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Role of CCL11 in Eosinophilic Lung Disease during Respiratory Syncytial Virus Infection

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Respiratory syncytial virus (RSV) is a major viral pathogen of infants and the elderly. Significant morbidity is caused by an overexuberant mixed lung cell infiltrate, which is thought to be driven by chemokines. One of the main chemotactic mediators responsible for the movement of eosinophils is CCL11 (eotaxin). Using a mouse model of eosinophilic bronchiolitis induced by RSV, we show here that treatment in vivo with a blocking antibody to CCL11 greatly reduces lung eosinophilia and disease severity. In addition, anti-CCL11 caused a striking inhibition of CD4-T-cell influx and shifted cytokine production away from interleukin-5 without reducing the resistance to viral replication. These results suggest that in addition to influencing eosinophil diapedesis and survival, anti-CCL11 has an action on T cells. These studies strengthen the case for anti-CCL11 treatment of Th2-driven diseases.

Respiratory syncytial virus (RSV) causes common colds in adults and bronchiolitis in infants. Viral bronchiolitis leads to the hospitalization of 1.5 to 2% of all infants in the developed world and poses a considerable threat to health worldwide. It is estimated that 73,400 to 126,300 infants in the United States are hospitalized each year for bronchiolitis or pneumonia due to RSV (31). Bronchiolitis develops relatively late during the course of infection, when viral replication is in decline, and seems to be largely the result of immune response overexuberance (27). Additionally, RSV bronchiolitis is strongly associated with the development of asthma and wheezing in later life (32), although it is not clear whether bronchiolitis is a marker for susceptibility to asthma or predisposes people to it (35). For these reasons, the development of a vaccine against RSV is a major priority. However, early trials of a formalin-inactivated RSV vaccine preparation were spectacularly unsuccessful and resulted in substantially enhanced pathology and some mortality in vaccine recipients who subsequently became infected with RSV. This enhancement was associated with pulmonary eosinophilia (18). Eosinophilic RSV pathology dependent on the genetic background has been observed in sensitized susceptible mouse strains (13).

An important factor in the inappropriate eosinophil response is the chemokine eotaxin (CCL11) (16). CCL11 administered in vivo induces a selective accumulation of eosinophils (38) that is predominately mediated by CCR3 (3). In addition to being a major eosinophil chemoattractant, CCL11 activates eosinophil effector functions and enhances eosinophil mobilization and migration. Constitutive low-level expression is required for normal eosinophil homeostasis, but this can be substantially upregulated by diverse proinflammatory stimuli, particularly the Th2 cytokines interleukin-4 (IL-4) and IL-13 (28) and the synergistic action of the proinflammatory cytokines tumor necrosis factor and IL-1β (5). In contrast, the Th1 cytokine gamma interferon (IFN-γ) is a potent inhibitor of CCL11 induction (25). Primary nasal epithelial cell cultures produce CCL11 in response to influenza A virus infection (17), and the intranasal infection of mice with RSV results in the upregulation of CCL11 in the lungs (9).

We therefore investigated the role of CCL11 in pulmonary disease enhancement in an established mouse model of RSV disease (27). We show here that in mice that were previously sensitized by use of a vaccinia virus expressing the RSV G protein (rVV-G), an anti-CCL11 antibody given during a challenge infection with RSV decreased acute illness and lung eosinophilia. This attenuation of disease was accompanied by a decrease in $CD4^+$-T-cell infiltration into the site of infection but not by impaired humoral immunity or reduced protection against RSV replication.

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MATERIALS AND METHODS

Mice and virus stocks. Eight- to 10-week-old female BALB/c mice (Harlan Olac Ltd., Bicester, United Kingdom) were kept under pathogen-free conditions. RSV and a recombinant vaccinia virus expressing the attachment protein (rVV-G), the fusion protein (rVV-F) of RSV, or control β-galactosidase (rVV-β gal) were grown in HEp-2 cells and assayed for infectivity as previously described (10).

Mouse infection and treatment. Mice were scarified on the rump on day 0 with 3×10^6 PFU of rVV-G, rVV-F, or rVV-β gal in a final volume of 10 μl (four or five mice per group); on day 14, the mice were challenged intranasally with 3×10^6 PFU of RSV (A2 strain) in a 50-μl volume. When indicated, mice were injected intraperitoneally with 20 μg of the purified immunoglobulin (Ig) fraction

of rabbit anti-eotaxin in 100 μl of phosphate-buffered saline (PBS) or with an isotype-matched control antibody from days 0 to 5 of the RSV challenge. The appearance and weight of mice were monitored daily. Mice were killed on day 5 by the injection of 3 mg of pentobarbitone and were exsanguinated via the femoral vessels. For the anti-T1/ST2 antibody (MAb 3E10; provided by A. J. Coyle of Millennium Pharmaceuticals, Inc.), rVV-G-primed mice were infected intranasally with RSV A2 and were given either 100 μg of 3E10 or 50 μg of anti-eotaxin intravenously daily from days 0 to 5 of the challenge. Control mice received 100 μg of rabbit immunoglobulin.

Assessment of illness. Since weight loss is a convenient index of disease severity in the mouse, animals were weighed prior to RSV challenge and daily thereafter. In addition, clinical illness scores were estimated daily by an independent observer as described previously (37), using the following values and symptoms: 0, healthy; 1, barely ruffled fur; 2, ruffled fur, but active; 3, ruffled fur and inactive; and 4, ruffled, inactive, hunched posture, and gaunt.

Cell recovery. Bronchoalveolar lavage (BAL) fluids, lung tissues, and serum samples were harvested as described previously (15). 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 was then kept on ice. BAL fluid (100 μl) was centrifuged onto glass slides and stained with hematoxylin and eosin. The remainder of the BAL fluid was centrifuged, the supernatant was retained at −80°C, and the pellet was resuspended at 10^6 cells/ml. Lungs and spleens were homogenized by passage through 100-μm-pore-size cell strainers (Falcon), red blood cells were lysed in ammonium chloride buffer, and the remaining cells were washed and resuspended in RPMI medium with 10% fetal calf serum. Viable cell numbers were determined by trypan blue exclusion.

Analysis of cell types. For the detection of intracellular cytokines, 10^6 cells/ml were incubated with 50 ng of phorbol myristate acetate (Sigma)/ml, 500 ng of ionomycin (Calbiochem)/ml, and 10 μg of brefeldin A (Sigma)/ml for 4 h at 37°C. The cells were stained with Quantum Red-conjugated CD8 and phycoerythrin-conjugated CD4 (Sigma) for 30 min on ice and then fixed for 20 min at room temperature with 2% formaldehyde. The samples were then permeabilized with 0.5% saponin in PBS containing 1% bovine serum albumin and 0.1% azide for 10 min. Fluorescein isothiocyanate-conjugated anti-IFN-γ (XMG1.2; Pharmingen GB) was added for a further 20 min at room temperature, washed with PBS containing 1% bovine serum albumin and 0.1% sodium azide, and analyzed on a Coulter EPICS Elite flow cytometer, with data being collected from at least 40,000 lymphocytes. Eosinophils were counted on hematoxylin-and-eosin-stained cytocentrifuge preparations.

Cytokine ELISA. IL-4, IL-5, and IFN-γ were assessed in lung lavage supernatants and serum samples by an enzyme-linked immunosorbent assay (ELISA) performed according to the manufacturer's instructions (Becton Dickinson-Pharmingen). Briefly, Immunosorb ELISA plates (Nunc) were coated with a capture antibody and left overnight at 4°C. The wells were then washed five times with PBS–0.05% Tween 20 and blocked with PBS–10% fetal bovine serum for 1 h at room temperature. One hundred microliters of a sample or standard was added to blocked wells for 2 h at room temperature. Bound cytokine was detected with a biotinylated anti-cytokine antibody, avidin-horseradish peroxidase, and tetramethylbenzidine. Color development was blocked with 2 N H_2SO_4, and optical densities were read at 490 nm. The concentration of cytokine in each sample was determined from a standard curve.

RSV-specific antibody ELISA. Antibodies in sera were assessed by ELISA as described previously (12). The ELISA antigen was prepared by infecting HEp-2 cells with RSV strain A2 at 1 PFU/cell. When a significant cytopathic effect was observed, the infected cells were harvested, centrifuged at 400 × *g*, resuspended in 3 ml of distilled water, and then subjected to 2 min of sonication (Ultrawave Ltd., Cardiff, Great Britain), and 50-μl aliquots were stored at −20°C until required. Microtiter plates were coated overnight with 100 μl of a 1:200 dilution of either sonicated RSV or HEp-2 cells alone. After blocking with 2% normal rabbit serum for 2 h, dilutions of test samples (diluted in PBS containing 1% HEp-2 lysate) were added for a further 1 h at room temperature. Bound antibodies were detected with peroxidase-conjugated rabbit anti-mouse Ig, with *o*-phenylenediamine as a substrate. Color development was blocked with 2 N H_2SO_4, and optical densities were read at 490 nm. The amount of RSV-specific antibody was determined by subtracting the absorbance obtained by incubating serum on RSV-coated plates from that obtained for the same sample incubated on HEp-2-coated plates.

Cytokine production from peptide-stimulated lungs. G-primed mice were treated with anti-eotaxin or control Ig during RSV infection. Five days after the challenge, whole lungs were removed and single mononuclear cell suspensions were prepared by passage through 100-mm-pore-size meshes, followed by separation over Ficoll-Paque. The cells (5×10^4) were cocultured in triplicate with 2 mM G184-197 (AICKRIPNKKPGKK) or medium alone. Twenty-four hours later, 100 ml of medium was removed from each well for a cytokine ELISA.

RSV titers in the lungs. The clearance of RSV was assessed in lung homogenates 4 days after the virus challenge. Lungs were removed from four mice per group and then were homogenized. After centrifugation at 1,500 × *g* for 4 min, the supernatants were titrated in doubling dilutions on HEp-2 cell monolayers in 96-well flat-bottomed plates. Twenty-four hours later, the monolayers were washed and incubated with a peroxidase-conjugated goat anti-RSV antibody (Biogenesis, Poole, Great Britain). Infected cells were detected with 3-amino-9-ethylcarbazole, with infectious units being enumerated by light microscopy.

Statistical analysis. The statistical significance between experimental groups was determined by Student's *t* or Mann-Whitney U test as appropriate.

RESULTS

Neutralization of CCL11 attenuates illness and pulmonary eosinophilia following RSV infection. The expression of the CCL11 protein was upregulated in the lungs of rVV-G-primed mice compared to rVV-β gal-primed mice by day 5 of the RSV challenge (data not shown). To determine whether CCL11 played a role in the increased severity of disease in rVV-G-primed mice, we injected mice intravenously with either rabbit anti-mouse CCL11 or normal rabbit IgG from days 0 to 5 of the RSV challenge. The treatment of mice with anti-CCL11 during RSV challenge reduced the disease severity, as measured by weight loss and morbidity (assessed by an independent observer who was blinded to treatments) (Fig. 1a and b). The most consistent effect of treatment with anti-CCL11 was a significant reduction in both the proportion and the total number of eosinophils recruited (Fig. 1c and d). Mice that were given anti-CCL11 displayed an average of >50% inhibition of pulmonary eosinophilia, decreasing from $12.7 \times 10^4 \pm 2.4 \times 10^4$ total eosinophils recovered by BAL to $5.2 \times 10^4 \pm 1.2 \times 10^4$.

Anti-CCL11 treatment alters recruitment of $CD4^+$ T cells to the lungs. Total cellular recruitment to the lungs (as sampled by BAL) was decreased in anti-CCL11-treated mice more than could be accounted for by the reduction in eosinophil recruitment alone (Fig. 2a). The accumulation in the lungs of $CD4^+$ T cells, but not of $CD8^+$ T cells, was also markedly suppressed in mice that were treated with anti-CCL11 relative to their control-treated littermates (Fig. 2 b and c). This differential inhibition of the recruitment of T lymphocytes resulted in a shift in the mean $CD4^+$ to $CD8^+$ ratio in the lungs from 2.23 for the control group to 1.44 for the treated group, similar to that produced by a disruption of the dominant class II epitope of G (33). Lymph node T cells were also examined, and their numbers did not vary between the different rVV-G-primed groups, suggesting that the expansion of $CD4^+$ T cells in secondary lymphoid tissues was not impaired by the administration of anti-CCL11.

To further investigate the effects of anti-CCL11 on T cells, we examined the expression of cytokines by T cells in BAL fluid by intracellular cytokine staining. Treatment with anti-CCL11 did not significantly alter the proportion of either $CD4^+$ or $CD8^+$ T cells that were primed to secrete IFN-γ. However, when the total numbers of $CD4^+$ and $CD8^+$ T cells were calculated, there were more IFN-γ-expressing $CD8^+$ Tc1 cells in anti-CCL11-treated animals than in control animals. These cells are responsible for downregulating Th2 responses and for dictating the T-helper phenotype of secondary RSV disease in F-primed mice (12). In terms of the levels of IL-4,

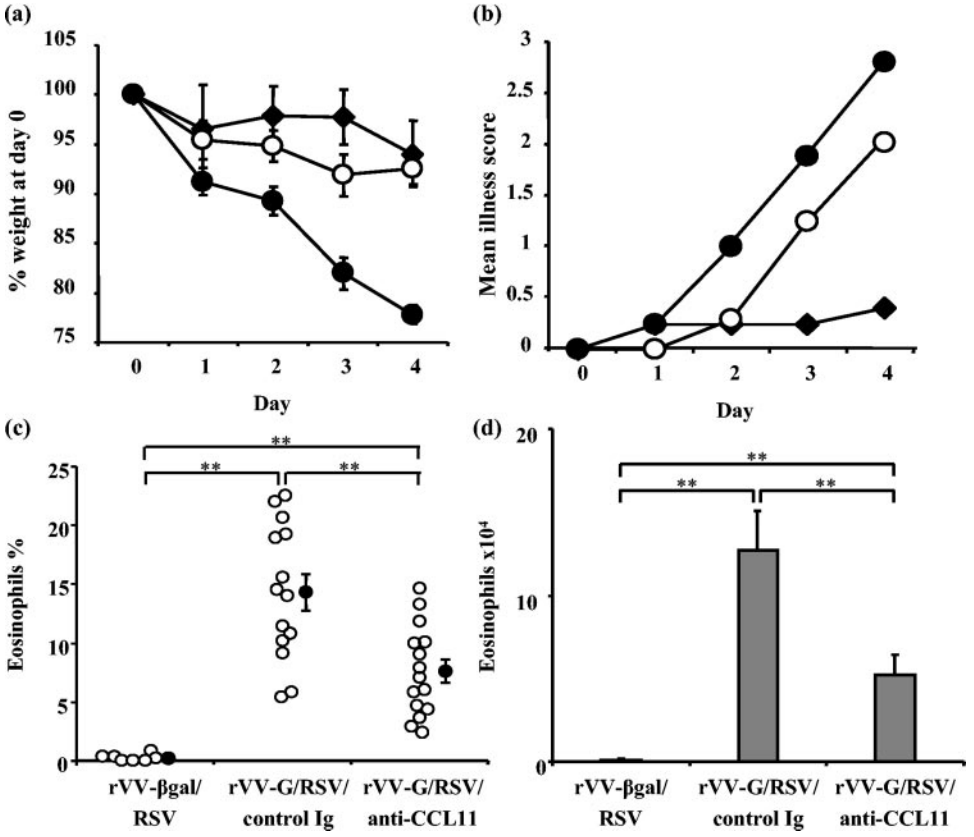

FIG. 1. Treatment with anti-CCL11 during RSV challenge of rVV-G-primed mice reduces severity of illness and pulmonary eosinophilia. rVV-β gal (♦)- or rVV-G-primed mice were challenged with RSV on day 0. rVV-G-primed animals were given anti-CCL11 (○) or control Ig (●) daily. Mean weight loss (a) and mean illness scores (b) are presented for four to five mice per group. These data are representative of three separate experiments. Differential counts were performed with hematoxylin-and-eosin-stained cytospin preparations of BAL cells from individual mice and were used to determine proportions (c) and total numbers (d) of eosinophils. Combined data from four independent experiments are shown, together with means ± SEM for each group. **, $P < 0.01$.

IL-5, and IFN-γ in cell-free BAL supernatants, there was a reduction in the amount of the Th2 cytokine IL-5 present in BAL fluids from mice that had received anti-CCL11 compared to those from rVV-G-primed, control antibody-treated animals (Fig. 3), and the level of IL-5 in rVV-G-primed, anti-CCL11-treated mice was not statistically different from that in rVV-β gal-primed mice. Statistically similar amounts of IFN-γ were detected in the lungs of anti-CCL11- and control-treated rVV-G-primed mice.

To confirm the helper phenotype of the differentially recruited fraction of T lymphocytes, we tested cells from anti-CCL11- and control-treated rVV-G-primed mice on day 5 of the RSV challenge for proliferation in response to the G184-197 peptide. This peptide was chosen because it is the dominant Th2-inducing epitope of the RSV G protein (33). Cells from control-treated animals yielded higher proliferative responses overall than did those from the anti-CCL11-treated group (mean proliferation indices of 24.2 ± 6.2 versus 14.8 ± 4.2), although due to the substantial variability within the assay, this difference did not reach statistical significance. The secretion of IFN-γ by G184-197-stimulated lung mash cells was similar for both the control- and anti-CCL11-treated groups (221.3 ± 55.4 versus 187.4 ± 29.8 pg/ml). Differences emerged in the production of Th2 cytokines. Pulmonary lymphocytes from mice treated with anti-CCL11 secreted smaller amounts of both IL-4 (46.8 ± 8.8 versus 25.8 ± 12.3 pg/ml) and IL-5 (46.25 ± 8.2 versus 30 ± 8.1 pg/ml) than those from the control group, although again, these reductions were not statistically significant. Together, these data suggested that the $CD4^+$-T-cell fraction excluded from the lungs by treatment with anti-CCL11 was (at least partially) specific for G and more Th2 than Th1 in its profile of cytokine secretion. The pattern of pulmonary inflammation induced by the administration of anti-CCL11 was therefore consistent with the suspected elimination of CCR3-expressing $CD4^+$ Th2 cells.

To confirm that neutralizing CCL11 diminished the recruitment of Th2 cells, we administered anti-CCL11 to mice that were primed with rVV-β gal or rVV-F during the RSV challenge. As previously described, pulmonary inflammation under these conditions is characterized by classical antiviral Th1-type responses, with Th2 cells and Th2 cytokines not being readily detected (34). Therefore, if the chemoattractant activity of CCL11 for lymphocytes were restricted to Th2 cells, depletion by anti-CCL11 would not be expected to alter $CD4^+$-T-cell recruitment to the lungs during either of these infections. This was confirmed to be the case, since the anti-CCL11 treatment

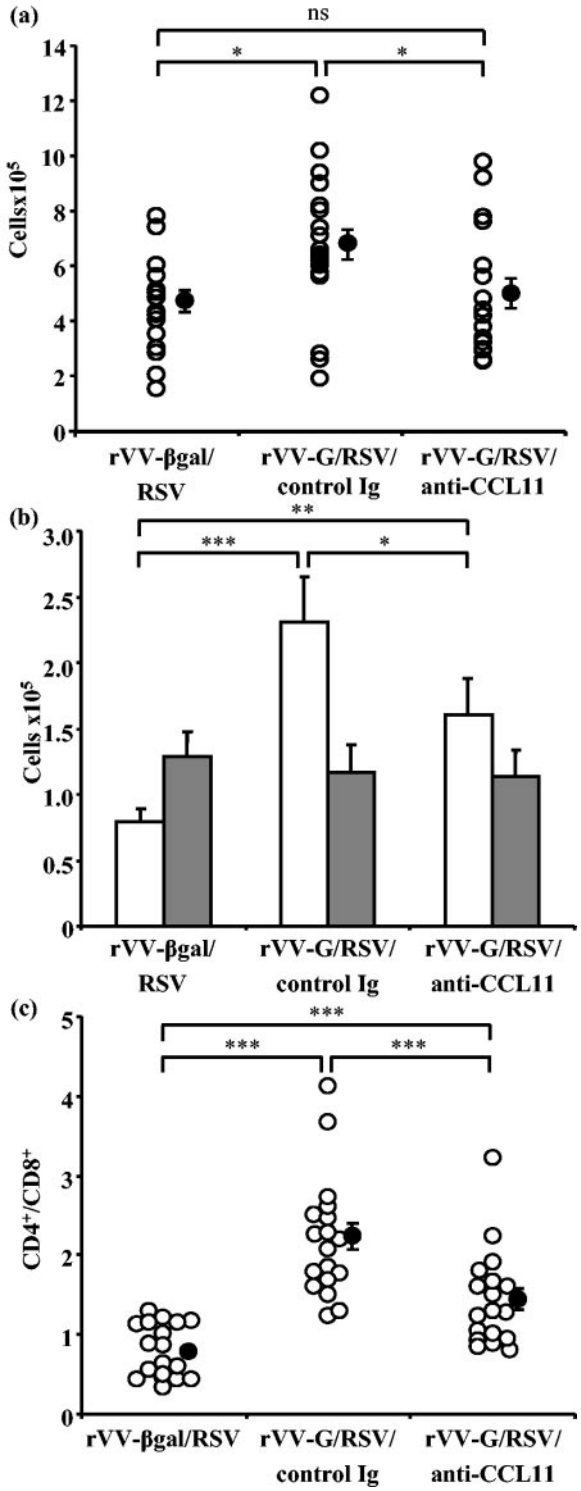

FIG. 2. Treatment with anti-CCL11 during RSV challenge of rVV-G-primed mice alters the balance of $CD4^+$ and $CD8^+$ T cells recruited to the lungs. Combined data from four independent experiments show total BAL cell infiltrates (a), numbers of $CD4^+$ (open bars) and $CD8^+$ (filled bars) T cells recovered from BAL fluid (b), and ratios of $CD4^+$ to $CD8^+$ cells (c). Means ± SEM for each group are shown. *P* values are represented as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

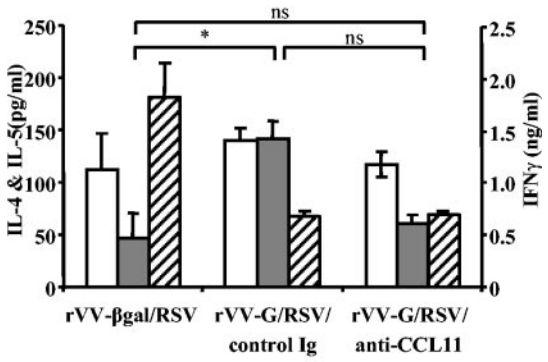

FIG. 3. Treatment with anti-CCL11 reduces IL-5 in the lungs. The levels of IL-4 (open bars; left axis), IL-5 (filled bars; left axis), and IFN-γ (hatched bars; right axis) in cell-free BAL supernatants were measured by ELISA. *P* values are shown for differences in IL-5 production between groups, with *P* values represented as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Similar data were obtained in other independent experiments.

had no effect on Th1-dominated RSV disease after rVV-F priming. Additionally, both control and anti-CCL11-treated animals showed identical patterns of pulmonary inflammation in terms of numbers and proportions of $CD4^+$ and $CD8^+$ T cells recruited, and crucially, similar numbers of BAL lymphocytes produced IFN-γ. These data confirm that treatment with anti-CCL11 does not alter the recruitment of Th1 (or indeed of Tc1) cells during infection with RSV.

Anti-CCL11 treatment does not impair humoral immunity or protection. $CD4^+$ T cells are required for B-cell class switching, which in mice is directed toward IgG2a expression by IFN-γ and toward IgG1 expression by IL-4 (36). Since the experiments described above showed a reduction in Th2 cytokines in the lungs, we examined the generation of RSV-specific antibodies (Fig. 4a to c). The results were not indicative of a difference in the nature of T-helper responses, although in this regard it is important that the major difference in BAL cytokine detection was observed for IL-5, whereas the levels of IL-4 and IFN-γ were similar regardless of the treatment. Consistent with this observation, the levels of RSV-specific whole immunoglobulin in sera were broadly similar for both rVV-G-primed groups, and a mild reduction in both IgG1 and IgG2a production in animals treated with anti-CCL11 was not significant.

The efficient generation of humoral immunity was reflected in the ability of antibody-treated mice to eliminate infection. Importantly, a treatment with anti-CCL11 did not compromise protection against a challenge with whole RSV, showing that sufficient antiviral immunity was achieved despite the beneficial reduction in inflammatory infiltrates (Fig. 4d). Thus, a disease-exacerbating fraction of inflammatory leukocytes that was not essential for viral clearance was selectively eliminated by the treatment with anti-CCL11. RSV-specific antibody isotypes were unaffected by the anti-CCL11 treatment.

Comparison of treatments with anti-CCL11 and anti-T1/ST2. T1/ST2 is a marker of Th2 $CD4^+$ T cells. $T1/ST2^+$ cells were identified in pulmonary infiltrates of rVV-G-primed, RSV-challenged mice by flow cytometry with anti-T1/ST2 at low levels up to about 12% of the total infiltrating $CD4^+$ T

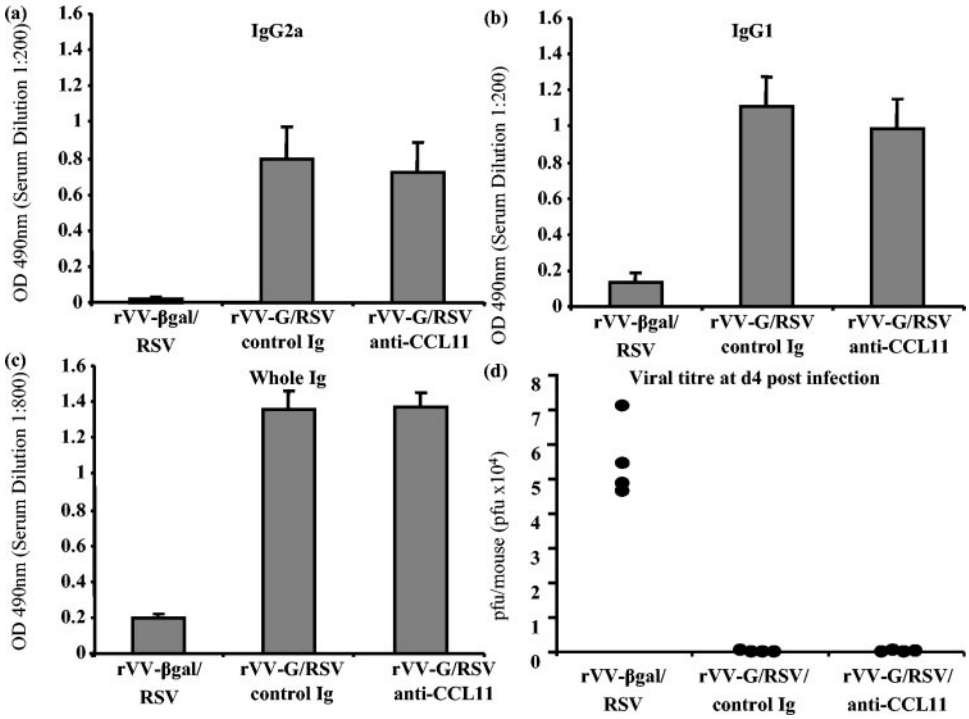

FIG. 4. Treatment with anti-CCL11 does not compromise protection. Levels of RSV-specific IgG1 (a), IgG2a (b), and Ig (c) were measured by ELISA. The data presented are mean absorbance values ± SEM of four mice per group at a serum dilution of 1:200 (a and b) or 1:800 (c). Similar results were obtained at other serum dilutions. (d) Recovery of infectious virus from whole lung homogenates of individual mice, primed and treated as indicated, was measured by titration on HEp-2 monolayers 4 days after challenge with RSV. Each data point represents a single mouse.

cells on day 5 of challenge. We have previously shown that anti-T1/ST2 can reduce this Th2 immunopathology (39). We therefore wanted to do a direct comparison with anti-CCL11. To this end, we infected rVV-G-primed mice intranasally with RSV A2 and administered either 100 μg of anti-T1/ST2 or 50 μg of anti-CCL11 intravenously daily from days 0 to 5 of the challenge. Control mice received 100 μg of rabbit immunoglobulins.

These experiments revealed considerable similarities between the administration of anti-T1/ST2 and that of anti-CCL11 in terms of effects on both illness and pulmonary inflammation, although in general the former treatment produced the most benefit. This was especially apparent from illness profiles, which showed that anti-T1/ST2-treated mice consistently suffered less weight loss than those that received anti-CCL11 (Fig. 5a). As shown in the figure, RSV infections in unprimed control mice (given an rVV encoding beta-galactosidase) caused weight loss due to viral replication between days 4 and 6. However, the early enhanced weight loss in rVV-G-primed mice was attenuated by either anti-T1/ST2 or anti-CCL11 treatment, which reduced the total inflammatory cell influx, affecting not only eosinophils and $CD4^+$ T cells, but also $CD8^+$ T cells (Fig. 5b to d). However, as described above, such a reduction in $CD8^+$-T-cell accumulation upon anti-CCL11 treatment was atypical and was not seen in a second experiment that was performed identically. Anti-T1/ST2, on the other hand, did reproducibly decrease the recruitment of $CD8^+$ as well as $CD4^+$ T cells and eosinophils (compared to control-treated mice) in several independent experiments. Substantial differences in leukocyte recruitment were also observed histologically in frozen lung sections stained with hematoxylin and eosin. A treatment with either anti-CCL11 or anti-T1/ST2 had similar effects on the production of inflammatory cytokines in this system.

DISCUSSION

In the model of virus-induced lung eosinophilia described here, the neutralization of CCL11 reduced eosinophil accumulation to the lung by about 50% and produced a beneficial effect which was seen upon pulmonary histology. Treatment with anti-CCL11 also perturbed the recruitment of $CD4^+$ T cells and reduced the production of the Th2 cytokine IL-5, but not that of IFN-γ, suggesting an important shift in the inflammatory T-helper phenotype. The decrease in lymphocyte accumulation appeared to be the result of reduced trafficking rather than an impaired expansion of effector cells and was accompanied by a shift in the local cytokine milieu towards a more Th1-like environment. These findings are in keeping with studies of the role of eosinophils in allergic disease using neutralizing antibodies (7) or knockout mice (29), but to our knowledge we are the first to report a role for CCL11 in virus-induced lung immunopathology. The finding that anti-CCL11 is beneficial in this setting suggests that therapies designed to interfere with CCL11 effects might be useful for such conditions.

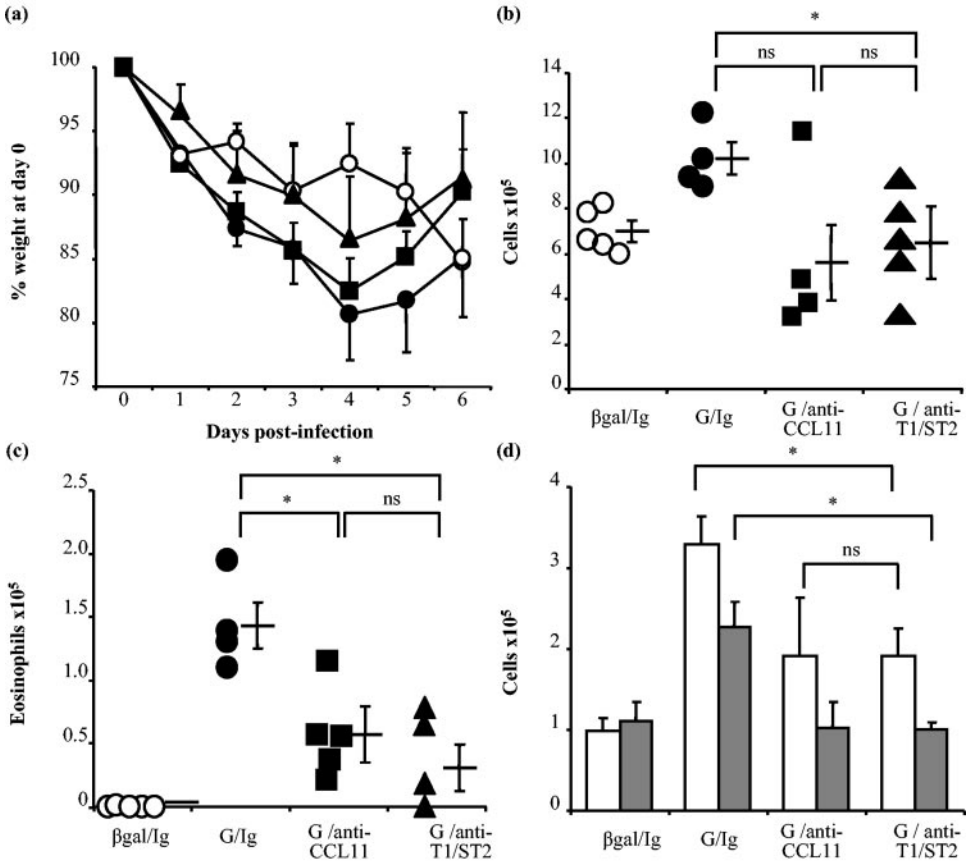

FIG. 5. Treatment with anti-T1/ST2 or anti-CCL11 has similar beneficial effects upon eosinophilic RSV disease. (a) Weight changes were monitored during RSV challenge of rVV-β gal (open symbols)- or rVV-G (filled symbols)-primed mice that were either mock treated (100 mg of normal rabbit Ig; circles) or given daily doses of 50 mg of anti-CCL11 (squares) or 100 mg of anti-T1/ST2 (triangles). Pulmonary infiltrates were sampled by BAL on day 6, and total cells (b), eosinophils (c), and $CD4^+$ and $CD8^+$ T cells (d; open and filled bars, respectively) were enumerated as described previously. Means ± SEM of four mice per group are shown. *P* values are represented as follows: *, $P < 0.05$; ns, not significant.

Although the observation of a decrease in $CD4^+$ T cells was initially unexpected, a role for CCL11 in the recruitment of CCR3-expressing Th2 cells has been shown by Lloyd et al., who used a model of allergic airway inflammation in which polarized effector Th2 cells were adoptively transferred into naïve mice prior to sensitization with ovalbumin (19). In this system, treatment with anti-CCL11 specifically inhibited the initial phase of Th2 cell recruitment in the first few days immediately after aerosol challenge, with recruitment via the CCR4/MDC (monocyte-derived chemokine) axis comprising the dominant chemoattractive potential within 1 week of the challenge. The administration of anti-CCL11 inhibited the accumulation of antigen-specific Th2 cells by at least 50% on day 4 after an ovum challenge and significantly reduced both eosinophil infiltration and IL-4 production. This study conclusively demonstrated a role for the CCL11/CCR3 axis in mediating the early recruitment of Th2 cells.

The present data do not rule out secondary effects on $CD4^+$-T-cell recruitment due to the presence of reduced numbers of infiltrating eosinophils. Such effects include a reduced secretion of eosinophil-derived type 2 cytokines, reduced antigen presentation by endobronchial eosinophils trafficking to the local lymph nodes, and dampening of inflammatory responses elicited by damage to pulmonary epithelium by toxic eosinophil granule proteins. Indeed, some or all of these mechanisms may contribute to the relief from pulmonary $CD4^+$-T-cell accumulation. A recent investigation confirmed that antigen-loaded eosinophils are able to amplify Th2 responses and, after adoptive transfer into naïve mice, prime de novo Th2-driven allergic lung disease (23). Nevertheless, these data are also consistent with a role for the CCL11/CCR3 axis in mediating the recruitment of $CD4^+$ T cells to the lungs of infected mice during an RSV challenge. Other groups have been able to selectively inhibit pulmonary eosinophilia (by the neutralization of macrophage inflammatory protein 1α and RANTES, which act predominately via CCR1) without a concomitant reduction in antigen-specific lymphocytes, suggesting that decreased T-cell accumulation is not an obligate consequence of lowered eosinophil recruitment (21). To the best of our knowledge, this represents the first report of an involvement of CCL11 signaling in the trafficking of $CD4^+$ T cells in a physiological setting. While previous studies have examined the trafficking of strongly committed Th2 cells induced by repeated stimulation under polarizing conditions in vitro (19), this study suggests a possible role for CCL11 in the migration of T cells

generated in vivo during the course of a normal immune response.

Despite considerable efforts, it was not possible to convincingly prove that the differential recruitment of $CD4^+$ T cells in the present study was due to a selective effect upon Th2-type, CCR3-expressing cells. Attempts to confirm the recruitment of such cells to the lungs of rVV-G-primed mice during RSV infection by direct staining with either anti-T1/ST2 or rabbit antibodies specific for murine CCR3 (kindly provided by P. Ponath of LeukoSite, Inc., Cambridge, Mass.) were unsuccessful. In this regard, it may be relevant that responding Th2 cells appear to progressively lose their expression of CCR3 (2, 19) and consequently may have downregulated this receptor by the time of sampling. Likewise, analyses of IL-4 and IL-5 production by ex vivo-stimulated, G-specific $CD4^+$ T cells did not prove any quantitative differences in Th2 cell accumulation in anti-CCL11-treated mice. Previous studies have suggested that only about 12% of $CD4^+$ T cells in the BAL fluids of rVV-G-primed mice are $T1/ST2^+$ (i.e., Th2 cells) on day 5 of an RSV challenge (39). The reduction in $CD4^+$-T-cell accumulation upon neutralization of CCL11 was, on average, closer to 25%, suggesting that the recruitment of both Th2 and non-Th2 subsets is affected by an anti-CCL11 treatment (Fig. 3). This is surprising, since CCR3 expression by activated lymphocytes is believed to be restricted to type 2 cells (40). Treatment with anti-CCL11 did not affect RSV illness or inflammatory cell accumulation in rVV-F- or rVV-β gal-primed mice, in which inflammation was restricted to a Th1 phenotype, confirming that the CCL11/CCR3 axis is not directly involved in Th1 cell recruitment. It is possible that the elimination of non-Th2 cells is a secondary effect due to the decreased eosinophil and Th2 cell recruitment induced by the disruption of the CCL11/CCR3 axis.

Activated eosinophils secrete several granule proteins that can damage the respiratory epithelium (6). Such damage might exacerbate local inflammation, leading to an augmented recruitment of non-Th2 $CD4^+$ T cells, especially in the context of an ongoing viral infection. The relief of tissue damage upon depletion of CCL11 may therefore result in a dampening of the exuberant immune response, thus explaining the smaller accumulation of $CD4^+$ T cells. Interestingly, a similar reduction in IFN-γ-producing cells upon anti-CCL11 treatment was observed during experimental mycobacterial antigen-elicited granuloma formation (30). These granulomas, which are normally characterized by the presence of neutrophils and of T cells secreting IFN-γ, but not of eosinophils (i.e., type 1 lesions), were reduced in size by an anti-CCL11 treatment. Furthermore, IFN-γ production by local draining lymph node cells was increased in a dose-dependent manner by exogenous CCL11 protein (30). The mechanism by which this is achieved is unclear (since Th1 cells do not express CCR3), but it may well contribute to the observed reduction in Th1 cell recruitment in the present study.

Although we could not conclusively demonstrate the effects of anti-CCL11 on the recruitment of Th2 cells, the neutralization of a single chemokine during ongoing complex inflammation with mixed Th1, Th2, and $CD8^+$-T-cell components is likely to have a limited effect. Furthermore, the neutralization of CCL11 is not equivalent to a complete blockade of CCR3, as several other ligands, including CCL11-2, RANTES, and MCP-3, can also engage this receptor and may compensate to some degree for the absence of its main ligand (41). Several studies have been unable to detect CCR3 on Th2 cells in vivo (4, 8), and Th2 cells both develop normally and appear to accumulate efficiently at allergic sites in $CCR3^{-/-}$ mice, suggesting that adequate compensatory mechanisms for Th2 recruitment exist (22); these may include the expression of CCR4 (19) or CCR8 (1). It remains to be seen whether blocking signaling via these receptors will further impede $CD4^+$-T-cell accumulation in rVV-G-primed, RSV-challenged mice.

A comparison of anti-CCL11 and anti-T1/ST2 treatments revealed several intriguing similarities, suggesting that overlapping Th2 cell subsets might express both T1/ST2 and CCR3, thereby leading to reductions in eosinophilia, illness, $CD4^+$-T-cell accumulation, and IL-5 production. The two treatments were not equivalent, however. Importantly, the treatment with anti-T1/ST2 produced a profound suppression not only of $CD4^+$ but also of $CD8^+$-T-cell recruitment, and it significantly reduced the amount of IFN-γ in BAL fluid (39). T1/ST2 is expressed predominately on type 2 cytokine-secreting cells, so the treatment was expected to eliminate only the relatively small fraction of Th2 and Tc2 cells. However, treatment with anti-T1/ST2 consistently produced far greater effects upon total T-cell accumulation than might have been predicted. This may again be due to the reduction in pulmonary damage, as suggested for anti-CCL11: the proportionately greater effect of anti-T1/ST2 presumably reflects the relative efficiency of each of these treatments at reducing Th2 cell accumulation.

The observation that IL-5, but not IL-4, was significantly reduced in BAL supernatants from mice that were treated with either anti-CCL11 or anti-T1/ST2 is intriguing. This may reflect the fact that CCR3 and T1/ST2 are expressed on Th2 cells at different stages of their differentiation, and the subsets of $CD4^+$ T cells affected by either treatment might be those which predominately produce IL-5 rather than IL-4. This hypothesis is supported by a report that individual $CD4^+$ T cells in vivo have different levels of commitment to a certain T-helper phenotype and can be distinguished by the expression of one or more type 2 cytokines and/or T1/ST2 (20). T1/ST2 expression has recently been demonstrated on Tr1 regulatory $CD4^+$ T cells expressing high levels of IL-10 in the respiratory tract (24). These cells mediate the suppression of Th1 responses in response to *Bordetella pertussis* and unrelated pathogens. No role for Tr1-type cells during experimental infection with RSV has been reported to date, although IL-10-producing cells are certainly detected in both primary and secondary disease. Our data describing the global downregulation of both type 1 and type 2 cytokines and of $CD4^+$ and $CD8^+$ T cells by an anti-T1/ST2 treatment suggest that effective Tr1 responses are not induced by G-enhanced RSV disease, a conclusion which is supported by the florid nature of the illness.

Together, our data suggest that selectively targeting the eosinophilic Th2 arm of a mixed type 1 and 2 pulmonary inflammation can have profound effects on global cellular recruitment. This selective targeting can be achieved by the depletion of either CCL11 or $T1/ST2^+$ cells. This compares favorably with the elimination of pulmonary eosinophilia in G-enhanced RSV disease after the administration of IL-12, which skews the local T-helper response in favor of Th1 but can increase weight loss, pulmonary inflammation, and illness (14). The present

data suggest that selectively targeting Th2 responses by treatment with anti-CCL11 will produce beneficial effects on pulmonary eosinophilia without the attendant risks of augmented disease or viral replication. The clinical potential of this study is supported by the fact that one of the chemokine receptors for CCL11, CCR5 (26), may play a role in the severity of bronchiolitis in humans (11).

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