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The Attachment (G) Glycoprotein of Respiratory Syncytial Virus Contains a Single Immunodominant Epitope That Elicits Both Th1 and Th2 CD4+ T Cell Responses

Steven M. Varga,* Erika L. Wissinger,† and Thomas J. Braciale2*†

BALB/c mice immunized with a vaccinia virus expressing the attachment (G) glycoprotein of respiratory syncytial virus (RSV) develop a virus-specific CD4+ T cell response that consists of a mixture of Th1 and Th2 CD4+ T cells following intranasal infection with live RSV. Recent work has shown that both Th1 and Th2 CD4+ T cells are elicited to a single region comprising aa 183–197 of the G protein. To more precisely define the CD4+ T cell epitope(s) contained within this region, we created a panel of amino- and carboxyl-terminal truncated as well as single alanine-substituted peptides spanning aa 183–197. These peptides were used to examine the ex vivo cytokine response of memory effector CD4+ T cells infiltrating the lungs of G-primed RSV-infected mice. Analysis of lung-derived memory effector CD4+ T cells using intracellular cytokine staining and/or ELISA of effector T cell culture supernatants revealed a single I-Eα-restricted CD4+ T cell epitope with a core sequence mapping to aa 185–193. In addition, we examined the T cell repertoire of the RSV G-peptide-specific CD4+ T cells and show that the CD4+ T cells directed to this single immunodominant G epitope use a restricted range of TCR Vβ genes and predominantly express Vβ14 TCR. The Journal of Immunology, 2000, 165:6487–6495.

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3 Abbreviations used in this paper: RSV, respiratory syncytial virus; FI, formalin-inactivated; vv, vaccinia virus; vvG, vv expressing the G glycoprotein of RSV; vB-gal, recombinant vv expressing β-galactosidase; p.i., postinfection; HEL, hen egg lysozyme.
memory CD4+ T cells. Thus, these observations implicate RSV G-specific memory CD4+ T cells as likely mediators of the immunopathology observed in FI-RSV-vaccinated individuals following natural RSV infection.

Despite the importance of RSV G-specific memory CD4+ T cells in mediating experimental RSV vaccine-enhanced disease, very little quantitative information currently exists concerning this population at the site of RSV infection, the lung. In addition, the fine specificity of the RSV G-specific memory CD4+ T cell population is presently undefined. To address both of these important issues, we sought to better characterize the RSV G protein-specific memory CD4+ T cell response in terms of both its magnitude and its fine specificity. Intracellular cytokine analysis of lung-derived memory effector CD4+ T cells from vvG-sensitized mice infected with RSV revealed that >40% of the CD4+ T cells produced IFN-γ or TNF-α following stimulation with a peptide spanning the immunodominant region of the G protein of RSV. Using a series of analogue peptides truncated at either the amino- or carboxyl-terminal end of the immunodominant region of RSV G183–197, we demonstrate that there is a single I-Eα-restricted CD4+ T cell epitope with a core 9-aa sequence of 185–193 that is recognized by both Th1 and Th2 memory effector CD4+ T cells. Strikingly, we also report that the RSV G-specific memory effector CD4+ T cells that have entered the lung following pulmonary RSV infection predominantly express Vδ14 TCR. These results demonstrate for the first time that RSV G-specific effector CD4+ T cells are directed to a single immunodominant I-Eα-restricted G epitope and employ TCR with a highly conserved TCR Vβ-chain.

Materials and Methods

Mice
Female BALB/cAnNTac (H-2b) mice were purchased from Taconic Farms (Germantown, NY) and used at 8–12 wk of age for all experiments. Mice were housed in a pathogen-free environment.

Viruses and infection of mice
Recombinant vv expressing the attachment (vvG) glycoprotein of RSV was a gift from J. L. Beeler (Food and Drug Administration, National Institutes of Health, Bethesda, MD). The generation and characterization of this virus have been described previously (25). Recombinant vv expressing β-galactosidase (vvβ-gal) was used as a negative control. The A2 strain of RSV was a gift from P. L. Collins (National Institute of Allergy and Infectious Diseases, National Institutes of Health). RSV was grown in Hep-2 cells (American Type Culture Collection, Manassas, VA). Groups of four mice were infected with 3 × 10^6 PFU of vvG or vvβ-gal in a 10-μl volume by scarification with a 25-gauge needle at the base of the tail. Three weeks after priming, mice were lightly anesthetized with metapine (Malincrodt Veterinary, Mundelein, IL) and intranasally inoculated with 2 × 10^6 PFU of RSV in 50 μl. At various times postinfection (p.i.), control and infected mice were sacrificed by cervical dislocation.

Peptides
The following peptides were used: hen egg lysozyme (HEL)108–119 (27) in addition to the RSV G protein-derived peptides shown in Tables II and III. All peptides were synthesized by the University of Virginia Biomedical Research Facility using standard techniques of F-moc chemistry.

Preparation of lung-derived lymphocytes
The lung vascular bed was flushed via the right ventricle with 4–5 ml of PBS containing 10 U/ml heparin (Sigma, St. Louis, MO) to remove any blood. The lungs were removed aseptically; carefully dissected away from the heart, the thymus, and the bronchial lymph nodes; and placed into RPMI medium (Life Technologies Laboratories, Grand Island, NY) supplemented with 10% FCS (Atlanta Biologicals, Norcross, GA), 10 U/ml penicillin G, 10 μg/ml streptomycin sulfate, 2 mM L-glutamine, 5 × 10^-5 M 2-ME, 1 mM sodium pyruvate (Life Technologies), 0.1 mM nonessential amino acids (Life Technologies), and 10 mM HEPES (Life Technologies). The tissue was finely minced and pressed through a wire screen. Particulate matter was removed by quick centrifugation at 10 000 rpm. Lung mononuclear cells were obtained by preparing single-cell suspensions from a pool of four mice per group. Cells were counted and resuspended at the given cell concentrations for the appropriate in vitro assay.

Flow cytometric analysis and intracellular cytokine staining
For multicolor FACS analysis, approximately 2 × 10^6 cells were stained with PE-conjugated anti-CD4 (clone RM4-5) and one of the following FITC-conjugated mAb: anti-LFA-1 (clone 2D7), anti-CD25 (clone 7D4), anti-CD44 (clone IM7), anti-CD62L (clone MEL-14), anti-CD69 (clone H1.23F), rat IgM (clone R4-22), rat IgG2a (clone R35-95), rat IgG2b (clone A95-1), hamster IgG (clone A19-3) in PBS supplemented with 2% FCS and 0.02% NaN3, (staining buffer). All mAb used in FACS staining were purchased from PharMingen (San Diego, CA). Stained cells were fixed and E lysed with FACS lysing solution (Becton Dickinson, San Jose, CA) washed, resuspended in staining buffer, and analyzed in two-color mode using a Becton Dickinson FACScalibur flow cytometer (Mountain View, CA). For intracellular cytokine staining approximately 2 × 10^6 cells were stimulated for 5 h in the presence of 1 μg/ml of brefeldin A (Sigma) in the presence (1 μM) or the absence of one of the RSV G protein-derived peptides or (control I-Eα-restricted peptide derived from HEL and/or mouse hepatitis virus). In some experiments, an anti-I-Aα (clone 34-5-3) or anti-I-Aβ/I-Eα (clone M5/114,15.2) mAb was added to the culture for the duration of the stimulation. The cells were subsequently washed in staining buffer, blocked with purified anti-FeRγRIII/II mAb (clone 2.4G2, Phar-Mingen), and stained with CD4-allophycocyanin. After fixation with FACS lysing solution, the cells were washed in permeabilization buffer (staining buffer containing 0.5% saponin; Sigma) and stained with PE-conjugated anti-cytokine mAb or an isotype-matched control mAb (all from PharMingen). The following mAb were used: anti-IL-2 (clone JES6- 5H4), anti-IL-3 (clone MP2-8F8), anti-IL-4, (clone BV4D-11D11), anti-IL-5 (clone TRFK5), anti-IL-6 (clone MP5-20F3), anti-IL-10 (clone JES55- 16E3), anti-GM-CSF (clone MP1-22E9), anti-IFN-γ (clone XMG1.2), anti-TNF-α, rat IgG1 (clone R3-34), rat IgG2a (clone R35-95), and rat IgG2b (clone A95-1). Control cells known to express the cytokine of interest were used in all experiments as positive controls (Mick-1, Mick-2, and Mick-3; PharMingen). Background staining with the appropriate isotype-matched control mAb was subtracted from each individual. Single-color controls were used in all multiparameter FACS analyses for electronic compensation, and between 60,000 and 80,000 events were acquired from each preparation. Lymphocyte populations were first gated based on forward scatter and 90° side scatter and then analyzed using CellQuest software (Becton Dickinson).

Restimulation in vitro and cytokine ELISA
Cultures of 2 × 10^6 lung mononuclear cells pooled from four mice per group were incubated in the presence or the absence of one of the RSV G-derived peptides and incubated at 37°C in 1-ml total volume of RPMI-10% FCS supplemented as described above in 24-well plates (Costar, Cambridge, MA). Supernatants were harvested at 24, 48, and 72 h and stored at −80°C before being assayed for cytokines by ELISA according to the manufacturer’s instructions. Briefly, Immulon 2 plates (Dynatech, Chantilly, VA) were coated overnight at 4°C with anti-cytokine mAb, washed the next day with PBS-0.05% Tween-20 (Sigma), and blocked with PBS-5% (v/v) FCS. Samples were added at 50 μl/well, and a standard curve was constructed for each plate by using eight 2-fold dilutions of recombinant cytokine. Following the addition of the samples, the plates were incubated overnight at 4°C before the addition of biotinylated anti-cytokine mAb. Avidin-peroxidase (Sigma) followed by the peroxidase substrate, 3,3’,5,5’-tetramethyl-benzidine dihydrochloride (Sigma) were used to develop the ELISA. The reaction was stopped with 2 N H2SO4. Plates were read at 450 nm using an EL 340 plate reader and analyzed using DeltaSoft 3 software (both from Bio-Tek Instruments, Winooski, VT). The following pairs of mAb were used: anti-IL-2, JES6-1A12 and biotinylated JES6-5H4; anti-IL-4, 11B11 and biotinylated BVD6-24G2; anti-IL-5, TRFK5 and biotinylated TRFK4; anti-IL-6, MP5-20F3 and biotinylated MP5-32C11; anti-IL-10, JES5-2A5 and biotinylated SXC-1; anti-IFN-γ, R4-6A2 and biotinylated XMG1.2 (all from PharMingen). Peroxidase and biotinylated anti-IL-13 mAb were purchased from R&D Systems (Minneapolis, MN). Recombinant murine IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ (PharMingen), and IL-13 (R&D Systems) were used as standards. The limit of detection was...
Enzyme-linked immunospot assays

Nitrocellulose-based 96-well microtiter plates (Millititer HA, Millipore, Bedford, MA) were coated overnight at 4°C with 5 µg/ml of either anti-IL-5 (clone TRFK5, PharMingen) or anti-IFN-γ (clone R4-6A2, PharMingen) mAb diluted in PBS, washed the next day with PBS, and blocked with RPMI-10% FCS. After washing with PBS, lung mononuclear cells pooled from four mice per group were added to the wells (1 × 10^6 to 7.8 × 10^5 cells/well as responders with 2 × 10^6 peptide-pulsed irradiated (2000 rad) splenocytes as stimulators) in RPMI 1640–10% FCS in a total volume of 200 µl/well. Following the addition of the samples, the plates were incubated overnight at 37°C for 48 h, washed with PBS-0.05% Tween-20 (Sigma) before the addition of biotinylated anti-cytokine mAb (anti-IL-5 clone TRFK4 and anti-IFN-γ clone XMG1.2, both from PharMingen), followed by avidin-peroxidase (1/400 dilution; Sigma). Spots were visualized by developing with the substrate 3-amino-9-ethylcarbazole (Sigma). All assays were performed in triplicate, and mean number of cytokine-secreting cells was calculated from the triplicate assays.

Results

Phenotype and quantitation of the lung-derived CD4^+ T cells following RSV infection of vvG-primed BALB/c mice

Examination of activation marker expression on CD4^+ T cells infiltrating the lungs of vvG-primed mice challenged with RSV revealed that most of the CD4^+ T cells in the lungs express a LFA-1^highCD44^highCD62L^low activated phenotype by days 5–6 p.i. (Fig. 1). Expression of the early activation markers CD25 and CD69 peaked earlier on days 3–4 p.i. In contrast, mice that had been vvG-primed but not infected with RSV did not have CD4^+ T cells capable of producing the cytokines IL-3 and GM-CSF on day 5 p.i. This was also the peak in the total number of CD4^+ IFN-γ^+ cells in the lung. The mean numbers of total CD4^+ IFN-γ^+ cells from four mice per group were as follows: day 3, 2.1 × 10^6; day 4, 1.5 × 10^6; day 5, 7.4 × 10^5; day 6, 5.1 × 10^5; day 7, 3.5 × 10^5; and day 9, 1.9 × 10^5. We also observed a peak in the percentage of CD4^+ T cells capable of producing the cytokines IL-3 and GM-CSF on day 5 p.i. In addition, by day 6 p.i., >40% of the lung CD4^+ T cells produced another Th1-associated cytokine, IL-2 following peptide stimulation. We were unable to detect CD4^+ T cells secreting the Th2-associated cytokines IL-4 and IL-5 in response to the G183–197 peptide by intracellular cytokine staining (data not shown). However, we were able to detect a slight (<1.5%) increase in the percentage of IL-10-producing CD4^+ T cells. Interestingly, we did observe a more significant 10–15% increase in the percentage of IL-6-producing CD4^+ T cells that was not dependent on peptide stimulation (data not shown). The percentage of cytokine-secreting CD4^+ T cells in response to peptide was low (<3%) in vvG-primed mice without a challenge infection with RSV or in mice undergoing a primary RSV infection (vvB-gal-primed, RSV day 5 p.i.). Thus, these results demonstrate that RSV infection of vvG-primed mice results in a substantial influx of memory effector CD4^+ T cells into the lung that predominantly express an activated cell phenotype and that as many as 40% are capable of producing the effector cytokines IFN-γ and/or TNF-α following stimulation with the G183–197 peptide.

We have recently shown that CD4^+ T cells producing both Th1 and Th2 cytokines are elicited to a single RSV G-derived peptide encompassing aa 183–197 (24). To quantitate the frequency of RSV G peptide-specific CD4^+ T cells in vvG-primed mice challenged with RSV, we examined cytokine production at the single-cell level using intracellular cytokine staining of lung mononuclear cells stimulated with the RSV G183–197 peptide. Table I demonstrates that a high frequency of cytokine-producing CD4^+ T cells are elicited to this single peptide. By day 5 p.i., >40% of the CD4^+ T cells made IFN-γ or TNF-α following peptide stimulation. The peak in the percentage of CD4^+ IFN-γ^+ cells was on day 5 p.i. This was also the peak in the total number of CD4^+ IFN-γ^+ cells in the lung. Thus, as expected, the majority of the CD4^+ T cells fitting the criteria for a memory cell phenotype on day 5 p.i. compared with the accelerated memory response that occurred by day 3 p.i. in vvG-primed mice. Thus, as expected, the majority of the CD4^+ T cells fitting the criteria for a memory cell phenotype on day 5 p.i. compared with the accelerated memory response that occurred by day 3 p.i. in vvG-primed mice. Lung cells were stained with anti-CD4 and anti-LFA-1, anti-CD25, anti-CD44, anti-CD62L, or anti-CD69 mAb. A CD4 gate was applied, and the histograms represent the cell surface expression of the indicated mAb on the gated CD4^+ T cells. The dotted line represents staining with an isotype-matched control mAb. The number in each histogram represents the mean fluorescence intensity for the indicated mAb. Data shown are representative of two separate experiments with a pool of two mice per experiment.

FIGURE 1. Expression of activation markers on lung-derived memory effector CD4^+ T cells during RSV infection of vvG-primed mice. Lung cells were stained with anti-CD4 and anti-LFA-1, anti-CD25, anti-CD44, anti-CD62L, or anti-CD69 mAb. A CD4 gate was applied, and the histograms represent the cell surface expression of the indicated mAb on the gated CD4^+ T cells. The dotted line represents staining with an isotype-matched control mAb. The number in each histogram represents the mean fluorescence intensity for the indicated mAb. Data shown are representative of two separate experiments with a pool of two mice per experiment.
Identification of the minimal core sequence recognized by RSV G peptide-specific memory effector CD4\(^+\) T cells

The above experiments demonstrate the presence of memory effector CD4\(^+\) T cells at a high frequency in the lungs of vvG-primed mice after intranasal RSV challenge using intracellular cytokine staining to detect IFN-\(\gamma\) production following stimulation with a peptide representing aa 183–197 of the G glycoprotein of RSV. Therefore, we chose to use this sensitive single-cell assay to more finely map the CD4\(^+\) T cell epitope(s) contained within the 183–197 region of RSV G. To verify that we were using an optimal peptide concentration to stimulate cytokine production by lung-derived effector CD4\(^+\) T cells in the intracellular IFN-\(\gamma\) assay, we performed a peptide dose-response curve using the RSV G\(_{183–197}\) peptide. Fig. 2 shows that the maximal percentage of IFN-\(\gamma\)-producing CD4\(^+\) T cells is elicited at a peptide concentration of 1 \(\mu\)M and higher. In contrast, no increase in the percentage of IFN-\(\gamma\)-producing CD4\(^+\) T cells was observed following stimulation with a control I-E\(\alpha\) binding peptide derived from HEL.

The 183–197 peptide contains three overlapping sequences that fit the I-E\(\alpha\) motif (28). To more clearly define the CD4\(^+\) T cell epitope(s) within this region, a series of truncated synthetic peptides of different lengths spanning aa 183–197 were synthesized (Table II), and tested for their capacity to stimulate the production of IFN-\(\gamma\) by lung-derived memory effector CD4\(^+\) T cells from vvG-primed mice 5 days after intranasal challenge infection with RSV. Fig. 3 demonstrates that peptides with successive aa deletions from the N-terminus are stimulatory until the isoleucine at aa position 185 is deleted. Consecutive deletions from the C-terminus revealed that loss of the lysine at position 193 ablated the ability to stimulate IFN-\(\gamma\) production by these memory effector CD4\(^+\) T cells. Fig. 3 also demonstrates that T cell responsiveness was not critically dependent on peptide length, since both the non-stimulatory peptide 183–192 and the stimulatory 184–193 peptide are 10 aa residues in length. These results suggest that there is a single CD4\(^+\) T cell epitope within RSV G requiring a minimal 9-aa core sequence spanning residues 185–193 (see underlined amino acids in Table II). Furthermore, to obtain a significant response, at least two additional amino acids at the N- and C-termini of the core 9 aa sequence are needed (Fig. 3, see peptide 183–195). However, since we could not detect Th2 cytokine production using the intracellular cytokine stain following peptide stimulation of lung effector CD4\(^+\) T cells ex vivo, we used the enzyme-linked immunospot assay to determine the relative frequency of IFN-\(\gamma\)- and IL-5-secreting cells specific to the RSV G\(_{183–195}\) peptide. The

Table I. Percentage of lung CD4\(^+\) cytokine\(^+\) T cells during RSV infection

<table>
<thead>
<tr>
<th>Day p.i.</th>
<th>IL-2</th>
<th>IL-3</th>
<th>IFN-(\gamma)</th>
<th>TNF-(\alpha)</th>
<th>GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>vvG</td>
<td>1.5</td>
<td>0.1</td>
<td>0</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>vvB-gal+RSV Day 5</td>
<td>1.9</td>
<td>0.1</td>
<td>1.3</td>
<td>2.5</td>
<td>0.4</td>
</tr>
<tr>
<td>vvG+RSV Day 3</td>
<td>1.2</td>
<td>0.3</td>
<td>4.2</td>
<td>2.6</td>
<td>1.2</td>
</tr>
<tr>
<td>vvG+RSV Day 4</td>
<td>6.8</td>
<td>1.7</td>
<td>16.6</td>
<td>13.9</td>
<td>9.3</td>
</tr>
<tr>
<td>vvG+RSV Day 5</td>
<td>10.2</td>
<td>2.8</td>
<td>42.4</td>
<td>41.4</td>
<td>23.7</td>
</tr>
<tr>
<td>vvG+RSV Day 6</td>
<td>21.3</td>
<td>1.3</td>
<td>39.3</td>
<td>43.1</td>
<td>15.7</td>
</tr>
<tr>
<td>vvG+RSV Day 7</td>
<td>18.5</td>
<td>0.8</td>
<td>34.9</td>
<td>32.4</td>
<td>9.9</td>
</tr>
<tr>
<td>vvG+RSV Day 9</td>
<td>9.3</td>
<td>0.3</td>
<td>25.4</td>
<td>29.5</td>
<td>4.0</td>
</tr>
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</table>

Table II. Sequences of RSV G protein-derived peptides used in this study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV 183–197</td>
<td>WAICKRIPNKKPGKK</td>
</tr>
<tr>
<td>RSV 183–195</td>
<td>WAICKRIPNKKPG</td>
</tr>
<tr>
<td>RSV 183–193</td>
<td>WAICKRIPNKK</td>
</tr>
<tr>
<td>RSV 183–192</td>
<td>WAICKRIPNKK</td>
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<tr>
<td>RSV 184–193</td>
<td>ACKRIPNKK</td>
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<tr>
<td>RSV 184–192</td>
<td>ACKRIPNKK</td>
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<tr>
<td>RSV 185–197</td>
<td>ICKRIPNKKPGKK</td>
</tr>
<tr>
<td>RSV 186–197</td>
<td>CRIPNKKPGKK</td>
</tr>
<tr>
<td>RSV 187–197</td>
<td>KRRIPNKKPGKK</td>
</tr>
</tbody>
</table>

FIGURE 2. Dose response of lung-derived memory effector CD4\(^+\) T cells to the RSV G\(_{183–197}\) peptide. Lung mononuclear cells from a pool of four vvG-primed mice 5 days after RSV infection were stimulated with the given doses of either a control peptide (HEL\(_{108–119}\)) or RSV G\(_{183–197}\) in the presence of brefeldin A for 5 h and stained for intracellular IFN-\(\gamma\) as described in Materials and Methods. One representative of three separate experiments is shown.

FIGURE 3. Identification of the N- and C-terminal ends of the CD4\(^+\) T cell epitope contained within RSV G\(_{183–197}\). Lung mononuclear cells from a pool of four vvG-primed mice 5 days after RSV infection were stimulated with 1 \(\mu\)M of the indicated G protein-derived peptide in the presence of Brefeldin A for 5 h and stained for intracellular IFN-\(\gamma\) as described in Materials and Methods. The means ± SD from three separate experiments with a pool of four mice per experiment are shown (n = 12/group).
The above results suggest that there is a single I-E<sup>d</sup>-restricted CD4<sup>+</sup> T cell epitope within RSV G<sub>183–197</sub> with a 9-aa core sequence of 185–193. To further analyze the specificity of the RSV-specific memory effector CD4<sup>+</sup> T cells, 12 variant peptides were synthesized in which individual residues within the optimal 183–195 sequence (see Fig. 3) were replaced by alanine to determine the amino acids critical for T cell stimulation (Table III). These alanine-substituted peptides were used to stimulate IFN-γ production by RSV G-specific memory effector lung CD4<sup>+</sup> T cells using the intracellular IFN-γ assay. Fig. 6 shows that the substitution of alanine for isoleucine at position 185 or arginine at position 188 greatly abolished T cell recognition and IFN-γ production, consistent with the predicted roles of these two aa as I-E<sup>d</sup> anchor residues. Alanine substitution at the two other predicted I-E<sup>d</sup> anchor residues (i.e., aa 190 and 193) also results in significant inhibition of IFN-γ production. However, of particular note was the finding that alanine substitution at position 187, 189, 191, or 192 dramatically diminished T cell recognition and IFN-γ production. This result was striking, since these residues probably represent TCR contact residues. While substitution at one of several different TCR contact residues might be expected to affect the recognition of a single CD4<sup>+</sup> T cell clone and its progeny, a heterogeneous population of effector CD4<sup>+</sup> T cells derived from different clones would be expected to employ a diverse array of TCR with differing sensitivities to substitution at TCR contact residues and therefore might not be expected to be similarly affected by substitution in individual TCR contact residues. For this reason it is noteworthy that substitution at positions outside the minimal core sequence (i.e., position 183, 194, or 195) did not significantly alter recognition by the memory effector CD4<sup>+</sup> T cells, although in some instances the composition of a residue flanking the core epitope has been reported to affect CD4<sup>+</sup> T cell recognition (29–31). Similar results were observed using ELISA of cell culture supernatants for the Th2 cytokines IL-5, IL-6, IL-10, and IL-13 following in vitro stimulation with the same panel of alanine-substituted peptides (data not shown).

TCR V<sub>B</sub> repertoire of RSV G peptide-specific memory effector CD4<sup>+</sup> T cells

Although residues 187, 189, 191, and 192 could represent secondary MHC anchors contributing to the stability of the peptide/MHC complex, the finding that a high proportion of the memory effector T cells secreting Th2 cytokines also recognize the RSV G<sub>185–193</sub> epitope. Cultures of lung mononuclear cells from a pool of four vvG-primed mice 5 days after RSV infection were stimulated with 1 μM of the indicated G protein-derived peptide as described in Materials and Methods, and culture supernatants were removed at the various times indicated and assessed for cytokine production by ELISA. One representative of three separate experiments is shown.

FIGURE 4. Memory effector CD4<sup>+</sup> T cells secreting TCR V<sub>B</sub> repertoire of RSV G peptide-specific memory effector CD4<sup>+</sup> T cells
CD4+ T cells was equally sensitive to alanine substitutions at G\textsubscript{185–193} core residues other than the four predicted I-E\textsuperscript{d} anchor sites suggested that these substitutions may be disrupting potential TCR contact residues necessary for the response of an oligoclonal T cell population. To examine this possibility, we challenged vvG-primed mice with RSV and stained lung-derived effector CD4+ T cells following stimulation with the RSV G\textsubscript{183–197} peptide for intracellular IFN-\gamma and TCR V\beta usage using a panel of V\beta-specific mAb. The TCR V\beta repertoires of the IFN-\gamma\textsuperscript{+} and IFN-\gamma\textsuperscript{−} CD4+ T cell populations are shown in Fig. 7. Surprisingly, among the activated CD4+ T cells infiltrating the lungs of vvG-primed, RSV-infected mice, both the IFN-\gamma\textsuperscript{+} and IFN-\gamma\textsuperscript{−} subsets of CD4+ T cells are highly enriched for V\beta14+ cells. Since we have demonstrated above that both Th1 and Th2 effector CD4+ T cells are elicited to the same I-E\textsuperscript{d}-restricted epitope, it is likely that the IFN-\gamma\textsuperscript{−} V\beta14+ population reflects Th2 CD4+ T cells that are unable to detect in the intracellular cytokine assay. Thus, RSV infection of vvG-primed BALB/c mice results in the generation and recruitment of a RSV-specific CD4+ T cell population that predominantly expresses a V\beta14 TCR.

**Discussion**

The BALB/c mouse model of RSV infection has proven to be a valuable tool in dissecting the mechanisms of vaccine-enhanced disease. Although it has been known for some time that CD4+ T cells directed against the RSV G protein may play an important role in mediating vaccine-enhanced immunopathology (32, 33), the specificity and character of the G protein-specific CD4+ T cell response in lungs have remained poorly defined. In this report we set out to examine in detail the effector CD4+ T cell response to the G protein of RSV, and as a result of our analyses we made three important observations. First, RSV infection of vvG-primed mice results in a high frequency of MHC class II-restricted virus-specific memory effector CD4+ T cells that enter the site of infection, the lung. These G-specific memory effector CD4+ T cells are directed to a single I-E\textsuperscript{d}-restricted immunodominant epitope with a 9-aa core sequence at G\textsubscript{185–193}.

Second, both Th1 and Th2 memory effector CD4+ T cells are directed against the same immunodominant RSV G epitope. Third, the RSV G-specific memory effector CD4+ T cells express a highly restricted V\beta TCR repertoire.

The study of virus-specific T cell populations has been greatly aided by recent technological advances, including the development of tetrameric complexes of MHC glycoprotein loaded with peptides and the staining for intracellular cytokines in cells stimulated with peptides (34, 35), both of which have allowed the direct visualization and quantitation of Ag-specific T cell populations (36). For the most part, these techniques have been used to analyze MHC class I-restricted CD8+ T cells in several different viral and tumor model systems (34, 35, 37–44). These studies have all shown that the magnitude of the Ag-specific CD8+ T cell response is greater than previously recognized. Interestingly, recent results from our laboratory using the influenza model suggest that the use of tetramers to quantitate the frequency of virus-specific CD8+ T cell populations may still result in an underestimate of the total virus-specific CD8+ T cell frequency (45). Nevertheless, the advent of the tetramer technology and the intracellular cytokine stain

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**Table III. Sequences of RSV alanine-substituted peptides spanning RSV G\textsubscript{183–195}**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>W 183 A</td>
<td>AAIKRPNKKG</td>
</tr>
<tr>
<td>I 185 A</td>
<td>WAACKRPNNKLG</td>
</tr>
<tr>
<td>C 186 A</td>
<td>WAIARPNKKG</td>
</tr>
<tr>
<td>K 187 A</td>
<td>WAICARPNNKKG</td>
</tr>
<tr>
<td>R 188 A</td>
<td>WAICAIPIPNKKG</td>
</tr>
<tr>
<td>I 189 A</td>
<td>WAIKRAIPNNKKG</td>
</tr>
<tr>
<td>P 190 A</td>
<td>WAICKRIANWKKG</td>
</tr>
<tr>
<td>N 191 A</td>
<td>WAICKRIPIPKKG</td>
</tr>
<tr>
<td>K 192 A</td>
<td>WAICKRPNNKKG</td>
</tr>
<tr>
<td>K 193 A</td>
<td>WAICKRPNNKKG</td>
</tr>
<tr>
<td>P 194 A</td>
<td>WAICKRIPNKKG</td>
</tr>
<tr>
<td>G 195 A</td>
<td>WAICKRIPNKKP</td>
</tr>
</tbody>
</table>
have led to a better understanding of the kinetics, phenotype, and frequency of virus-specific CD8\(^+\) T cell populations.

In contrast to the great number of studies examining CD8\(^+\) T cell responses using these new techniques, much less is known about virus-specific CD4\(^+\) T cells. For technical reasons, it is far easier to create stable peptide-loaded MHC class I tetramers than MHC class II tetramers. As was the case with virus-specific CD8\(^+\) T cells (35, 46), recent work performed in the lymphocytic choriomeningitis virus system has demonstrated that the virus-specific CD4\(^+\) T cell frequency is much greater than previously thought (47). In the murine RSV model, there is little information on the frequency of cytokine-secreting virus-specific CD4\(^+\) T cells during either an acute RSV infection or a memory response in RSV Ag-primed mice undergoing challenge infection with RSV. One study examined the frequency of MHC class II-restricted CTL by limiting dilution analysis following acute RSV infection, reporting frequencies of <1% for virus-specific T cells (48).

Here we show that by 5 days after RSV infection of vvG-primed mice, lung mononuclear cells from a pool of four vvG-primed mice 5 days after RSV infection were stimulated with 1 \(\mu\)M RSV G\(_{183-197}\) peptide in the presence of brefeldin A for 5 h and stained with Abs to CD4, with a panel of TCR \(\beta\)-specific Abs, and with Abs to IFN-\(\gamma\) as described in Materials and Methods. A lymphocyte gate was applied, and cells were subsequently gated on either CD4\(^+\)IFN-\(\gamma\)^- or CD4\(^+\)IFN-\(\gamma\)^+ cells. The data are the percentage of gated CD4\(^+\)IFN-\(\gamma\)^- or CD4\(^+\)IFN-\(\gamma\)^+ cells that express the given \(\beta\)-TCR. One representative of three separate experiments is shown.

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** TCR repertoire of lung-derived memory effector CD4\(^+\) T cells during RSV infection of vvG-primed mice. Lung mononuclear cells from a pool of four vvG-primed mice 5 days after RSV infection were stimulated with 1 \(\mu\)M RSV G\(_{183-197}\) peptide in the presence of brefeldin A for 5 h and stained with Abs to CD4, with a panel of TCR \(\beta\)-specific Abs, and with Abs to IFN-\(\gamma\) as described in Materials and Methods. A lymphocyte gate was applied, and cells were subsequently gated on either CD4\(^+\)IFN-\(\gamma\)^- or CD4\(^+\)IFN-\(\gamma\)^+ cells. The data are the percentage of gated CD4\(^+\)IFN-\(\gamma\)^- or CD4\(^+\)IFN-\(\gamma\)^+ cells that express the given \(\beta\)-TCR. One representative of three separate experiments is shown.
for the Th1 and Th2 cytokine response in vGp-primed mice undergoing challenge RSV infection.

In the present study we demonstrate that most of the RSV G peptide-specific CD4+ T cells that can be detected using the intracellular IFN-γ assay express Vβ14 TCR. In addition, we show that approximately 30% of the activated CD4+ T cells that fail to make IFN-γ following peptide stimulation also express Vβ14 TCR. Many of these IFN-γ− cells may represent virus-specific Th2 CD4+ T cells that are not easily detected using the intracellular cytokine stain. This seems likely, since we have shown here that Th2 cytokines are produced in short term in vitro cultures using the CD4+ epitope we have defined in this report (see Fig. 4).

In addition, in vitro activation of lung-derived CD4+ T cells isolated from vGp-primed mice with a Vβ14 TCR-specific mAb induces the production of both Th1 and Th2 cytokines (S. M. Varga and T. J. Braciale, unpublished observations). Thus, it is possible that CD4+ T cells expressing a Vβ14 TCR directed against the immunodominant epitope we have defined in this study may differentiate into either Th1 or Th2 CD4+ T cells in vivo.

A previous study examining CD4+ T cells specific to an I-E\(^d\)-restricted nucleoprotein epitope of mouse hepatitis virus demonstrated that a single epitope could elicit both Th1 and Th2 CD4+ T cells (27).

However, the mouse hepatitis virus nucleoprotein-specific Th1 and Th2 CD4+ T cells expressed TCR with distinctly different Vβ gene usage (27). At present we do not know whether the restricted Vβ usage of the G\(_{185-193}\) specific CD4+ T cells reflects the selection of Vβ14+ T cells with a unique TCR β-chain, i.e., a single unique complementarity-determining region 3 or TCR β-chains with limited complementarity-determining region 3 length and sequence diversity. Also, since TCR Vβ usage by the memory effectors CD4+ T cells has not been analyzed as yet, we cannot determine whether these responding lung effector CD4+ T cells represent a single clone or a panel of memory CD4+ T cell clones with limited TCR diversity. Experiments are in progress to address each of these issues.

A great deal of evidence indicates that MHC class II-restricted CD4+ T cells play an important, yet often unappreciated role in antiviral immunity (2, 62–69). During RSV infection, CD4+ T cells can contribute to the antiviral immune response by providing help for B cells to make antiviral Abs as well as secreting cytokines such as IFN-γ. However, during a RSV memory response, G protein-specific CD4+ T cell responses can also be detrimental to the host by mediating immunopathology and enhancing disease (32, 33, 70). In this report we show that G protein-specific memory CD4+ T cells are directed against a single I-E\(^d\)-restricted immunodominant epitope. This epitope elicits a memory response yielding both Th1 and Th2 effector CD4+ T cells. While we cannot formally exclude the possibility that the same effector CD4+ T cell in the lungs is producing both Th1 and Th2 cytokines, our evidence to date supports the view that Th1 and Th2 cytokines are produced by different effector CD4+ T cells. Both these effector cell types may contribute to the enhanced pathology observed in immune individuals. In addition, we have demonstrated for the first time that the majority of RSV G protein-specific CD4+ T cells express a restricted Vβ14 TCR. Such a focused T cell response eliciting both Th1 and Th2 effector cells and capable of mediating enhanced disease could be related to the finding that only a subset of the children who received the FI-RSV vaccine in the 1960s went on to exhibit enhanced disease upon RSV infection (11–15).

Our future studies in the murine RSV model will examine whether the CD4+ T cells that exclusively express Vβ14 TCR are capable of mediating RSV vaccine-enhanced disease. Our results suggest that a comparable analysis of CD4+ T cell specificity, effector activity, and TCR usage may be warranted in the human.

Acknowledgments

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