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Mucosal Delivery of a Respiratory Syncytial Virus CTL Peptide with Enterotoxin-Based Adjuvants Elicits Protective, Immunopathogenic, and Immunoregulatory Antiviral CD8⁺ T Cell Responses

Cameron P. Simmons,* Tracy Hussell,† Tim Sparer, † Gerhard Walzl, † Peter Openshaw, † and Gordon Dougan* 

In an effort to develop a safe and effective vaccine against respiratory syncytial virus (RSV), we used Escherichia coli heat-labile toxin (LT), and LTK63 (an LT mutant devoid of ADP-ribose transferase activity) to elicit murine CD8⁺ CTL responses to an intranasally codelivered CTL peptide from the second matrix protein (M2) of RSV. M282–90-specific CD8⁺ T cells were detected by IFN-γ enzyme-linked immunospot and ⁵¹Cr release assay in local and systemic lymph nodes, and their induction was dependent on the use of a mucosal adjuvant. CTL elicited by peptide immunization afforded protection against RSV challenge, but also enhanced weight loss. CTL-mediated viral clearance was not dependent on IFN-γ since depletion using specific mAb during RSV challenge did not affect cellular recruitment or viral clearance. Depletion of IFN-γ did, however, reduce the concentration of TNF detected in lung homogenates of challenged mice and largely prevented the weight loss associated with CTL-mediated viral clearance. Mice primed with the attachment glycoprotein (G) develop lung eosinophilia after intranasal RSV challenge. Mucosal peptide vaccination reduced pulmonary eosinophilia in mice subsequently immunized with G and challenged with RSV. These studies emphasize that protective and immunoregulatory CD8⁺ T cell responses can be mucosally elicited using enterotoxin-based mucosal adjuvants but that resistance against viral infection may be accompanied by enhanced disease. The Journal of Immunology, 2001, 166: 1106–1113.

Respiratory syncytial virus (RSV), a paramyxovirus, causes relatively mild common cold symptoms in immunocompetent adults but can cause severe lower respiratory tract disease in infants and is now regarded as a significant cause of morbidity and mortality in the elderly. RSV bronchiolitis is the most common single cause of hospitalization of infants in developed countries. Furthermore, RSV lower respiratory tract illnesses in early childhood are an independent risk factor for the subsequent development of wheezing up to age 11 years (1). There is no effective human vaccine with which to prevent RSV infection. Indeed, parenterally administered, formalin-inactivated RSV causes a dramatic increase in the severity of naturally acquired disease (2, 3). Children with fatal vaccine-enhanced RSV disease developed pulmonary and blood eosinophilia. The exacerbation of disease in humans immunized with formalin-inactivated RSV has been modeled in murine studies using recombinant vaccinia virus (rVV) engineered to express the RSV attachment protein G (rVV-G). BALB/c mice primed with rVV-G and challenged with RSV are more resistant to infection, but develop enhanced weight loss and an immunopathology characterized by pulmonary eosinophilia (4, 5). In mice, CD4⁺ T cells secreting type 2 cytokines are necessary for this response since their depletion eliminates eosinophilia (6). Eosinophilia in mice can be prevented if RSV-specific CD8⁺ T cells are activated before or at the same time as CD4⁺ T cells (7). Although BALB/c mice develop lung eosinophilia after G protein priming, normal C57BL/6 mice do not. Removal of functional CD8⁺ T cells using specific Ab or TAP-1, β₂-microglobulin, or CD8 gene knockout mice renders C57BL/6 mice susceptible to eosinophilia (8). CD8⁺ T cells are therefore critical regulators of Th2-driven eosinophilic lung disease and it has been proposed that vaccines which drive RSV-specific CD8⁺ T cells may prevent immunopathology caused by CD4⁺ Th2 cells (8).

CD8⁺ T cells are associated with immunity to RSV. RSV-specific CD8⁺ T cells can be detected in the blood of previously infected adults in whom CTL responses are associated with reduced clinical symptoms (9). CD8⁺ T cells also mediate resistance to RSV in murine models. BALB/c mice primed with rVV expressing the RSV second matrix protein (M2) mount a CD8⁺ T cell response and develop protective immunity (10). In BALB/c mice, the H-2Kb-restricted peptide corresponding to residues 82–90 of the M2 protein, which is conserved in subgroups A and B RSVs, is necessary and sufficient to induce protective CD8⁺ T cell responses (11, 12). Successful RSV vaccination strategies designed to exploit the protective role of M282–90-specific CD8⁺ T cells include DNA vaccines (13) and intranasal (i.n.) administration of a chimeric M2 peptide (14).
Although strong RSV-specific CTL can mediate resistance to infection, viral clearance can also be associated with acute and sometimes fatal pulmonary disease (15).

Intranasal vaccination against RSV represents an attractive approach since host immune cells are likely to be primed in relevant lymphoid tissues. CD8+ T cell responses are, however, typically difficult to induce by simple introduction of nonliving Ag into the respiratory tract. We have previously shown that i.n. coadministration of peptide or protein Ags with bacterial enterotoxins like Escherichia coli heat-labile (LT) enterotoxin augments Ag-specific CD8+ CTL responses (16). Induction of CD8+ CTL responses using LT as an adjuvant is independent of functional TNF (C.P.S. and G.D., unpublished observations), IL-12, IFN-γ, and CD4+ T cell help (16). The adjuvant activity of enterotoxins for CTL induction extends to nontoxic mutant derivatives of LT containing attenuating single amino acid substitutions in their enzymatically active A subunits (e.g., LTK63). LTK63 is molecularly defined (17), has well described mucosal adjuvant properties (18–20), and may represent a safe adjuvant for use in humans.

We have employed a mucosal vaccination strategy that exploits the adjuvant properties of LT and LTK63 to elicit CD8+ CTL responses to the dominant CTL epitope in the M2 protein of RSV. The CTL elicited by immunization-mediated resistance to RSV challenge via a mechanism independent of IFN-γ. Furthermore, mucosally elicited M2_{82–90}-specific CD8+ T cells reduced pulmonary eosinophilia in mice subsequently primed with the G protein and challenged with RSV. These are the first studies describing induction and characterization of protective CTL responses against an RSV Ag by combining mucosal immunization with defined mucosal adjuvants.

Materials and Methods

Mice and virus stocks

BALB/c mice were purchased from Harlan Olac (Bicester, U.K.) and used when 8 to 10 wk old. RSV and recombinant vaccinia virus expressing the rVV-G. matrix protein (M2), or control β-galactosidase (rVV-β-gal) were grown in HEp-2 cells and assayed for infectivity as previously described (21). All stocks were free of Mycoplasma infection (determined by DNA hybridization; Gen-Probe, San Diego, CA).

Ags and Abs

A peptide with sequence SYGSINNI, corresponding to residues 82–90 of the RSV M2 protein, was purchased from Zinsser Analytic (Maidenhead, Berkshire, U.K.) and was >95% pure. Wild-type porcine LT and LTK65 were kindly provided by M. Pizza and R. Rappuoli (Chiron Vaccines, Siena, Italy). The neutralizing anti-IFN-γ Ab from clone XMG1.2 was purified from ascites fluid.

Immunizations and challenge

For i.n. immunization, groups of mice were lightly anesthetized and 30 μl of Ag in PBS was applied to the nasal nares on days 0 and 14. In control groups, mice were scarified once on the rump on day 0 with 3 × 10^6 PFU rVV-M2 or rVV-β-gal in a final volume of 10 μl. Three to four mice from each group were killed on day 28 when superficial cervical lymph nodes and spleens were removed for CTL and enzyme-linked immunosoprot (ELISPOT) assays. The remaining mice were challenged i.n. with 3 × 10^6 human RSV (A2 strain, 50 μl). On selected days postchallenge, four to five mice were killed by injection of 3 mg pentobarbital.

Cytokine depletion

Depletion of IFN-γ was performed by i.p. injection of 0.25 mg rat anti-mouse IFN-γ Ab (clone XMG1.2) starting 2 days before RSV challenge and then every other day for 7 days. Control mice received an equivalent amount of an irrelevant, isotype-matched mAb on the same days.

Cell recovery

Bronchoalveolar lavage (BAL) was performed on selected days postchallenge by methods described previously (8). Briefly, the lungs of each mouse were inflated six times with 1 ml of 12 mM lidocaine in DMEM. A total of 100 μl of this BAL fluid from each mouse was retained for cytokin analysis and the rest was immediately diluted into ice-cold RPMI 1640 containing 10% FCS (Sigma, St. Louis, MO), 2 mMl/l L-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin (complete RPMI, cRPMI). Cells were harvested by centrifugation and resuspended at 10^5 cells/ml for flow cytometric analysis. The BAL supernatant was stored at −70°C for subsequent analysis of cytokine content.

Flow cytometric analysis of intracellular and cell surface Ags

Cells were stained with rat anti-mouse fluorochrome-conjugated Abs to CD4 (QR), CD8 (PE), and CD45RB (FITC) (PharMingen, Oxfordshire, U.K.) for 30 min on ice, washed, and then fixed for 20 min at room temperature with 2% formaldehyde. All samples were analyzed on a Coulter Epics Elite flow cytometer (Coulter, Palo Alto, CA) collecting data on at least 40,000 lymphocytes.

Enumeration of eosinophils

Eosinophils were enumerated by microscopic examination of Giemsa-stained cytospin centrifuge preparations. At least 300 cells/sample were examined.

Lung virus titer

RSV titers were assessed in lung homogenates on day 2, 4, or 7 after virus challenge as previously described (22). Briefly, lungs were removed from four to five mice per group and homogenized. After centrifugation at 7000 rpm for 1 min, 100 μl of supernatant was removed for cytokin analysis. The remaining supernatant was titrated in doubling dilutions on 60–80% confluent HEp-2 cell monolayers in 96-well flat-bottom plates. Twenty-four hours later, monolayers were washed and incubated with peroxidase-conjugated goat anti-RSV Ab (Biogenesis, Poole, U.K.). Infected cells were detected using 3-aminio-9-ethylcarbazole, infectious units being enumerated by light microscopy.

ELISPOT for murine cytokines

The ELISPOT assays were performed as previously described (23) and all Abs were purchased from PharMingen. Briefly, nitrocellulose-based 96-well flat-bottom plates (Multiscreen-HA; Millipore, Hertfordshire, U.K.) were coated overnight at 4°C with 50 μl/well of either anti-IFN-γ (4 μg/ml; R46A2) or anti-IL-5 (4 μg/ml; TRFK5) Ab diluted in carbonate buffer (pH 9.6). After washing three times with filtered PBS, all wells were blocked with 200 μl of CRPMI for 2–3 h at 37°C. Following removal of the blocking media, 3-fold serial dilutions of spleen or lymph node cells (mesiastinal or superficial cervical) from individual mice were added to the wells in duplicate (maximum 5 × 10^5 cells/well in 200 μl of medium) and incubated for 20 h at 37°C in 5% CO2 in the presence of 1 μM M2_{82–90} peptide. In some experiments, cells were also incubated in the presence of 1 μM of the H-2K^d-binding peptide corresponding to residues 91–99 of listeriolysin O from Listeria monocytogenes. Similarly, in some experiments, cells were incubated in the presence of 50 μl of culture supernatant from hybridoma cultures of either GK1.5 (anti-mouse CD4) or YTS169 (anti-mouse CD8). Cells were removed by washing three times with PBS, followed by an additional three times with PBS/Tween 20 (0.05% v/v), then 50 μl of the biotinylated anti-IFN-γ (XMG1.2) or anti-IL-5 (TRFK4) Abs (1 μg/ml in filtered PBS/Tween 20) was added to each well for 2 h. After washing plates five times with filtered PBS/Tween 20, a 1/1000 dilution of extravidin-alkaline phosphatase (Sigma) was added to all wells for 1–2 h at room temperature. Finally, after washing three times with PBS/Tween 20 and once with PBS alone, a solution of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (fast 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Sigma) was added as substrate. Spots representing single IFN-γ- or IL-5-producing cells were counted using a dissecting microscope. The number of peptide-specific spot-forming cells (SFCs) was determined by subtracting the number of spots obtained with cells stimulated with media from those stimulated with peptide.

Cytokine ELISA

Capture ELISAs for the detection of TNF were performed using Maxisorp 96-well plates (Nunc, Roskilde, Denmark). An OptEIA sandwich ELISA (PharMingen) was used to detect TNF according to the manufacturer’s instructions. Ab pairs for TNF were G281-2626 (capture) and MP6-XT3 (detection). Bound Abs were detected using streptavidin-HRP followed by tetramethylbenzidine and hydrogen peroxide. Optical densities were read at 450 nm. The concentration of cytokine in each sample was calculated from a standard curve generated with recombinant cytokine. The detection limit for TNF was 25 pg/ml.
Spleens and superficial cervical lymph nodes were aseptically removed and single-cell suspensions were prepared by passing organs from individual mice through 100-μm nylon sieves (Marathon Laboratories, London, U.K.). After lysis of splenic erythrocytes with Tris-ammonium chloride, a total of 3.5 × 10^7 leukocytes from the spleen were cultured for 6 days in upright T25 tissue culture flasks in 10 ml of cRPMI in the presence of 3 × 10^6 RSV-infected P815 cells. P815 cells were infected with RSV A2 at a multiplicity of infection of 10:1 for 2 h at 37°C and then washed three times with cRPMI before addition to splenocytes. Graded numbers of effector cells, consisting of cells that remained viable after the 6-day culture period, were harvested and cocultured in triplicate with 10,000 Na^51 CrO_4-labeled P815 cells or P815 cells pulsed with 1 μM M282–90. In some experiments, effector cells were also cultured with P815 cells pulsed with the H-2Kd-binding peptide residues 91–99 of listeriolysin O from L. monocytogenes. Effector cells were cocultured with target cells for 4–5 h at the indicated E:T ratios in 200 μl of cRPMI. A total of 100 μl of culture supernatant was then removed into 100 μl of OptiPhase Hisafe scintillation mixture (Fisher, Leicestershire, U.K.) and the amount of Na^51 Cr O_4 was measured using a 1450 Microbeta liquid scintillation counter (Wallac, Milford, Conn.). The percentage of killing was calculated using the following formula: ( [experimental release – spontaneous release] / [maximum release – spontaneous release] ) × 100, where spontaneous release represents the counts obtained when the target cells were incubated in culture media without effectors and maximum release represents the counts obtained when the target cells were lysed with 1% Triton X-100. Each experiment was repeated at least twice.

Statistical analysis

Mean IFN-γ ELISPOT numbers were compared using the nonparametric Mann-Whitney U test. A p value of <0.05 was regarded as significant.

Results

The mucosal adjuvants LT and LTK63 augment CD8^+ CTL responses to the dominant CTL epitope from the M2 protein of RSV

We have previously shown that enterotoxin-based mucosal adjuvants can augment CTL responses to i.n. coadministered peptide (16). To assess the immunogenicity of M282–90, BALB/c mice were immunized i.n. on day 0 and 14 with 20 μg of M282–90 alone or 20 μg of M282–90 coadministered with 1 μg of LT or 5 μg of LTK63. In a previous study, coadministration of 5 μg of LTK63 with CTL peptides was necessary for effective CTL induction (16). To compare the magnitude of the response elicited by mucosal immunization with an established potent method of eliciting M2-specific CTL, separate groups of mice were immunized with 3 × 10^6 PFU of rVV expressing the M2 protein or an irrelevant Ag, β-gal. Coadministration of M282–90 in the presence of either LT or LTK63 elicited splenic T cells which produced IFN-γ in an ELISPOT assay upon secondary restimulation with the M282–90 peptide (Fig. 1A). The responses in immunized mice were specific for the M282–90 peptide as cells did not respond when stimulated with a H-2Kd-binding peptide from L. monocytogenes (data not shown). Addition of an anti-CD8 mAb, but not an anti-CD4 mAb, to ELISPOT wells largely abrogated spot formation, indicating that the responding cells were CD8^+ T cells (data not shown). The number of splenic peptide-specific IFN-γ SFCs elicited by i.n. coadministration of peptide with adjuvant was similar regardless of whether LT or LTK63 was used, but was significantly greater than the response measured in mice immunized with toxin alone or peptide alone. The number of M282–90-specific IFN-γ SFCs elicited by parenteral vaccination with rVV-M2 exceeded the number obtained by mucosal immunization in the spleen (p = 0.002) (Fig. 1A), but not significantly in the superficial cervical lymph node (Fig. 1B). To examine the longevity of the mucosally induced T cell response, IFN-γ SFCs were measured 103 days after vaccination. The M282–90-specific CD8^+ T cell responses elicited by two mucosal immunizations remained detectable, albeit at a reduced frequency, 103 days after the initial immunization (Fig. 1C).

FIGURE 1. LT and LTK63 can adjuvant long-lived M282–90–specific T cell responses after i.n. immunization. Mice were immunized i.n. on days 0 and 14 with 20 μg of M282–90 admixed with either 1 μg of LT or 5 μg of LTK63. Control mice received toxin alone or M282–90 alone. Mice immunized with rVV-M2 or rVV-β-gal were scarified once on day 7 with 3 × 10^6 PFU of virus. The data depict the number of M282–90–specific IFN-γ SFCs per million splenocytes obtained from individual immunized mice on day 28 (A). Additionally, a symbol depicting the mean and SD of the group is shown next to the relevant individual points. There were significantly fewer M282–90–specific IFN-γ SFCs in LT/M282–90 or LTK63/M282–90 immunized mice compared with mice immunized with rVV-M2 (p = 0.002 for both groups), but significantly more SFCs compared with control immunized mice (*, p = 0.002 for LT vs LT/M282–90; *, p = 0.01 for LTK63 vs LTK63/M282–90). B, Mucosal and parenteral immunization elicits M282–90–specific T cell responses in superficial cervical lymph nodes which drain the upper respiratory tract. The data depict the mean number (plus SD) of M282–90–specific IFN-γ SFCs per 10^6 lymph node cells obtained from individual immunized mice (n = 3) on day 28. There were significantly more M282–90–specific IFN-γ SFCs in rVV-M2 and LTK63/M282–90–immunized mice compared with control immunized mice (**, p < 0.001 for rVV-M2 vs M282–90; **, p < 0.001 for LTK63/M282–90 vs M282–90). C, Mucosally elicited M282–90–specific T cell responses are long-lived. The data depict the mean number (plus SD) of M282–90–specific IFN-γ SFCs per million splenocytes obtained from immunized mice (n = 4) on day 103. There were significantly more M282–90–specific IFN-γ SFCs in LT/M282–90 and LTK63/M282–90–immunized mice compared with control immunized mice (**, p < 0.005 for LT/M282–90 vs M282–90; *, p < 0.05 for LTK63/M282–90 vs M282–90).

In ^51Cr release assays using restimulated splenocytes from immunized mice, M282–90–specific CD8^+ T cells were cytolytic as shown by efficient killing of M282–90 peptide-pulsed P815 cells, but not unpulsed cells (Fig. 2). These data show that M2-specific
CD8\(^+\) T cells are induced, cytolytic, and long-lived when LT or LTK63 are used as adjuvants.

**Mucosally elicited CD8\(^+\) CTLs mediate resistance to RSV challenge**

Since LT/LTK63 were able to adjuvant M2-specific CTL, we evaluated whether these CTL were protective against RSV challenge. Mice immunized as previously described were i.n. challenged with RSV A2 to determine whether mucosally elicited CD8\(^+\) T cells accelerate the natural rate of viral clearance, which normally occurs by day 7 postchallenge. On day 2 post-RSV challenge, the viral load in lung tissue was similar (between 10\(^1\) and 10\(^2\) PFU/lung) in all mice irrespective of the immunization regimen used. On day 4 however, mice immunized with rVV-M2 had a significantly reduced viral burden compared with mice receiving rVV expressing \(\beta\)-gal (p < 0.001). Mucosally elicited CTL also accelerated RSV clearance. Mice i.n. immunized with the M2\(_{82-90}\) peptide along with LT or LTK63 had significantly lower viral loads compared with mice that received peptide alone or adjuvant alone (Fig. 3A).

Flow cytometric analysis of cells recovered from BAL was performed to determine whether resistance to RSV infection in immunized mice correlated with a lymphocyte infiltrate into the lung. Mice i.n. immunized with M2\(_{82-90}\) plus LT, or mice immunized with rVV-M2, had significantly more CD8\(^+\) T cells with an activated/memory phenotype (CD45RB\(^{hi}\)) recovered from BAL on day 4 post-RSV challenge compared with mice immunized with either peptide alone or toxin alone (Fig. 3B). The number of activated/memory CD4\(^+\) T cells recovered from BAL was statistically similar in all groups. Additionally, in some experiments, individual CTL-primed mice lost weight during the course of viral infection (data not shown).

**Resistance to RSV challenge in peptide-immunized mice is independent of IFN-\(\gamma\)**

mAb depletion of IFN-\(\gamma\) was performed to assess the role of this cytokine in resistance to RSV infection and vaccine-enhanced weight loss in mice primed mucosally with M2\(_{82-90}\). Mice were i.n. immunized with M2\(_{82-90}\) plus LTK63, M2\(_{82-90}\) alone, or LTK63 alone on days 0 and 14 and challenged with RSV A2 on day 28. Just before, and during the challenge infection, half the mice in each group were administered an anti-IFN-\(\gamma\) mAb. The remaining mice received an isotype control mAb. Mice i.n. immunized with M2\(_{82-90}\) plus LTK63 and treated with the control mAb had significantly lower RSV lung titers on day 4 postchallenge compared with control animals (Fig. 4A). Depletion of IFN-\(\gamma\) similarly immunized mice did not abrogate vaccine-induced resistance to infection (Fig. 4A). Furthermore, IFN-\(\gamma\) depletion did not significantly affect the T cell infiltrate into the pulmonary airways as measured by flow cytometric analysis of cells recovered from BAL (data not shown). Although IFN-\(\gamma\) depletion did not affect viral clearance, it did abrogate the marked decline in body weight and ruffled appearance of nondepleted mice mucosally primed with M2\(_{82-90}\) and RSV challenged (Fig. 4B).

**IFN-\(\gamma\) enhances expression of a variety of soluble factors from immune cells, one of which, TNF, is associated with immunologically driven weight loss.** The amount of TNF in supernatants of lung homogenates was measured to determine whether IFN-\(\gamma\) depletion affected TNF levels in RSV-infected tissue. TNF concentrations were significantly lower in M2\(_{82-90}\)-primed mice depleted of IFN-\(\gamma\) compared with identically immunized, but nondepleted mice (Fig. 4C). Levels of IL-4 and IL-5 were below detectable limits, suggesting there was a not a shift toward an increase in Th2-type cells.

**Mucosally elicited M2\(_{82-90}\)-specific CD8\(^+\) T cells prevent eosinophilia in G-primed and RSV-challenged mice: evidence for decreased Th2 response**

In mice, sensitization to the RSV G protein results in severe inflammatory lung disease during subsequent infection with RSV (24, 25). A characteristic feature of this disease is pulmonary eosinophilia. One potential approach for preventing pathogenic immune responses in mice, and potentially in humans, is via the priming of RSV-specific CD8\(^+\) T cell responses before Ag sensitization/vaccination. To this end, the ability of mucosally and parenterally elicited M2\(_{82-90}\)-specific CTL to prevent eosinophilia in rVV-G primed mice was compared. To mucosally prime M2\(_{82-90}\)-specific CTL, BALB/c mice were i.n. immunized with M2\(_{82-90}\) plus LTK63 on days 0 and 14. Parenterally primed mice received 3 \(\times\) 10\(^6\) PFU of rVV-M2 intradermally (i.d.) on day 7. All mice were scarified with rVV-G on day 21, then challenged with RSV on day 35. Consistent with previous results (Fig. 3B), flow cytometric analysis of cells recovered from BAL on day 7 postchallenge demonstrated a lymphocyte infiltrate consisting predominantly of activated CD8\(^+\) T cells in CTL-primed mice (data not shown). At the same time point, Giemsa staining of cells recovered from BAL indicated that mice mucosally immunized with M2\(_{82-90}\) alone or LTK63 alone had pulmonary eosinophilia (Fig. 5). However, five of six mice mucosally immunized with M2\(_{82-90}\) plus LTK63 had fewer numbers of eosinophils, but this difference did not reach statistical significance (p = 0.06) for the whole group (Fig. 5). Vaccination of mice with rVV-M2 2 wk before sensitization with the RSV G protein completely suppressed the development of pulmonary eosinophilia, as has been shown previously (7) (Fig. 5). All mice, regardless of the immunization regimen used, had undetectable levels of RSV in lung tissue (data not shown). CD4\(^+\) Th2 cells that recognize residues 193–205 of the G protein are responsible for the pulmonary eosinophilia and enhanced illness which develops in mice previously sensitized to G and challenged with RSV (26). Cytokine ELISPOTs were used to determine whether the reduction in eosinophilia observed in M2\(_{82-90}\)
primed mice was due to a decrease in the frequency of Th2 cells present in the lymph nodes draining the lung. Concordantly, there were significantly fewer T cells producing IL-5 in the mediastinal lymph nodes of M282–90-primed mice compared with mice with substantial pulmonary eosinophilia (Fig. 6, top). Conversely, there were significantly more cells making IFN-γ in M282–90-primed mice compared with mice with pulmonary eosinophilia (Fig. 6, bottom). These data show that a dual vaccination regimen with both a Th1- and Th2-inducing vaccine can decrease the immunopathological effects of Th2 vaccine-enhanced pulmonary eosinophilia.

Discussion

In mice, the ability of enterotoxin-based mucosal adjuvants to promote local and systemic Ab responses to coadministered bystander proteins has been extensively reported (27–29). We have recently complemented these studies by describing CD8+ CTL responses to peptide or protein Ags coadministered with several different mucosal adjuvants (16). These studies showed that LT augments Ag-specific CTL responses independently of TNF (C. P. Simmons, R. Fowler, M. Pizza, and G. Dougan, unpublished observations), IL-12, IFN-γ, and CD4+ T cell help (16). The present study extends these observations by describing the induction of protective CD8+ CTL responses to a dominant RSV epitope by mucosal vaccination and the modulation of RSV-induced immunopathology.

The requirements of an RSV vaccine are stringent. An ideal RSV vaccine should reduce the incidence and severity of RSV A- and B-related disease in adult and infant populations, must be efficacious in the presence of maternal Abs, and would be compatible with existing pediatric vaccines. Importantly, any RSV vaccine must not induce enhanced pathology when the host is infected with homologous or heterologous viruses. In murine studies, successful RSV vaccination approaches include DNA vaccines (13, 30), purified protein vaccines (31, 32), peptide vaccines (33, 34), and live attenuated viruses (35). The novelty of the approach used in this study is that it combines reasonably efficient CTL induction with mucosal delivery of nonliving Ag. Furthermore, unlike other studies, this is the first to examine the influence of different immunization regimens on previously well-characterized aspects of RSV immunopathology. Given the history of RSV vaccines (2, 3), this represents an important but neglected aspect of studies of experimental RSV vaccines in mice.
at least 3 mo after vaccination. The M282–90-specific T cells elicited by peptide vaccination proliferated when incubated with RSV-infected targets in secondary CTL assays, suggesting these T cells also recognized naturally processed M2. The magnitude of the M282–90-specific T cell responses elicited using LT or LTK63 was similar to that previously achieved in mice immunized with the dominant H-2Kb-binding epitope from OVA (16). The mechanisms through which bacterial enterotoxins mediate their adjuvant affects for i.n. coadministered Ags remains unclear. Nonetheless, clues are provided in studies defining the APC responsible for presentation of i.n. administered peptides. Porgador et al. (36) showed that after i.n. coimmunization of Ova257–264 with cholera toxin, only ex vivo dendritic cells from the nasal-associated lymphoid tissue could present Ova257–264 to Ova-specific MHC I-restricted T cells in vitro. These results suggest that bacterial enterotoxins directly or indirectly modulate dendritic cells in the nasal-associated lymphoid tissue such that they become competent for productive T cell priming.

Mice with mucosally or parenterally elicited M282–90-specific T cell responses were more resistant to RSV challenge. The levels of resistance achieved via mucosal vaccination in this study is of a similar magnitude to that achieved in previous studies using either DNA immunization (13) or i.n. administration of a chimeric peptide containing the M282–90 sequence (14). Resistance to infection in immunized mice correlated with the magnitude of the M282–90-specific T cell response as measured by IFN-γ ELISPOT. Mice immunized with rVV-M2 mounted the strongest M282–90-specific T cell responses, and, concordantly, had the lowest lung RSV titers. Immunity to infection in these mice has previously been shown to occur solely through the actions of M282–90-specific CD81 T cell responses (11, 37).

Resistance to RSV infection in mucosally immunized mice was achieved despite Ab depletion of IFN-γ. This suggests that mucosally elicited M282–90-specific T cells mediate their effector function primarily via killing of infected host cells and/or expression of cytokines other than IFN-γ. These results are in contrast to those of Hsu et al. (13), who showed that IFN-γ was essential for resistance to RSV when M282–90-specific T cells were elicited by DNA vaccination. The contrasting routes of immunization, the method and duration of cytokine depletion (minimal in the study by Hsu et
Figure 5. Priming of M282-90-specific CD8 T cells by mucosal or parenteral vaccination reduces the severity of eosinophilia in G-vaccinated and RSV-infected mice. Mice were i.n. immunized on days 0 and 14 with either LTK63 (5 µg), M282-90 peptide (20 µg), or LTK63 (5 µg) admixed with M282-90 (20 µg). A separate group of mice received 3 x 10^6 PFU of rVV-M2 i.d. on day 7. All mice were sacrificed with rVV-G on day 21, then challenged with RSV on day 35. The data depict the percentage of eosinophils detected in BAL of individual mice 7 days after RSV challenge. Eosinophils were undetectable in rVV-M2-primed mice. There were fewer eosinophils detected in BAL of individual mice 7 days after RSV challenge compared with control groups (p = 0.06 vs peptide alone; p = 0.17 vs LTK63 alone).

Figure 6. Suppression of eosinophilia in M282-90-primed mice is associated with a significantly reduced frequency of cells making IL-5 and a significant increase in cells producing IFN-γ. The data depict the mean number (plus SD) of IL-5-producing cells (top) and IFN-γ-producing cells (bottom) in the mediastinal lymph nodes of mice immunized and challenged with RSV as described in the legend to Fig. 5. There were significantly fewer IL-5 SFCs (p < 0.05) and significantly more IFN-γ SFCs (p < 0.05) in M282-90-primed mice (rVV-M2 or LTK63/M282-90) compared with either peptide alone or LTK63-immunized mice.

al. (13)), the timing of the RSV challenge, and the magnitude of the elicited M282-90-specific CD8 T cell response may all contribute to the apparent differential requirement for IFN-γ in these studies.

CD8 T cells are central to the two different immunopathologies described in the murine model of RSV. In persistently infected γ-irradiated BALB/c mice, adoptive transfer of high numbers of a CTL line or clone was accompanied by an acute and sometimes lethal respiratory disease characterized by lung hemorrhage and frequent neutrophil efflux (15). A contrasting immunopathology, characterized by Th2 immune responses and pulmonary eosinophilia, occurs in mice primed with the RSV G protein and challenged with RSV. RSV-specific CD8 T cells producing IFN-γ have been proposed as negative regulators of this Th2 response (8).

In the studies described here, resistance to RSV in CTL-primed mice occurred at the expense of weight loss, although this was variable between experiments. Depletion of IFN-γ during RSV challenge of M282-90-primed mice successfully prevented weight loss without adversely affecting viral clearance, suggesting an immunological basis for enhanced disease. TNF may represent a prime immunological candidate for this wasting phenomenon since M282-90-primed mice depleted of IFN-γ, which did not suffer weight loss, had significantly reduced levels of TNF in supernatants of lung homogenates. TNF, whose expression is augmented by IFN-γ, is produced by monocytes, macrophages, and T lymphocytes and plays a critical role in inflammation and infection. However, exuberant TNF induction during viral infection can have detrimental consequences which include weight loss and wasting, thymic atrophy, and an increase in serum glucocorticoids (38). We hypothesize that TNF expression, partly driven by IFN-γ-producing M282-90-specific CD8 T cells, may play a critical role in the wasting illness that occurs in M282-90-primed mice challenged with RSV.

Numerous studies implicate CD4 T cells expressing Th2 cytokines as being central to enhanced disease in mice vaccinated with rVV-G and subsequently infected with RSV (8). It has been proposed that vaccines designed to enhance CD8 T cell recognition of RSV Ags might avoid disease caused by CD4 T cells (8). In this study, the priming of M282-90-specific CD8 T cells before rVV-G vaccination modulated the magnitude of the pulmonary eosinophilia and Th2 T cell response which occurred during subsequent RSV challenge. We hypothesize that IFN-γ produced by RSV-specific CD8 T cells during challenge infection suppresses eosinophilia by limiting the expansion of G-specific Th2 cells and down-regulating expression of the potent eosinophil chemoattractant eotaxin. IFN-γ is recognized as a potent inhibitor of eotaxin synthesis (39). These results are consistent with previous studies in which the priming of RSV fusion protein-specific CD8 T cells before rVV-G vaccination suppressed the development of Th2 T cells and eosinophilia during challenge infection (7, 8). However, our results are the first to show that immune-modulating CD8 T cells can be elicited by mucosal peptide vaccination. Furthermore, our results argue that the anatomical site of CD8 T cell priming may be relatively unimportant in the context of limiting the development of G-specific CD4 T cells.

The results of this study could help in the design of vaccination strategies which aim to prime protective, multivalent immune responses to RSV Ags while limiting immunopathological disease during natural infection. Conceivably, a vaccination strategy which fulfills these aims would also impact on the frequency of childhood wheezing resulting from RSV lower respiratory tract illness in infancy. Taken together, our results highlight the fine balance which exists between the protective and disease-producing...
effects of RSV-specific T cells. Indeed, a major hurdle for RSV vaccine development will be to completely dissociate these two elements.

References


