Storm PhosphorImager; Molecular Dynamics). Results were normalized to two housekeeping genes (L32 and glyceraldehyde phosphate-3-dehydrogenase genes). CCL5 enzyme-linked immunosorbent assay was performed with matched antibody pairs (R&D) according to the manufacturer’s instructions.

**Lung RSV titer.** Clearance of RSV was assessed in lung homogenates 4 days after virus challenge. Lungs were removed and homogenized. After 4 min of centrifugation at 4,000 × g, supernatants were titrated in doubling dilutions on HEp-2 cell monolayers in 96-well flat-bottom plates. Twenty-four hours later, monolayers were washed, fixed with methanol, and incubated with peroxidase-conjugated goat anti-RSV antibody (Biogenesis; Poole, United Kingdom). Infected cells were detected using 3-aminio-9-ethylcarbazole and infectious units enumerated by light microscopy.

**CCL5 expression during primary RSV infection and Th2 T-cell-mediated immunopathology.** To study the cellular sources and kinetics of CCL5 production during primary and Th2 immune-augmented RSV infection, mice were sensitized to RSV by priming with rVV-G or the control, rVV-βGal, previously demonstrated to not affect primary infection.

In all groups, lung CCL5 mRNA levels increased 12 h after RSV infection compared to preinfection controls, peaked at 18 to 24 h, and declined by 48 h (Fig. 1A; also not depicted). In rVV-G primed mice, this early response was significantly greater than that with rVV-βGal (P < 0.01). In both groups at all time points, the CCL5 mRNA level was significantly greater than that for the preinfection control (P < 0.01). Seven days after infection, CCL5 mRNA was still expressed at levels significantly above background in all groups (Fig. 1A; also data not depicted). The CCL5 protein was detected in the BAL fluid of RSV-infected mice, peaking 24 h after infection, and then declined rapidly (Fig. 1B) and remained low on days 2 to 4, an effect seen consistently in independent experiments (not shown). The profile of CCL5 production in primary infection was not significantly different from that for rVV-βGal-treated mice (results not depicted).

We wished to determine whether CCL5 production was due to resident or infiltrating cells and if this production was RSV specific. Since CCL5 production was highest in rVV-G primed mice, we isolated lung cells from rVV-G-primed, RSV-infected mice on various days after infection and examined ex vivo CCL5 production by ELISPOT. In vitro stimulation of lung cells from mice that were uninfected or early in infection with live RSV resulted in a detectable but low frequency of produc-
tion of CCL5. However, cells from mice 7 days after RSV infection showed a large increase in CCL5 production after live RSV restimulation, compared to unstimulated cells and compared to cells from uninfected mice (Fig. 2A). This response required replicating virus, since UV-inactivated RSV did not lead to augmented CCL5 production. Levels of protein production in stimulated culture supernatant showed similar effects (not depicted). This directly measured virus-specific local production was not evident in the first 4 days of infection.

Since this response was present only in the lungs of infected mice during later stages of infection, we hypothesized that the source of CCL5 was infiltrating cells. Intracellular staining for CCL5 and fluorescence-activated cell sorter (FACS) analysis on lung cells taken from mice scarified with rVV-G and infected with RSV confirmed that CCL5 was produced by both CD4+ (27% of infiltrating CD4+ T cells in the example shown in Fig. 2B) and CD8+ (21% of infiltrating CD8+ T cells; Fig. 2C) T cells. Interestingly, CD8+ cells that were not high CCL5 producers contained no detectable CCL5, whereas all CD4+ cells contained low levels of stainable CCL5. Although few T cells were present in the early stages of infection (days 0 to 4), those that were recovered contained no detectable CCL5. Therefore, we observed two phases of CCL5 production: the first we believe to be from resident cells and the second from an infiltrating population of CD4 or CD8 T cells that secrete CCL5 in a virus-specific manner.

**Effect of Met-RANTES on RSV disease.** Having seen that RSV influences levels of CCL5 in the lung, we wished to determine whether blocking CCL5 would affect cell recruitment and viral clearance. Met-RANTES is a competitive blocker of the CCL5 receptors CCR1 and -5. Treatment of RSV-infected mice with Met-RANTES reduced the recruitment of CD4+ T cells to the BAL on day 4 of infection and of BAL CD8+ T cells over the last 3 days of infection, as measured by FACS (Fig. 3A and B). There was no difference in activation of these cells as determined by the level of CD45RB expression, and B-cell numbers were not altered by Met-RANTES treatment (not depicted).

However, there was a large increase in RSV titer recovered from the lung 4 days after infection (Fig. 3C). This was accompanied by a >25% decrease in the proportion of lung cells secreting IFN-γ 7 days after RSV infection (measured by ELISPOT), both spontaneously and after ex vivo RSV stimulation (Fig. 3D). Because Met-RANTES reduced cellular infiltration during primary infection, we wished to test if it would be effective as a treatment for established “exacerbated” pathology. Therefore, rVV-G-primed RSV-infected mice were treated with Met-RANTES on days 5 and 6 days after RSV challenge. Treatment with Met-RANTES reduced cell recruitment in both lung (not shown) and BAL on day 7 (Fig. 4A). Lower numbers of naive CD4+ and CD8+ T cells were found in both the lung and BAL (Fig. 4B), and the total numbers of IFN-γ- and IL-5-secreting cells in the lung, both with and without ex vivo RSV stimulation, were decreased (P < 0.05) (Fig. 4C and D).

**Effect of Met-RANTES treatment on immunity to a subsequent RSV challenge.** Given that Met-RANTES appears to control the recruitment of CD4+ and CD8+ T cells, it was hypothesized that Met-RANTES might affect the formation of RSV-specific memory. To test this, mice were treated with Met-RANTES during primary RSV infection and rechallenged with RSV 4 weeks later. Paradoxically, mice treated with Met-RANTES during the first infection showed an increased cellular response 7 days after a subsequent RSV chal-
challenge compared to the response with a standard secondary infection (Fig. 5A and B). This was reflected in increased CD4+ and CD8+ T-cell numbers in the lung, measured by FACS (Fig. 5C), and a higher proportion of cells of the CD62Llo/CD44hi "effector memory" phenotype, although "naive" CD8+ T-cell numbers also increased (Fig. 5C). There were also more cytokine-secreting lung cells than with primary infection, but Met-RANTES treatment did not alter this. There was no difference in RSV-induced illness assessed by weight loss and illness scores, nor was there any difference in the ability of the two groups to clear RSV.

**DISCUSSION**

We found that CCL5 expression after RSV infection occurs in two phases: the first, seen during the first 48 h of infection, appears to be an innate response to viral challenge by resident lung cells arising from epithelial cells (10) and lung macrophages (21). Since lymphocyte recruitment is minimal before day 3 (24), this early peak in CCL5 transcription is unlikely to be due to infiltrating lymphocytes. The second peak occurs 7 days after infection. By this time, replicating virus is virtually eliminated, but infiltrating inflammatory cells are numerous (24).

This biphasic pattern resembles that observed previously (21), but the source of the second phase of production has not been demonstrated. From the results of intracellular cytokine staining, we believe that CD4+ and CD8+ T cells are primarily responsible for the second wave of CCL5 production, although resident cells may also increase CCL5 production as a response to virus-induced inflammation.

To further delineate the role of CCL5 in vivo, we treated mice with the CCL5 analogue, Met-RANTES. We found that Met-RANTES inhibited the recruitment of both CD4+ and
CD8⁺ T cells to the bronchial epithelium, as sampled by BAL. It is possible that Met-RANTES blocks and/or downregulates expression of the CCL5 receptors (CCR1 and CCR5) on both CD4⁺ and CD8⁺ T cells. Alternatively, recruitment and activation of dendritic cells may be blocked by Met-RANTES treatment, therefore lessening T-cell responses (1, 35).

Met-RANTES treatment caused a striking increase in RSV replication during primary infection in vivo. This may be a direct consequence of the reduced recruitment of antiviral T cells but could also be due to inhibition of innate mechanisms of viral clearance. CCL5 can act as a chemoattractant for NK cells (which are recruited early in this RSV infection model (13) and can also block apoptosis of alveolar macrophages (18), an effect that may be important in viral clearance (10). In addition, pretreatment of HEP-2 cells with CCL5 or Met-RANTES has been shown to reduce virus growth (5). Howev...

Studies of T-cell responses in asthma often show local helper T cells that preferentially make IL-4, IL-5, IL-9, and IL-13. Such “Th2” cells are thought to mediate eosinophilia, goblet cell hyperplasia, and bronchial hyperresponsiveness. However, there is growing evidence that Th1 cells may act in concert with Th2 cells to enhance inflammation in asthma or may themselves be immunopathogenic. In humans, it is notable that pulmonary bacterial and viral infections (which normally induce Th1-like immune responses) frequently trigger asthmatic exacerbations. Indeed, upper respiratory viral infections are present in 80 to 85% of asthma exacerbations in school-age children (15). This may be explained by the observation that Th1-polarized T cells can drive the recruitment of Th2 cells into the lung, even in the absence of Th2 antigen (29, 34). Pulmonary viral infections can also drive recruitment of allergen-specific T cells during infection or enhance subsequent sensitization to allergen (19, 22, 31).

We found that CCL5 was induced both in primary (Th1-driven) and in Th2-mediated immunopathology. It is possible that CCL5 production is enhanced by the Th2 cytokine IL-13 (38), which could explain the higher CCL5 mRNA we observed in the Th2-dominated responses seen in rVV-G primed mice. Holtzman’s “epithelial-viral-allergic paradigm” asserts that CCL5 produced by virus-infected epithelial cells drives recruitment of both Th1 and Th2 proinflammatory cells in asthma (10). Our model of rVV-G priming followed by RSV infection allowed us the opportunity to use Met-RANTES treatment in an exacerbated inflammatory response, where both antiviral responses and Th2 immunopathology occur. When CCL5 signaling was blocked later during infection of rVV-G-primed animals, T-cell recruitment to the lung was attenuated and both IFN-γ- and IL-5-secreting cells were equally reduced in number. It has been shown that CD8⁺ cells contain CCL5 bound to proteoglycans within intracellular granules, which may be released upon antigen-specific stimulation (26). This may explain why we found elevated CCL5 mRNA with only low levels of the CCL5 protein in the BAL fluid on day 7 and release of CCL5 on antigen-specific restimulation. An alternative possibility is that the IFN-γ produced by the CD8⁺ cells indirectly stimulates the lung epithelial cells to produce CCL5 at this later time point.

Others have shown that CCL5 can reduce IL-12 production in primary RSV infection (38). However, in our model of rVV-G priming (in which IL-12 administration can inhibit eosinophilia [12]), Met-RANTES had no effect on eosinophilia or weight loss but did reduce lung inflammation. Interestingly, clinical studies of asthma exacerbation show that CCL5 and T-cell recruitment to the lung correlates with clinical symptoms...
but not with eosinophilia (3). We have recently shown that CCL11 plays a major role in governing eosinophilia and Th2 cytokine production in this model (20). Therefore, CCL5 production does not selectively bias Th1 or Th2 immunopathology but recruits multiple subsets of lymphocytes to the lung.

Surprisingly, we found that mice treated with Met-RANTES during primary RSV infection showed significantly increased cell recruitment during secondary RSV rechallenge. This paradoxical effect was evident in recruitment of both CD4+ and CD8+ T cells to the lung; the proportion of cells bearing the effector memory (CD62Llo/CD44hi) phenotype increased, but CD4+ cells and macrophages results in production of CCL5 by resident, accessible local sites, particularly in noninflamed tissues. Synthetic chemokine and is therefore unlikely to reach less-accessible sites, particularly in noninflamed tissues.

Consequently, the results suggest that infection of epithelial cells and macrophages results in production of CCL5 by resident cells during the first 48 h of infection. This CCL5 recruits CD4+ and CD8+ T cells and plays an important role in controlling viral replication. Later, CCL5 from resident cells disappears to be replaced by virally induced CCL5 from recruited T cells. Blocking CCL5 with Met-RANTES at this time reduces immunopathology but does not influence the Th1/Th2 balance. This second action is important both during primary infection and during augmented immunopathological responses. Our data suggest that CCL5 plays a central role in driving inflammation in RSV lung disease and that blocking its effect may prove beneficial in bronchiolitis. To this we should also add a note of caution: chemokine blockade might be beneficial in bronchiolitis. To this we should also add a note of caution: chemokine blockade might be beneficial in bronchiolitis. To this we should also add a note of caution: chemokine blockade might be beneficial in bronchiolitis. To this we should also add a note of caution: chemokine blockade might be beneficial in bronchiolitis.

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