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Mutating the CX3C Motif in the G Protein Should Make a Live Respiratory Syncytial Virus Vaccine Safer and More Effective

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ABSTRACT Respiratory syncytial virus (RSV) belongs to the family Paramyxoviridae and is the single most important cause of serious lower respiratory tract infections in young children, yet no highly effective treatment or vaccine is available. Through a CX3C chemokine motif (182CWAIC186) in the G protein, RSV binds to the corresponding chemokine receptor, CX3CR1. Since RSV binding to CX3CR1 contributes to disease pathogenesis, we investigated whether a mutation in the CX3C motif by insertion of an alanine, A186, within the CX3C motif, mutating it to CX4C (182CWAIAC187), which is known to block binding to CX3CR1, might decrease disease. We studied the effect of the CX4C mutation in two strains of RSV (A2 and r19F) in a mouse challenge model. We included RSV r19F because it induces mucus production and airway resistance, two manifestations of RSV infection in humans, in mice. Compared to wild-type (wt) virus, mice infected with CX4C had a 0.7 to 1.2 log10-fold lower virus titer in the lung at 5 days postinfection (p.i.) and had markedly reduced weight loss, pulmonary inflammatory cell infiltration, mucus production, and airway resistance after challenge. This decrease in disease was not dependent on decrease in virus replication but did correspond to a decrease in pulmonary Th2 and inflammatory cytokines. Mice infected with CX4C viruses also had higher antibody titers and a Th1-biased T cell memory response at 75 days p.i. These results suggest that the CX4C mutation in the G protein could improve the safety and efficacy of a live attenuated RSV vaccine.

IMPORTANCE RSV binds to the corresponding chemokine receptor, CX3CR1, through a CX3C chemokine motif (182CWAIC186) in the G protein. RSV binding to CX3CR1 contributes to disease pathogenesis; therefore, we investigated whether a mutation in the CX3C motif by insertion of an alanine, A186, within the CX3C motif, mutating it to CX4C (182CWAIAC187), known to block binding to CX3CR1, might decrease disease. The effect of this mutation and treatment with the F(ab\’)2 form of the anti-RSV G 131-2G monoclonal antibody (MAb) show that mutating the CX3C motif in the G protein should make a live respiratory syncytial virus vaccine safer and more effective. J Virol 91:e02059-16. https://doi.org/10.1128/JVI.02059-16.

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KEYWORDS CX3C motif, G proteins, live attenuated vaccines, respiratory syncytial virus, vaccines, virology

Respiratory syncytial virus (RSV), a pneumovirus of the family Paramyxoviridae, is a leading cause of lower respiratory tract disease in infants and young children worldwide (1). Infection can lead to bronchiolitis and viral pneumonia (1–5), and serious
infection in young children has been associated with later development of asthma (6). Its disease burden worldwide has made it a high priority for disease prevention. Immune prophylaxis is available for high-risk young infants but requires monthly doses, is costly, and does not prevent the large burden of disease in young children without a high risk (7–9). Good infection control practices, the other available preventive measures, are effective in preventing nosocomial transmission in health care settings (10). The most effective preventive, a safe and effective vaccine, is still not available despite over 50 years of research.

The first candidate vaccine, a formalin-inactivated RSV (FI-RSV) vaccine, was tested in children in the 1960s and predisposed young RSV-naive recipients to serious enhanced respiratory disease (ERD) with later natural infection, resulting in a marked increase in hospitalizations and two deaths (11–14). Concern that any other nonlive RSV vaccine might also predispose to ERD has so far limited vaccine development for young, potentially RSV-naive children to live attenuated RSV or virus vector vaccines. Since natural infection does not lead to ERD, live attenuated vaccines have been considered safe for and tested in young children, and none led to ERD, but none have yet been sufficiently promising to move to licensure (15, 16).

Our studies of pathogenesis of RSV disease led us to hypothesize that a mutation in the CX3C motif in the G protein might improve the safety and efficacy of a live attenuated RSV vaccine. The G protein is one of two surface glycoproteins that induce protective immunity and also modulate host immune responses and contribute to pathogenesis of disease with infection. The other surface glycoprotein, the F protein, induces a higher titer of neutralizing antibodies and better cross protection among different RSV strains and has most often been the focus for developing an RSV vaccine (17).

The G protein’s contribution to disease pathogenesis, however, led us to explore its role in developing a live attenuated vaccine. In earlier studies, we have shown that the presence of the G protein during infection in mice contributes to pulmonary inflammation, weight loss, lung mucus production, increased airway resistance, depressed respiratory rate, altered adaptive immune responses, and FI-RSV vaccine-induced ERD (18–22). In these studies, we also show that binding G with a monoclonal antibody (MAb), 131-2G, which reacts to the central conserved region in G, prevents these signs of disease.

Importantly, the G protein contains a CX3C chemokine motif in its central conserved region, and through this motif, RSV binds to the CX3C receptor, CX3CR1 (23–25). We have previously shown that G binding to CX3CR1 induces leukocyte migration in vitro similar to the one CX3C chemokine, fractalkine (23), and, in mouse studies, explains G-associated altered migration of T cells to RSV-infected lungs (26), depressed respiratory rates (22), and FI-RSV vaccine-induced ERD (20). Since G binding to CX3CR1 through the CX3C motif in G is important to RSV disease, mutations to the motif that prevent G binding to CX3CR1 might prevent disease. The finding that CX3CR1 is an important receptor for primary human airway epithelial cells (hAECs), though not for cell lines usually used to study RSV (24, 25, 27), suggests the CX3C motif is more important to human RSV disease than studies to date suggest. Consequently, we chose to investigate the potential benefit of mutating the wild-type (wt) CX3C motif in G to CX4C, which does not bind to CX3CR1 (24). We studied the effect of this mutation in the RSV A2 and RSV rA2 line 19F (r19F) strains in mice. The r19F strain, unlike A2, induces pulmonary mucus and airway resistance in mice (19, 28, 29). The effect of this mutation and treatment with the F(ab')2 form of the anti-RSV G 131-2G MAb show that mutating the CX3C motif to CX4C blocks much of the disease and immune modulation associated with the G protein and should improve the safety and efficacy of a live attenuated RSV vaccine.

RESULTS

The CX4C virus has reduced titers. All four viruses replicated after challenge and were detected on days 3 and 5 postinfection (p.i.), with the r19F virus growing to
slightly higher titer on day 5 p.i. (Table 1). No infectious virus was detected at day 8 p.i.

In the first experiment, we used a 10⁶ TCID₅₀ challenge dose for all the viruses. Since the CX4C viruses gave lower lung titers than the CX3C viruses (0.72 log₁₀-fold lower for A2 and 1.23 log₁₀-fold lower for r19F), we increased the challenge inoculum to 2.0 × 10⁶ TCID₅₀ for the CX4C viruses and kept the inoculum dose at 10⁶ TCID₅₀ for the CX3C viruses. In experiments with the higher inoculum for the CX4C viruses, the lung virus titers at 5 day p.i. were much closer, i.e., 0.16 or 0.20 log₁₀-fold lower for CX4C versus wt r19F viruses and 0.17 to 0.26 log₁₀-fold lower for the A2 viruses. The treatment with F(ab')₂ 131-2G did not affect the lung virus titer for any of the viruses (Table 1). The limit of virus detection in lung homogenates was a TCID₅₀ of greater than or equal to log₂ 0.9. These results suggest the CX4C mutation attenuates replication in mice. The RSV real-time PCR results for lung homogenates (3 mice/group) on days 3 and 5 p.i. gave results similar to those for virus infectivity (Fig. 1).

**CX4C viruses are associated with less weight loss.** The mice infected with wt viruses had significantly (P ≤ 0.001) lower weights than mock-infected mice on days 3,

---

**TABLE 1** Lung RSV titers

<table>
<thead>
<tr>
<th>Group</th>
<th>Titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day 5 p.i.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Study 1</td>
<td>Study 2</td>
<td>Study 3</td>
</tr>
<tr>
<td>Wt A2</td>
<td>4.57 ± 0.12</td>
<td>4.53 ± 0.07</td>
<td>4.60 ± 0.12</td>
</tr>
<tr>
<td>A2-CX4C</td>
<td>4.57 ± 0.08</td>
<td>4.52 ± 0.06</td>
<td>4.59 ± 0.08</td>
</tr>
<tr>
<td>Wt r19F</td>
<td>4.97 ± 0.21</td>
<td>4.95 ± 0.17</td>
<td>4.98 ± 0.21</td>
</tr>
<tr>
<td>r19F-CX4C</td>
<td>4.95 ± 0.12</td>
<td>4.92 ± 0.08</td>
<td>4.96 ± 0.18</td>
</tr>
<tr>
<td>Mock</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Wt A2 + F(ab')₂ 131-2G</td>
<td>4.55 ± 0.12</td>
<td>4.57 ± 0.02</td>
<td>4.58 ± 0.06</td>
</tr>
<tr>
<td>Wt r19F + F(ab')₂ 131-2G</td>
<td>4.98 ± 0.09</td>
<td>4.94 ± 0.65</td>
<td>4.97 ± 0.12</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mice were challenged intranasally with 10⁶ TCID₅₀ for all the viruses (study 1) or 10⁶ TCID₅₀ for the wt viruses and 2.0 × 10⁶ TCID₅₀ for the CX4C viruses (studies 2 and 3). Mock represents challenge with mock-infected tissue culture material (n = 3 mice/group). Anti-RSV G MAb 131-2G [F(ab')₂ form]-treated mice were given 300 μg of the MAb intraperitoneally 2 days before challenge. Lung samples were collected on days 3, 5, and 8 p.i., and the viral titer in lung homogenates was determined with a microtiter infectivity assay (5 mice/group) as described in Materials and Methods. Viral titers are represented as log values (TCID₅₀/g of lung). The data are means ± SEMs. ND, not determined.

<FIG 1> The level of RSV replication in lung tissue was determined by real-time RT-PCR (quantitative RT [qRT]-PCR) for M gene (relative genome level equivalent log PFU per gram of lung tissue). Mice were challenged intranasally with 10⁶ TCID₅₀ for all the viruses (study 1) or 1 × 10⁶ TCID₅₀ for the wt viruses and 2.0 × 10⁶ TCID₅₀ for the CX4C viruses (studies 2 and 3). Mock, challenged with mock-infected tissue culture material (n = 3 mice/group). Lung samples were collected on days 3 and 5 p.i., and viral titers in lung homogenates were determined with a real-time PCR (5 mice/group) as described in Materials and Methods. The data are means ± SEM from three independent studies.

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and 8 p.i.; wt A2-infected mice had more weight loss on day 3 than wt r19F-infected mice (P < 0.05). Note that mice challenged with either of CX4C viruses and mice given MAb F(\(ab′\))\(_2\) 131-2G before challenge with any of the viruses had no weight loss compared to mock-infected mice [Fig. 2A; data for MAb F(\(ab′\))\(_2\) 131-2G-treated mice not shown]. On day 5 p.i., the weights for 19F-CX4C- or A2-CX4C-infected mice were significantly (P < 0.05) higher than the corresponding weights for wt virus-infected mice (102.65% ± 2.44% versus 93.89% ± 2.91% and 103.93% ± 5.82% versus 93.89% ± 4.47% of preinfection weight, respectively). The increased weight for the CX4C-infected versus wt virus-infected mice persisted through day 8 p.i. (103.92% ± 2.53% versus 99.25% ± 3.65% for the 19F viruses and 104.33% ± 2.54% versus 99.70% ± 4.76% for the A2 viruses) (Fig. 2A). There was no difference in weight loss between the challenges with 1 × 10\(^6\) and 2 × 10\(^6\) TCID\(_{50}\) of CX4C viruses. These results show that the CX4C mutation attenuates disease, as manifested by weight loss, and this attenuation did not appear to be dependent on changes in virus replication.

**CX4C viruses are associated with less pulmonary inflammation.** The effects of the mutation at the CX3C site of RSV G protein on pulmonary inflammation are evident in bronchoalveolar lavage (BAL) fluid numbers on days 3, 5, and 8 p.i. At the peak

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**FIG 2** Effect of the CX4C mutation on disease after challenge. (A) Weight loss (n = 10 mice/group). (B) Pulmonary inflammation (n = 5 mice/group). (C) Pulsus paradoxus (n = 5 mice/group). (D) Muc5AC levels (n = 5 mice/group). Mice were challenged intranasally with the indicated viruses or mock infected as described in Materials and Methods. Significant decreases (*, P < 0.05, and †, P < 0.001 compared to wt RSV-infected mice as assessed by one-way ANOVA and post hoc Tukey’s HSD test) are indicated. The error bars indicate SEM. The results are mean values for one experiment and are representative of three independent experiments.
in BAL fluid cell numbers for wt RSV-infected mice on day 5 p.i., the CX4C-infected mice and 131-2G-treated wt-infected mice had significantly fewer BAL fluid cells than untreated, wt RSV-infected mice (P < 0.05) [Fig. 2B; data for MAb F(ab\')\(_2\) 131-2G-treated mice not shown]. There was a significant decrease in almost all types of cells in the BAL fluid (Table 2) between the wt- and CX4C-infected mice. The highest percent decrease for A2 and r19F viruses was in CD3\(^{-}\)Ly-6G/Gr-1\(^{+}\) cells (polymorphonuclear cells [PMNs]) with a 70% decrease for RSV A2 and an 81% decrease for r19F. These data suggest that, similar to treatment or prophylaxis with the anti-G MAb, 131-2G, the CX4C mutation substantially decreases pulmonary inflammation. The decrease in BAL fluid cell numbers with the CX4C versus wt viruses did not change with the increase from 1 \(\times\) 10\(^6\) TCID\(_{50}\) to 2.0 \(\times\) 10\(^6\)-TCID\(_{50}\) inoculum. The decrease was 74% compared to 72% for A2-infected mice and 70% compared to 71% for r19F-infected mice.

**CX4C viruses are associated with reduced airway dysfunction.** The effect of the CX4C mutation on airway dysfunction is of particular interest given the prominence of signs of airway obstruction, i.e., wheezing and diagnosis of bronchiolitis, in human RSV disease. As expected from previous studies (19, 30), wt r19F-infected mice had a significant (P = 0.05) increase in breathing effort, as indicated by an increase in pulsus paradoxus (PP) compared to mock-infected mice and wt A2-infected mice. The breathing effort in CX4C r19F-infected mice and mice given MAb F(ab\')\(_2\) 131-2G prophylaxis and infected with wt r19F was significantly (P = 0.001) decreased compared to untreated, wt r19F-infected mice [Fig. 2C; data for MAb F(ab\')\(_2\) 131-2G-treated mice not shown]. This decrease gave values similar to those for mock-infected mice (Fig. 2C). These results suggest that G binding to CX3CR1 contributes to the increased breathing effort seen with the r19F virus, and the CX4C mutation eliminates this effect. The decrease in breathing effort with the CX4C versus wt 19F RSV did not change with the increase in the inoculum. It was 77% for mice infected with 1 \(\times\) 10\(^6\) TCID\(_{50}\) and 78% for mice infected with 2.0 \(\times\) 10\(^6\) TCID\(_{50}\).

**CX4C viruses are associated with less pulmonary mucin production.** Another manifestation of RSV infection in humans is an increase in airway mucus. As previously reported, wt r19F-infected mice show increased mucin 5 subtype AC (Muc5AC) levels in lung homogenates and periodic acid-Schiff (PAS) staining (19, 30) but not in wt A2-infected mice, 131-2G-treated wt r19F-infected mice, and mock-infected mice [Fig. 2D; data for MAb F(ab\')\(_2\) 131-2G-treated mice not shown]. The CX4C mutation significantly decreased lung mucus production, as indicated by PAS histology and Muc5AC enzyme immunoassay (EIA) on day 8 p.i. (Fig. 2D and 3). The decrease in mucus production with CX4C compared to wt r19F RSV did not change with the increase in the inoculum. It was 41% for mice infected with 1 \(\times\) 10\(^6\) TCID\(_{50}\) and 44% for mice infected with 2.0 \(\times\) 10\(^6\) TCID\(_{50}\).

### Table 2: Types of cells in BAL fluid at 5 days p.i.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Mean no. of cells (10(^3)) ± SEM</th>
<th>% reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>30.46 ± 2.80</td>
<td>2.78(^a)</td>
</tr>
<tr>
<td>CD8</td>
<td>24.68 ± 2.18</td>
<td>1.90(^b)</td>
</tr>
<tr>
<td>B cells</td>
<td>7.89 ± 2.12</td>
<td>3.12(^c)</td>
</tr>
<tr>
<td>Macrophages</td>
<td>13.90 ± 4.20</td>
<td>3.40(^a)</td>
</tr>
<tr>
<td>PMNs</td>
<td>4.12 ± 2.12</td>
<td>1.23(^c)</td>
</tr>
<tr>
<td>NK</td>
<td>34.80 ± 2.39</td>
<td>2.30(^c)</td>
</tr>
</tbody>
</table>

\(^a\)Mice (5 mice/group) were challenged intranasally with the indicated viruses or mock infected, as described in Materials and Methods. The data are the mean total number of BAL cell subtype determined by flow cytometry with anti-CD3\(^+\) and anti-CD4\(^+\) indicating CD4 T cells; anti-CD3\(^+\) and anti-CD8\(^+\) indicating CD8 T cells; anti-CD3\(^-\) and anti-CD45R/B220\(^+\) indicating B cells; anti-CD3\(^-\)–anti-CD11b\(^+\) indicating macrophages, dendritic cells, or monocytes; anti-CD3\(^-\) and anti-mouse Ly06G/Gr-1\(^{+}\) indicating PMNs; and anti-CD3\(^-\)–anti-mouse CD49b/integrin alpha 2 (DX5\(^+\)) indicating NK cells. The percent reduction is relative to the corresponding untreated, wt virus-infected mice. Significant decreases in numbers compared to untreated, wt-infected mice are indicated.

\(^{b}\)P = 0.05 (ANOVA) compared to untreated, wt-infected mice.

\(^{c}\)P = 0.001 (ANOVA) compared to untreated, wt-infected mice.
CX4C viruses are associated with increased Th1 and decreased Th2 pulmonary cytokine responses. Characteristics of the lung cytokine and chemokine responses to infection were investigated to help explain the differences in disease. The effect of the CX4C mutation on cytokine and chemokine levels in lung was evident on days 3, 5, and 8 p.i. but was different for each day. Infection with the CX4C compared to wt viruses had significantly increased levels of alpha interferon (IFN-α) and IFN-β (3 p.i.) and of IFN-γ (5 p.i.) and a modest increase in tumor necrosis factor alpha (TNF-α) (day 5 p.i.) in lung homogenates (Table 3). We also noted an increase in interleukin 4 (IL-4) on day 5 p.i. for wt A2- and 19F-infected mice and in IL-13 on day 8 p.i. for wt r19F-infected mice and no increase in IL-4 or IL-13 for the CX4C viruses. There was no detectable difference in IL-6 cytokine levels between wt- and CX4C-infected mice. Treatment with F(ab')2 131-2G showed cytokine and chemokine levels similar to those for the CX4C viruses. The lower level of proinflammatory cytokines after RSV-CX4C mutated virus infection is consistent with fewer inflammatory cells in the lung. Since IL-13 has been
TABLE 3 Effect of CX4C mutation on pulmonary cytokine levels after RSV infection

<table>
<thead>
<tr>
<th>No. of days p.i.</th>
<th>Group</th>
<th>Level (pg/ml)*</th>
<th>IL-6</th>
<th>IFN-α</th>
<th>IFN-β</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>IL-4</th>
<th>IL-13</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Wt A2</td>
<td>109 ± 1</td>
<td>94 ± 1</td>
<td>28 ± 1</td>
<td>163 ± 1</td>
<td>86 ± 0</td>
<td>37 ± 1</td>
<td>56 ± 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A2-CX4C</td>
<td>106 ± 2</td>
<td>415 ± 3e</td>
<td>56 ± 1b</td>
<td>163 ± 2</td>
<td>104 ± 1</td>
<td>37 ± 1</td>
<td>41 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>r19F</td>
<td>112 ± 1</td>
<td>130 ± 4</td>
<td>22 ± 1</td>
<td>162 ± 1</td>
<td>91 ± 0</td>
<td>37 ± 1</td>
<td>38 ± 9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>r19F-CX4C</td>
<td>109 ± 2</td>
<td>492 ± 8b</td>
<td>53 ± 1b</td>
<td>163 ± 0</td>
<td>103 ± 1</td>
<td>37 ± 1</td>
<td>54 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mock</td>
<td>9 ± 0</td>
<td>22 ± 1</td>
<td>≤15.6</td>
<td>21 ± 1</td>
<td>32 ± 1</td>
<td>16 ± 1</td>
<td>62 ± 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wt A2 + F(ab’)2</td>
<td>109 ± 4</td>
<td>401 ± 4f</td>
<td>58 ± 2b</td>
<td>164 ± 1</td>
<td>81 ± 5</td>
<td>37 ± 1</td>
<td>53 ± 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>r19F-CX4C + F(ab’)2</td>
<td>110 ± 2</td>
<td>490 ± 5e</td>
<td>60 ± 7b</td>
<td>166 ± 3</td>
<td>86 ± 5</td>
<td>37 ± 1</td>
<td>58 ± 6</td>
<td></td>
</tr>
</tbody>
</table>

* Mice were challenged intranasally with the indicated virus or with virus plus MAb 131-2G (F(ab’)2 administered intraperitoneally 2 days before challenge or mock infected as described in Materials and Methods. The lung homogenates were prepared and tested by Luminox multiplex assay. The data are means ± SEM. Significant differences between wt and CX4C viruses or MAb-treated wt viruses are indicated. The results are representative of two independent experiments.

TABLE 4 Effect of CX4C mutation on antibody responses 75 days p.i.

<table>
<thead>
<tr>
<th>Group</th>
<th>Neutralization titer</th>
<th>IgG (total RSV)</th>
<th>IgG (G peptide) (aa 155–206)</th>
<th>IgG2a (total RSV)</th>
<th>IgG1 (total RSV)</th>
<th>IgG1/IgG2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt A2</td>
<td>63 ± 11</td>
<td>10,708 ± 1,980</td>
<td>834 ± 54</td>
<td>3,384 ± 412</td>
<td>3,387 ± 401</td>
<td>1.02 ± 0.2</td>
</tr>
<tr>
<td>A2-CX4C</td>
<td>110 ± 19b</td>
<td>14,284 ± 1712b</td>
<td>1,575 ± 34b</td>
<td>7,009 ± 1,102b</td>
<td>1,688 ± 581b</td>
<td>0.25 ± 0.1</td>
</tr>
<tr>
<td>Wt r19F</td>
<td>128 ± 11</td>
<td>10,428 ± 4,075</td>
<td>1,723 ± 28</td>
<td>3,304 ± 721</td>
<td>3,145 ± 792</td>
<td>1.20 ± 0.5</td>
</tr>
<tr>
<td>r19F-CX4C</td>
<td>188 ± 25b</td>
<td>16,740 ± 2691b</td>
<td>3,436 ± 21b</td>
<td>9,511 ± 1,265b</td>
<td>782 ± 176b</td>
<td>0.09 ± 0.03</td>
</tr>
</tbody>
</table>

*aBALB/c mice were treated or not with MAb F(ab’)2 131-2G 2 days before challenge with 10^6 TCID50 of wild-type A2 and r19F-CX3C and 2 × 10^4 TCID50 of A2 and r19F-CX4C or mock-infected tissue culture material (Mock). RSV- and RSV G peptide (aa 155 to 206)-specific antibody IgG and RSV-specific isotype IgG2a and IgG1 were measured by ELISA against RSV tissue culture lysate, as described in Materials and Methods. Data are titered calculated by dilution factors and end point calculations. The results are presented as averages of data from two separate experiments ± SEM from 5 mice per group. NA, not applicable.

bP < 0.05, a significant difference as determined by one-way ANOVA and post hoc Tukey’s HSD test, compared to A2 and r19F-CX3C with CX4C-mutated A2 and r19F.

associated with increased airway resistance and mucus production (19), the low levels in CX4C r19F-infected and wt A2-infected mice correlate with the lack of increased airway resistance and mucus production compared to wt r19F-infected mice. The higher level of Th1 cytokines during acute infection is consistent with the shift from a Th2 to a Th1 memory response with infection with the two CX4C viruses and is similar to that previously seen with MAb 131-2G prophylaxis of r19F-infected mice (31).

The CX4C mutation increased serum antibody levels and changed the isotype pattern from Th2 to Th1. At 75 days p.i., as previously shown for r19F infection, prophylaxis with F(ab’)2 forms of 131-2G increased the serum titer and shifted the antibody isotype titer pattern from Th2 to Th1 (31). The CX4C mutation had a similar effect, suggesting G binding to CX3C1 modulates the adaptive, in addition to the acute, response to infection. There was a 1.3- to 1.6-fold increase in IgG titers against virus lysate with the CX4C-infected mice and 131-2G F(ab’)2-treated, wt-infected mice (data for MAb F(ab’)2 131-2G-treated mice not shown) compared to untreated, wt-infected mice (Table 4). Mice infected with a CX4C virus also had higher titers of antibodies against a peptide from the central conserved region of G that includes the...
CX3C motif than mice infected with wt virus. The ratios of IgG1 to IgG2a isotype titers were 1.02 ± 0.24 and 1.20 ± 0.45 for the wt A2- and wt r19F-CX3C-infected mice and 0.25 ± 0.11 and 0.09 ± 0.03 for the CX4C A2- and CX4C r19F-infected and 0.20 ± 0.07 and 0.09 ± 0.01 for 131-2G F(ab')2-treated, wt-infected mice. Serum neutralizing antibody titers were 1.47- to 1.76-fold higher in mice infected with the CX4C viruses or in mice treated with 131-2G F(ab')2 and infected with wt viruses than in untreated, wt-infected mice.

To identify possible mechanisms for the enhanced antibody response with the CX4C mutation, we determined Tfh CD4 T cells (CXCR5+) and germinal center (GC) B cells (transcription factor Bcl-6 positive) at 75 days p.i. Tfh CD4 T cells guide antigen-stimulated B cells to the germinal center, and GC B cells give long-term antibody responses while non-GC B cells give short-term responses. As indicated in Fig. 4, mice infected with CX4C viruses showed increases in percentages of Tfh CD4 T cells and GC B cells compared to wt-infected mice. 131-2G prophylaxis had a similar effect [data for MAb F(ab')2 131-2G-treated mice not shown]. An increase in Tfh T cells and GC B cells suggests the CX4C viruses may induce a more durable antibody response.

The CX4C mutation shifted the T cell response from a Th2 to a Th1 pattern. Our previous studies showed that 131-2G prophylaxis of wt r19F-infected mice not only shifted the antibody isotope pattern from Th2 to Th1, but also shifted the memory T cell response from Th2 to Th1 (31). As shown above, CX4C-infected mice have a shift in the IgG isotype pattern from a Th2 to a Th1 bias at 75 days p.i. The memory T cell response was also shifted from Th2 to Th1 with both the CX4C mutation and prophylaxis with MAb 131-2G. The spleen cells from wt A2- and wt r19F-infected mice had a significantly (P ≤ 0.05) higher percentage of IL-4- and Gata-3-responding CD4 T cells and IL-4-responding CD8 T cells at day 75 p.i. than mock-challenged mice (Table 5 and Fig. 5). In contrast, CX4C A2- and CX4C r19F-infected mice or wt-infected mice given 131-2G prophylaxis infection had no significant increase in IL-4 or Gata-3. The CX4C-infected mice also had higher IFN-γ and T-bet responses than the wt-infected mice (Table 5 and Fig. 5). The Gata-3 and IL-4 responses are indicative of a Th2 response, and T-bet and IFN-γ are indicative of a Th1 response. Thus, the CX4C mutation was associated with a shift from a Th2 to a Th1 memory T cell response.

**DISCUSSION**

This study suggests the RSV G protein is likely important to the design of a live RSV vaccine. The G protein has usually been considered less important than the F protein in vaccine design because G vaccines induce lower titers of serum neutralizing antibodies and less protection from virus replication in animals and, with greater antigenic diversity, induce fewer cross strain neutralizing antibodies. We, however, hypothesized that G’s role in disease pathogenesis, not its ability to induce protection from virus replication, makes it an attractive target in vaccine design. The present study suggests this hypothesis is correct. We show that inserting an alanine into G’s CX3C chemokine motif gives a virus that, in mice, causes substantially less disease with infection and induces a more desirable immune response. If these findings in mice translate to humans, a live RSV vaccine with this mutation should be safer and more effective. The basis for these findings likely results from the CX4C mutation blocking G protein’s ability to bind to CX3CR1 on the cell surface. Since CX3CL1, or fractalkine, binding to CX3CR1 affects a range of immune responses, including leukocyte migration, Th2-type T cell responses, inflammation, cell survival, and adaptive immunity (32–37), it is not surprising that G protein binding to CX3CR1 has a substantial effect on RSV disease. CX3CL1, similar to RSV G, has a membrane form and a secreted form. CX3CL1 is expressed in a variety of cell types, including endothelial and epithelial cells, lymphocytes, neurons, microglial cells, and osteoblasts. The secreted form is chemotactic for immune cells, including monocytes, NK cells, T cells, and dendritic cells that express CX3CR1 on the cell surface. CX3CR1 has also been noted on B cells, neurons, microglia, smooth muscle cells, airway epithelial cells, and some tumor cells. The CX3CL1-CX3CR1 interaction has been implicated in inflammation and immune responses linked to
FIG 4 (A and B) Effect of CX4C mutation on the frequency of Tfh cells. Mice were challenged intranasally with the indicated virus or mock infected, as described in Materials and Methods. Spleen cells were harvested at day 75 p.i. and stimulated with RSV-specific CD4 G peptide (or albumin control peptide) for 6 h. Tfh cells were determined by staining with CD4, CXCR5, and Bcl-6 antibodies and flow cytometry. (A) Representative dot plots from flow cytometric analysis of CXCR5 and Bcl-6. (B) Percentages of CXCR5+ and Bcl-6+ CD4 T cells from corresponding dot plot quadrants. The values for control peptide stimulation (0.02% to 0.08%) were similar for all infection groups. The data are means and SEM. *, significant difference (P < 0.001) compared to untreated, CX3C-infected mice by one-way ANOVA and post hoc Tukey’s HSD test.
TABLE 5 Effect of the CX4C mutation on memory response by CD4 and CD8 splenocytes

<table>
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<tr>
<th>Group</th>
<th>CD4 Total</th>
<th>T-bet IFN-γ</th>
<th>Gata-3 IL-4</th>
<th>CD8 Total</th>
<th>T-bet IFN-γ</th>
<th>Gata-3 IL-4</th>
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<td>0 ± 0</td>
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</tr>
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</table>

*a*, wt A2; 2 A2-CX4C; 3, wt r19F; 4, r19F-CX4C; 5, wt A2 plus F(ab')2, 131-2G; 6, wt r19F plus F(ab')2, 131-2G; 7, mock infected.

Significant difference (p < 0.0001), as determined by one-way ANOVA and post hoc Tukey’s HSD test.
cardiovascular, neurologic, autoimmune, and pulmonary diseases, including asthma, and altered immunity with malignancies (32–37). These associations have included both increases and decreases in inflammation, depending on the context or environment of the interaction.

A potential role for the G protein-CX3CR1 interaction in RSV disease is indicated by studies showing the G protein's substantial influence on host responses to infection with RSV. Several studies have investigated the role of the G protein in RSV infection, with a focus on the CX3C motif. For example, mutations in the CX3C motif have been shown to affect the interaction between the RSV G protein and CX3CR1, leading to altered cellular responses.

**Figure 5** Representative dot plots from flow cytometric analysis of the effects of CX4C mutation on the frequencies of T-bet and IFN-γ or Gata-3 and IL-4 secretion in CD4 T cells. BALB/c mice were treated or not with MAb F(ab')2 131-2G 2 days before challenge with 10^6 TCID50 of wild-type A2 and r19F-CX3C and 2 × 10^6 TCID50 of A2 and r19F-CX4C or mock-infected tissue culture material (Mock). Spleen cells were harvested at day 75 p.i. and stimulated with RSV G protein-specific CD4 epitope peptide or ovalbumin (Ova) as a negative-control peptide.
and disease pathogenesis. In *in vitro* studies, the RSV G protein has been noted to stimulate AEC responses and to dampen macrophage and dendritic cell responses (38–48). In *vivo* in mice, RSV G protein has been associated with increased weight loss, numbers of pulmonary inflammatory cells, levels of pulmonary mucus and Th2 cytokines, airway obstruction, and enhanced respiratory disease in RSV-challenged, FI-RSV-vaccinated mice (18, 20, 21, 26, 31, 48–51). Studies in mice have also shown that it enhances cytotoxic T cell responses (44, 52), modifies adaptive immune responses (31), and, when given intravenously, suppresses respiratory rates (22).

We hypothesized that much of its effect on RSV disease is related to binding to CX3CR1. In earlier studies, we found that G binding to CX3CR1 led to a decrease in CX3CR1+/CD4+ T cell migration to the lungs of RSV-infected mice (26), enhanced pulmonary inflammation in FI-RSV-vaccinated mice challenged with RSV (20), and suppressed respiratory rates in mice given G intravenously (22). These effects were demonstrated through studies with anti-G MAbs, anti-CX3CR1 antibodies, and mutations to the CX3C motif. Polack et al. used anti-CX3CR1 antibodies to show that G binding to CX3CR1 is not responsible for G-induced suppression of IL-6 production in human monocytes (43).

In the present study, we extend disease likely linked to G binding to CX3CR1 by showing a decrease in disease when the CX3C motif in the wt virus is mutated to CX4C. The resulting G protein and virus do not bind to CX3CR1 (23, 24). We show in two strains of RSV that this mutation in the infecting virus markedly decreases weight loss and numbers of pulmonary inflammatory cells, most notably PMNs, in mice. Since one of these strains, r19F, induces mucus production and signs of airway obstruction in mice (19, 28), we were also able to show that the CX4C mutation decreases these two features of disease commonly seen with human infection (4). Thus, if this mutation has the same effect during human infection, it should decrease disease associated with replication of a live-attenuated vaccine. Since this decrease in disease is not dependent on a decrease in virus replication, the likely decrease in virus replication from CX4C not binding to CX3CR1, an important receptor for infection in primary human AECs, should further improve its safety profile in young children. Disease associated with replication has been a safety concern for live attenuated RSV vaccines (15).

Our data also show that the CX4C mutation may improve the efficacy of a live attenuated vaccine. In mice at day 75 p.i., the CX4C mutation is associated with a significant increase in RSV IgG and neutralizing antibodies and a shift in memory T cell responses from a Th2 bias to a Th1 bias. These results are similar to those previously described in mice given 131-2G prophylaxis prior to RSV r19F challenge (31). A higher titer of neutralizing antibodies is associated with protection from disease, and a Th1-biased memory response is the desired response for RSV vaccines (15). Thus, the CX4C mutation has the potential to also improve the quality and efficacy of the immune responses to a live attenuated RSV vaccine.

The effect of the CX4C mutation on the cytokine levels in lung homogenates during acute infection is consistent with the change in disease. The CX4C mutation and prophylaxis with MAb 131-2G affected the levels of all cytokines except IL-6. The increase in IFN-α, IFN-β, IFN-γ, and TNF-α and decrease in IL-4 and IL-13 are consistent with the shift from a Th2 to a Th1 response during acute infection, as well as later memory response. Interestingly, Polack et al. found that the RSV G protein’s role in increasing IL-6 production in human macrophages was not associated with CX3CR1 (43). Our findings are consistent with those with a cold-passaged virus, cp-52, which lacks the G protein, in cotton rats, nonhuman primates, and children (53). Cotton rats and nonhuman primates infected with cp-52 compared to the parent B1 virus have a >100-fold decrease in virus replication but only an ∼4-fold-lower titer of neutralizing and anti-F protein antibodies (54). Mice infected with cp-52 had less virus replication, lower levels of Th2 cytokines, and higher levels of Th1 cytokines in the lung than the parent B1 virus (49).

One consideration in interpreting the effect of the CX4C mutation on disease in mice is that less virus replication might account for the decrease in disease. When we
increased the virus inoculum for the CX4C viruses, virus replication in the lung increased, but this increase was not accompanied by any decrease in the effect of the mutation on disease. This suggests that the effect of the CX4C mutation on disease is not dependent on virus replication and that any decrease in virus replication would further attenuate the virus. Since CX3CR1 is an important receptor for infection of primary human AECs in vitro and presumably natural human infection, the CX4C virus should be further attenuated in humans by its inability to bind to CX3CR1. The fact that the CX4C mutation-associated decrease in disease is independent of virus replication was predicted by our earlier studies showing that the F(ab′)2 form of MAb 131-2G decreases disease by affecting virus replication (18, 19, 21).

Another concern is the relevance of these findings to infection in humans. Although this question will be answered only in clinical vaccine trials, in vitro studies suggest the findings are likely to be relevant to human infection. For example, in in vitro studies of human peripheral blood mononuclear cells and human AECs, we previously showed that the CX4C mutation affects immune responses to RSV. In this study, the CX4C mutation led to significant increases in levels of IFN-α and IFN-λ during infection of a human AEC line, an increase in IFN-α and TNF-α production by human plasmacytoid dendritic cells (pDCs) and monocytes, and an increase in IFN-γ production in effector/memory T cells (55). We also showed that the CX4C mutation affects many cytokines produced by RSV-infected primary human AECs (24). In this study, there was a significant increase IL-1RA, IL-15, and MCP-1 and a significant decrease in fractalkine, IL-8, RANTES, MIG, and MIP-1b that was likely not explained by lower levels of replication with the CX4C virus.

Though it is likely that blocking G binding to CX3CR1 explains the effect of the CX4C mutation on RSV disease and adaptive immune responses, it is possible that the mutation could also affect other sites on G that explain some of these effects. For example, sites near the CX3C motif might be affected by the CX4C mutation, including several sites implicated in Th2-type immune responses (56–58). Further, study is needed to define the mechanisms responsible for the various effects of this mutation on disease.

Thus, though we are not yet certain of the mechanisms, the likely benefit of the CX4C mutation in a live attenuated RSV vaccine is clear. Our data suggest it should make live attenuated RSV vaccines safer by decreasing disease with vaccination and more effective by inducing higher titers of antibodies and a Th1-biased memory response. Other studies suggest it will also decrease virus replication in the primary site for RSV infection in humans, AECs, which should further improve its safety. The CX4C mutation in a live attenuated RSV vaccine may be the missing piece in finally achieving a safe and effective live attenuated RSV vaccine.

**MATERIALS AND METHODS**

**Viruses.** The RSV A2 CX3C and A2 CX4C strains were developed as described previously (59). The RSV rA2 line 19F (r19F) CX3C and CX4C viruses were developed and provided by M. Moore with a previously described reverse genetics system (60). For both viruses, the wt strains have an intact CX3C motif (182CWAIAC187) in the G protein and are parental to the mutant strains (designated CX4C), which have an alanine (A186) insertion within the CX3C motif, i.e., 182CWAIAC187. All four virus stocks were prepared as previously described (19), and the G gene sequences were confirmed for each. Briefly, the viruses were inoculated onto subconfluent HEp-2 cells at a multiplicity of infection (MOI) of 0.01, incubated for 5 days at 37°C under 5% CO₂, frozen overnight at −80°C, thawed at 4°C, and centrifuged at 3,000 rpm for 5 min at 4°C to remove cellular debris, and the supernatants were pooled and purified by centrifugation on a sucrose cushion gradient (20% sucrose) at 16,000 × g for 4 h. The pellet was suspended in serum-free minimal essential medium (MEM), divided into aliquots, quick frozen in liquid nitrogen, and stored at −80°C. Virus infectivity titers were determined by a microinfectivity assay, with virus replication detected by EIA using a goat anti-RSV antibody (Millipore, Billerica, MA) to detect RSV antigens, as previously described (61). The infectivity titer was calculated using the Reed and Muench method.

**Monoclonal antibody preparation.** The anti-G protein monoclonal antibody 131-2G (62, 63) was purified from mouse ascitic fluid or hybridoma supernatant on G protein columns. The F(ab′)2 fragments were generated by pepsin digestion as previously described (19). The protein concentration was determined by a micro-bicinchoninic acid (BCA) protein assay (Pierce Protein Research Products, Rockford, IL) and endotoxin by Limulus amebocyte lysate chromogenic endpoint assay, according to the manufacturer’s instructions (Lonza, Atlanta, GA).
**Mice.** Mouse studies were performed according to a protocol approved by the Emory University (Atlanta, GA) Institutional Animal Care and Use Committee. Four- to 6-week-old, specific-pathogen-free female BALB/c mice (Charles River Laboratory, Wilmington, MA) were used in all experiments. The mice were housed in microisolator cages and fed sterilized water and food ad libitum. The studies were designed to evaluate the acute inflammatory response and disease and later adaptive immune response after primary RSV infection. The mice were infected intranasally with $10^6$ TCID$_{50}$ for all the viruses (study 1) or $1 \times 10^6$ TCID$_{50}$ for the wt viruses increased to $2.0 \times 10^6$ TCID$_{50}$ for the CX4C viruses (studies 2 and 3). The anti-RSV G MAb 131-2G (F(ab')$_2$) form-treated mice were given 300 $\mu$g of the MAb intraperitoneally 2 days before infection. The weight measurements, BAL fluid cell collection, and lung tissue harvesting were done on days 3, 5, and 8 p.i. Pulse oximetry measurements and Muc5AC detection were done 6, 8, and 10 days p.i. Spleen and serum samples were collected on day 75 p.i.

**Quantification of lung viral load.** The pulmonary viral load was assessed by measuring infectious virus in homogenized lung tissue. BeadBeater (Biospec Products, Bartlesville, OK) was used to homogenize the lungs as described previously (19). Briefly, lung tissue was homogenized in 2-ml deep 96-well plates (Axygen Scientific, Union City, CA) with a BeadBeater (Biospec Products, Bartlesville, OK), 1 ml of zirconium beads (Biospec Products, Bartlesville, OK), and 500 $\mu$l of autoclaved, sterile basal MEM. A small piece of the left lung lobe was weighed and placed into a well with beads, and 400 $\mu$l of basal MEM was added and homogenized with 12 cycles of 1 min of shaking followed by 1 min of cooling on ice and centrifugation at 2,000 rpm for 5 min at 4°C; the supernatants were harvested and aliquots were stored at $-80^\circ$C for future studies. Virus infectivity titers were determined by the microtiter infectivity assay described above.

**Quantification of viral RNA levels by RSV real-time PCR.** Total RNA was extracted from homogenized lung tissue using a Qiagen total-RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions and stored at $-80^\circ$C. Quantitative real-time PCR was performed by using an AgPath-ID one-step reverse transcription (RT)-PCR kit (Applied Biosystems, Foster City, CA) and a Stratagene 3000 detection system (Agilent Technologies, Santa Clara, CA). Thermal-cycling conditions included 10 min at 45°C, followed by 45 cycles of 15 s at 95°C and 1 min at 55°C. The primers and probes for the RSV matrix (M) gene were as follows: forward primer, 5'-GGG AAA TAT GGA AAT GCT GAA AAA-3'; reverse primer, 5'-TCT TTT TCT AGA TTG TAY TGA ACA G-3'; probe, 5'-6-carboxyfluorescein (FAM)-TGT CCG TCT TCT ACG CCC TCG TC-black hole quencher 1 (BHQ-1)-3'. Serial dilutions of known numbers of plaque-forming units (PFU) of RSV RNA were used to obtain a standard curve for quantitative real-time PCR. Threshold cycles ($C_T$) for each sample were converted to PFU equivalents/ml using the standard curve. Assays were performed for three different sets of mice.

**BAL specimens.** BAL specimens were collected from anesthetized and exsanguinated, euthanized mice by lavaging the lungs 3 times using 1 ml sterile 1× PBS each time. The BAL fluid cells were stained for extracellular markers using a microculture-staining protocol described by Tripp et al. (49) for anti-CD3 (17A2) (T cells), anti-CD4 (GK.1.5) (T cells), anti-CD8 (53-6.7) (T cells), anti-CD45R/B220 (RA3-6B2) (B cells), anti-CD11b (M1/70) (macrophages, dendritic cells, and monocytes), anti-mouse Ly6G/Gr-1 (RB6-8C5) (PMNs), anti-mouse CD49b/Integrin alpha 2 (DX5) (NK and NK T cells), and mouse isotype antibody controls (all from eBiosciences, San Diego, CA). The distributions and patterns of cell surface markers were determined for 30,000 lymphocyte-gated events analyzed on a BD LSRII flow cytometer (BD Biosciences, Mountain View, CA), and data were analyzed using FlowJo software (TreeStar, Ashland, OR).

**Pulse oximetry.** We used PP to assess lung function. PP results from the difference in cardiac output between inspiration and expiration, indicates the effort or force required for breathing, and provides an indicator of airway resistance. We used a rodent pulse oximeter (MouseOx; Starr Life Sciences Corp., Oakmont, PA) to measure PP, as described previously (30). The pulse oximeter determines changes in red absorbance, which correlates with blood volume in peripheral vessels (vessel distention) as a proxy for changes in cardiac output with respiration and, thus, PP. Mice (5 at a time) were anesthetized using an isoflurane (2.5%)-oxygen (2 liters/min) mixture provided by an anesthesia machine (Vet Equip, Pleasanton, CA) with isoflurane (2.5%)-oxygen (2 liters/min) mixture provided by an anesthesia machine (Vet Equip, Pleasanton, CA) in a Plexiglas box. Individual mice were removed from the box and placed on their backs with continued isoflurane-oxygen nose cone anesthesia; their feet were restrained; the MouseOx sensor was applied to their thighs; and $O_2$ saturation, heart rate, pulse rate, pulse distention, and breath distention were recorded approximately every 0.1 s (MouseOx software, version 4.0) for 1 to 5 min. Only time points at which all parameters were successfully measured were included in analyses, with 30 s to 2 min of measurement per mouse. The average recorded breath-associated change in vessel wall distention was used as the value for each mouse. Data were analyzed using Microsoft Excel.

**Determination of Muc5AC protein expression.** The Muc5AC concentration in lung homogenates was assayed by enzyme-linked immunosorbent assay (ELISA) for mouse mucin 5 subtype AC (USCN Life Science Inc., Wuhan, China), according to the manufacturer’s protocol, and the total protein concentration was determined with a BCA protein assay kit (Thermo Scientific, Rockford, IL). The concentration of Muc5AC in the sample was determined using an external standard curve according to the manufacturer’s instructions. Data are presented as the fold Muc5AC increase in tissue compared to mock-infected mice.

**Histopathology.** Lungs were fixed in 10% formalin for 1 h, followed by 70% ethanol, and embedded in paraffin blocks. Five-micrometer sections of lung tissue were deparaffinized in xylene, rehydrated with ethanol, and stained with PAS (Sigma-Aldrich, St. Louis, MO) as described previously. The PAS-stained slides were digitally scanned using a Hamamatsu Nanozoomer 2.0HT slide scanner (Meyer Instruments, Houston, TX) with a 20× objective using ImageJ software. Fifteen to 20 fields ($\times$20 magnification) were examined per tissue section.
Multiplex cytokine analysis. Cytokine levels were determined in the supernatants of centrifuged lung homogenates with a mouse cytokine Bio-Plex Pro Mouse cytokine 8-plex Luminex assay (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. IL-13 cytokine levels were determined in the supernatants of centrifuged lung homogenates with a mouse IL-13 ELISA kit (Abcam, Cambridge, MA). Concentrations were standardized to milligrams of protein determined by a BCA assay (Thermo, Rockford, IL). The concentration of each cytokine was determined by comparison to the standard curve according to the manufacturer’s instructions. The threshold of detection was 15.6 pg/ml for IFN-γ and IFN-β and 5 pg/ml for the rest of the cytokines used in the study.

Antibody responses. Neutralizing and binding antibodies were determined at 75 ± 3 days after infection for the various treatment groups. Anti-RSV IgG binding antibodies were determined by EIA as described previously (31). The cutoff value (absorbance of 0.252) was chosen as the mean optical density (OD) ± 3 standard deviations for the 1:500 dilution of sera from mock-infected mice for various IgG ELISAs. The highest dilution with absorbance above the cutoff value was estimated from a standard curve generated from the manufacturer-provided standards for the various IgG ELISAs using linear regression. Anti-RSV IgG binding antibodies were determined by EIA against RSV G peptide (amino acids [aa] 153 to 206) incubated at 4°C overnight. After blocking (2% dry milk) for 1 h at 37°C, 100 μl of 2-fold serial dilutions of serum samples in PBS containing 0.05% Tween 20 (PBST) was added to appropriate wells and incubated for 2 h at 37°C. Next, the plates were washed with PBST and then incubated for 1 h at 37°C with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (diluted 1:5,000) antibody (Thermo Scientific, Waltham, MA). After a second washing, the color was developed with TMB Microwell peroxidase substrate (KPL, Gaithersburg, MD) as indicated by the manufacturer. The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution, and absorbance was measured at a wavelength of 450 nm.

The RSV neutralizing antibody titer was determined using 2-fold serially diluted heat-inactivated (56°C for 30 min) sera beginning at a 1:20 dilution. Serum specimens (75 μl) were mixed with 100 TCID₉₀ of RSV (25 μl) in MEM-5% FBS, added to wells of a 96-well microtiter plate with HEp-2 cells (1.5 × 10⁶/well), and incubated for 3 days at 37°C. The plates were washed with PBS, and the cells were fixed with 80% acetone-20% PBS, followed by blocking with PBS-5% dry milk blocking buffer. RSV replication was detected with goat anti-RSV antibody (Millipore, Billerica, MA) and HRP-conjugated donkey anti-goat antibody (Jackson ImmunoResearch, West Grove, PA) as noted above for determining the infectivity titers of the virus preparations (19). Neutralization titers are expressed as the reciprocal of the serum dilution giving a 50% reduction in the number of RSV-positive wells.

Preparation of splenocytes. Mice were euthanized at day 75 postinfection, and spleens were removed and placed into complete RPMI supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific, Pittsburgh, PA), 2 mM l-glutamine (Gibco, Grand Island, NY), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 1 mM HEPES (Thermo Scientific, Waltham, MA), and 5,000 units/ml penicillin-streptomycin (Gibco, Grand Island, NY) and 0.5 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO) and minced and ground through a sterile steel mesh to obtain a single-cell suspension. The resulting cell suspension was treated with red blood cell (RBC) lysis buffer (BD Biosciences, Mountain View, CA) solution and Perm/Wash buffer (BD Biosciences, Mountain View, CA). Before intracellular staining, the cells were fixed and permeabilized with CytoFix/CytoPerm (BD Biosciences, Mountain View, CA) and data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Stimulation of splenocytes with RSV CD4- and CD8-specific peptides. The spleen cells (10⁶ cells/well) were treated with brefeldin A (BD GolgiPlug, San Jose, CA) for the last 4 h, incubated with FcR-blocking antibody (anti-CD16/CD32), and then stained for surface markers (data not shown). The stimulated spleen cells (10⁶ cells/well) were incubated with a mixture of TMB and ovalbumin control peptide is at aa 323 to 339. The stimulated spleen cells (10⁶ cells/well) were treated with brefeldin A (BD GolgiPlug, San Jose, CA) for the last 4 h, incubated with FcR-blocking antibody (anti-CD16/CD32), and then stained for surface markers (data not shown). Intracellular cytokines were detected using designated antibodies (Table 2). The distributions and patterns of cell surface markers were determined for 100,000 lymphocyte-gated events analyzed on a BD LSRII flow cytometer (BD Biosciences, Mountain View, CA), and data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Statistical analyses. Unless otherwise indicated, groups were compared by one-way analysis of variance (ANOVA) and post hoc Tukey’s honestly significant difference (HSD) test (P ≤ 0.05). A P value of ≤0.05 was considered statistically significant. All statistical analyses were performed using the statistical package R (R Developmental Core Team, 2012). The data are shown as means and standard errors of the mean (SEM).

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