Gene-gun DNA vaccination aggravates respiratory syncytial virus-induced pneumonitis

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A CD8+ T-cell memory response to respiratory syncytial virus (RSV) was generated by using a DNA vaccine construct encoding the dominant Kd-restricted epitope from the viral transcription anti-terminator protein M2 (M282–90), linked covalently to human β2-microglobulin (β2m). Cutaneous gene-gun immunization of BALB/c mice with this construct induced an antigen-specific CD8+ T-cell memory. After intranasal RSV challenge, accelerated CD8+ T-cell responses were observed in pulmonary lymph nodes and virus clearance from the lungs was enhanced. The construct induced weaker CD8+ T-cell responses than those elicited with recombinant vaccinia virus expressing the complete RSV M2 protein, but stronger than those induced by a similar DNA construct without the β2m gene. DNA vaccination led to enhanced pulmonary disease after RSV challenge, with increased weight loss and cell recruitment to the lung. Depletion of CD8+ T cells reduced, but did not abolish, enhancement of disease. Mice vaccinated with a construct encoding a class I-restricted lymphocytic choriomeningitis virus epitope and β2m suffered more severe weight loss after RSV infection than unvaccinated RSV-infected mice, although RSV-specific CD8+ T-cell responses were not induced. Thus, in addition to specific CD8+ T-cell-mediated immunopathology, gene-gun DNA vaccination causes non-specific enhancement of RSV disease without affecting virus clearance.

INTRODUCTION

Respiratory syncytial virus (RSV) causes common colds in adults, but is the major cause of viral bronchiolitis and pneumonia in infants and is increasingly recognized as a significant cause of morbidity and mortality in the elderly. Moreover, children who recover from severe RSV disease during early life are more likely to develop wheezing and asthma later in childhood (Stein et al., 1999). Natural infection induces a long-lasting immune response, but does not prevent reinfection; however, secondary infections tend to be less severe and rarely affect the lower respiratory tract (Murphy et al., 1994). A vaccine that confers better protection than naturally induced immunity is therefore required, but, to date, RSV remains an elusive vaccine target.

In the 1960s, attempts to vaccinate infants with formalin-inactivated RSV caused a dramatic increase in the severity of naturally acquired disease. In children with fatal vaccine-enhanced disease, a dense pulmonary cellular infiltrate was observed, which comprised activated macrophages, lymphocytes, polymorphonuclear leukocytes (PMNs) and eosinophils (Chin et al., 1969; Kapikian et al., 1969).

The mouse model has been used extensively to try to understand immunological and pathogenic effects of candidate RSV vaccines. Scarification of BALB/c mice with recombinant vaccinia virus (rVV) expressing the G, F or M2 proteins of RSV induces partial protection against RSV replication, but also leads to augmented disease during subsequent intranasal (i.n.) RSV challenge. The immune response induced and the pattern of vaccine-enhanced disease differ, depending on the protein used in immunization. rVV-G induces a T2-biased response with enhanced disease that is characterized by an extensive pulmonary eosinophilic infiltrate; by contrast, scarification with rVV-F or rVV-M2 induces a TH1/TH2-dominated response that leads to augmented disease, characterized by a pulmonary infiltrate of mononuclear cells and PMNs (Openshaw et al., 1992; Alwan & Openshaw, 1993).

CD8+ T cells exert antiviral effects against RSV in both humans (Isaacs, 1991) and mice (Kulkarni et al., 1993). rVV-M2-scarified BALB/c mice mount a strong CD8+ T-cell response and develop transient antiviral immunity...
The induced CD8+ T cells are almost all specific for a single, H-2Kb-restricted peptide that corresponds to residues 82–90 of the transcription anti-terminator protein M2 (M282–90) (Openshaw et al., 1990). RSV vaccination strategies that have been designed to induce M282–90-specific CD8+ T cells include i.n. administration of a chimeric M2 peptide (Hsu et al., 1998a), mucosal delivery of M282–90 synthetic peptide with enterotoxin-based adjuvant (Simmons et al., 2001) and minigene DNA vaccination (Hsu et al., 1998b; Iqbal et al., 2003). In the first two cases, a strong antiviral CD8+ T-cell response is associated with enhanced disease. Thus, as with the rVV-M2 vaccine, strong RSV-specific CD8+ T-cell responses accelerate virus clearance, but can also lead to fatal pulmonary disease enhancement. However, T112-inducing vaccines also have immunopathogenic effects. It is therefore probable that an ideal (non-pathogenic) RSV vaccine should generate neutralizing antibodies and either a balanced T111/T112 response or a weak T111/Tc1 response.

DNA vaccination is an efficient way of inducing CD8+ T-cell responses, although responses are generally weaker than those induced by live vectors. However, DNA vaccines offer several advantages. They are simple to store and administer and generate endogenous synthesis of antigen, allowing encoded proteins to enter the major histocompatibility complex (MHC) class I presentation pathway and securing efficient induction of CD8+ T cells (Li et al., 1998). Moreover, the weak CD8+ responses that they induce may cause reduced immunopathology on RSV infection, but still potentially be protective. Previously tested RSV DNA vaccines include intramuscular (i.m.) immunization with DNA encoding the F or G proteins of RSV (Li et al., 1998; Tripp et al., 1999) and intradermal (i.d.) and i.n. immunization with DNA encoding the M282–90 epitope (Hsu et al., 1998b; Iqbal et al., 2003). In all of these studies, the immunization regimes required large quantities of DNA (60–100 µg). Gene-gun DNA administration allows effective immune responses to be induced with much smaller quantities of DNA (1–3 µg DNA per immunization). Gene-gun immunization with DNA encoding the F or G proteins of RSV has been shown to protect against RSV infection (Bembridge et al., 2000). However, these authors found that gene-gun immunization was associated with an unwanted, T112-biased response to RSV infection, particularly in mice that were immunized with an empty plasmid vector.

We have recently shown that gene-gun immunization with a single epitope induces protective immunity against lymphocytic choriomeningitis virus (LCMV) (Bartholdy et al., 2003). The constructs used encoded MHC class I-restricted epitopes that were linked covalently to human β2-microglobulin (β2m) and a murine leader of β2m inserted ahead of an LCMV epitope (to ensure translation in the endoplasmic reticulum). Notably, this construct reverses the bias towards a type 2 profile that is associated with some gene-gun immunization protocols, including those mentioned above (Pertmer et al., 1996; Feltquate et al., 1997; Bembridge et al., 2000).

In the present report, we have assessed the vaccine potential of a DNA construct encoding the immunodominant epitope from the RSV transcription anti-terminator protein (M282–90) linked to human β2m in BALB/c mice. We found that gene-gun administration of this DNA construct induced an M282–90-specific CD8+ T-cell population that was smaller than that elicited by scarification with rVV-M2, but could be expanded rapidly after RSV infection and accelerated virus clearance from the lungs. However, gene-gun DNA vaccination also led to enhanced disease during RSV challenge. This was partly mediated by RSV-specific CD8+ T cells, but was also due to non-specific effects of gene-gun-administered DNA. These findings add to the multiplicity of mechanisms by which enhanced disease can occur in this model.

**METHODS**

**Mice.** Female BALB/c mice (8–10 weeks old) were purchased from Harlan Olac or Taconic. All animals were housed under specific pathogen-free conditions and all animal experiments were conducted according to national guidelines.

**DNA vaccine construction.** The M2β2m and NPβ2m vaccines comprised the eukaryotic expression vector pcDNA3.1/zeo+ (Invitrogen) containing the murine β2m leader followed by either the M282–90 RSV peptide epitope or the NP118–126 LCMV peptide epitope, respectively, tethered to human β2m by a 10 aa linker ([G3S9]GG). The constructs were generated by using a similar construct as template, murine β2m leader–GP33–41–10 aa linker–human β2m inserted as an Nhel/NotI fragment in pcDNA3.1/zeo+. The GP33–41 peptide sequence was exchanged for either the M282–90 or NP118–126 sequence by using PCR. A PCR product covering the last 28 bases of the leader (containing a HindIII site, the peptide, linker and human β2m) was generated by using the forward primer 5’-GGACCGAAGCTTGACCGGCTTGTATGCT/attatgGGA-3’ and a reverse primer that was situated 200 bp upstream of the insert (5’-CCACATATCTAGCTGCTTACTGGCTTATCGA-3’). The reverse primer 5’-GCAACCGCGGGGGTCTTGAAGCTTTTATGAGCTTACATCCACCTA-3’ (containing a NotI site) was used for both constructs. PCR products, containing some of the vector, the leader sequence and the peptide sequence, the linker and human β2m terminated by a stop codon, were subsequently cloned as HindIII/NotI fragments into the template vector.

The M2 construct without human β2m was amplified from M2β2m by using a forward primer that was situated 200 bp upstream of the insert (5’-CTCAGGAGTGGCTATGCA-3’) and a reverse primer that comprised some of the peptide sequence, a stop codon and an XbaI restriction site (5’-cctgctagatattatTTTATGATGTCAGGACAAGCGTAGC-3’). PCR products containing some of the vector, the leader sequence and the peptide sequence terminated by a stop codon were subsequently cloned as a HindIII/XbaI fragment into the template vector. Cells of *Escherichia coli* strain XL-1 Blue (Stratagene) were transformed with the constructs by electroporation. DNA sequencing using cycle sequencing, Big Dye Terminators and ABI 310 genetic analyzer (ABI Prism) identified positive clones. Primers were obtained from Hobolih DNA Synthes. Large-scale DNA preparations were produced by using Qiagen Maxi Prep.
Gene-gun immunization. DNA was coated on to 1·6 nm gold particles at a concentration of 2 μg DNA (mg gold)⁻¹. The DNA/gold complex was coated onto plastic tubes and 0·5 mg gold was delivered to the mouse per shot (1 μg DNA per shot). These procedures were performed according to the manufacturer’s instruction (Bio-Rad). Mice were immunized on the abdominal skin by using a hand-held gene-gun device employing compressed helium (400 p.s.i.) as the particle-motive force. Unless otherwise mentioned, mice were inoculated twice at an interval of 3 weeks and then allowed to rest for 3 weeks before further challenge/investigation.

Virus infection. Mice were lightly anaesthetized and challenged i.n. with 1×10⁶ p.f.u. human RSV A2 strain in 100 μl. For vaccinia infection, mice were sacrificed on the rump with 3×10⁶ p.f.u. (10 μl) rVV expressing the RSV M2 protein (rVV-M2) or the RSV G protein (rVV-G).

Depletion of CD8⁺ T cells. Mice received 50 μl clarified ascitic fluid containing mAb αCD8a 53-6.7 in 0·5 ml PBS intraperitoneally on days −1, 0, 2 and 5 relative to infection. Flow cytometry consistently showed <1% CD8⁺ T cells in spleen, bronchoalveolar lavage (BAL), mediastinal lymph nodes (MLNs) and lung mesh in depleted mice.

Cell recovery. Mice were terminally anaesthetized with pentobarbitone and bled via the femoral artery. BAL was collected as described previously (Hussell et al., 1997). Lungs were inflated six times with 1·5 ml 12 mM lidocaine in Earl’s balanced salts solution. Peritoneal cells were obtained by lavage with 5 ml ice-cold Hank’s balanced salts solution. Lungs and/or MLNs were removed aseptically and transferred to RPMI 1640 medium supplemented with 2-mercaptoethanol, L-glutamine and penicillin/streptomycin solution. Single-cell suspensions were obtained by pressing the organs through a fine sterile steel mesh. Cells were washed once and the cell concentration was adjusted in supplemented RPMI 1640 medium that contained 10% fetal calf serum.

Flow cytometry. Fluorescein isothiocyanate-conjugated rat anti-mouse CD44, peridinin-chlorophyll-protein complex (PerCP)- or phycoerythrin (PE)-conjugated anti-CD8a or CD8b and PE-conjugated anti-gamma interferon (IFN-γ) were purchased from Pharmingen. For visualization of peptide-specific, cytokine-producing CD8⁺ T cells, 2×10⁵ MLN cells were incubated with MHC K⁺-restricted M282-90 RSV peptide or L⁺-restricted NP118-126 LCMV peptide at a concentration of 1 μg ml⁻¹ for 5 h in the presence of interleukin 2 (50 U ml⁻¹) and monensin (3 μM). After incubation, cells were stained for surface markers with directly labelled mAbs in staining buffer (1% BSA, 0·1% NaNO₃, 3 μM monensin in PBS) for 20 min in the dark at 4°C, washed and fixed with 2% formaldehyde for 30 min. Subsequently, cells were permeabilized in 0·05% saponin, stained with cytokine-specific mAbs, washed and resuspended in staining buffer. Cells were analysed by using a FACSCalibur instrument (Becton Dickinson) and at least 10⁶ cells were gated by using a combination of low angle and side scatter to exclude dead cells and debris. Data analysis was conducted by using CellQuest or WinMDI software.

Clinical severity of infection. Mice were monitored daily for 7 days after i.n. RSV challenge. Weight loss was calculated as the weight on the indicated day relative to the initial weight.

Enumeration of lymphocytes, eosinophils and PMNs. BAL fluid (100 μl) from each mouse was cytospinrifuged onto glass slides and stained with Giemsa’s reagent for cytological analysis. Lymphocytes, eosinophils and PMNs were enumerated by microscopy. At least 300 cells per sample were counted.

Virus titration. RSV titres were assessed in lung homogenates as described by Stott et al. (1987). Briefly, lungs were removed on day 4 post-infection (p.i.) and snap-frozen in liquid nitrogen. For analysis, lungs were thawed and homogenized. After centrifugation at 7000 r.p.m. for 2 min, supernatants were titrated in doubling dilutions on 60–80% confluent HEp-2 cell monolayers in 96-well, flat-bottomed plates. After 24 h incubation at 37°C, monolayers were washed and fixed in absolute methanol for 20 min. The monolayer was subsequently incubated for 1 h at room temperature with biotin-conjugated goat anti-RSV antibodies (Biogenesis), washed and incubated with streptavidin–horseradish peroxidase for 30 min at room temperature. Infected cells were detected by using 3-amin-9-ethylcarbazole; infectious units were enumerated by light microscopy.

Statistical analysis. A non-parametric Mann–Whitney rank sum test was used to perform comparisons between groups. P values of <0·05 were considered to be statistically significant.

RESULTS

DNA vaccination induces epitope-specific CD8⁺ T-cell memory

We previously found that gene-gun DNA vaccination with a single immunogenic peptide linked to human β₂m induced long-lived, specific, CD8⁺ T-cell responses in vitro as well as in vivo (Christensen et al., 2002; Bartholdy et al., 2003). To see whether constructs encoding the immunodominant MHC class I-restricted epitope of the second matrix protein of RSV A2 (M282-90) had a similar effect, mice were gene-gun-vaccinated (i.d.) twice, 3 weeks apart, with a vaccine encoding the M282-90 epitope linked to human β₂m, a vaccine encoding the M282-90 epitope without the linkage to human β₂m or the empty vector as a control. To compare the magnitude of the response elicited by DNA vaccination with an established potent method of eliciting M282-90-specific CD8⁺ T cells (Kulkarni et al., 1993, 1995), other mice were immunized in parallel with 3×10⁶ p.f.u. rVV expressing the M2 protein (rVV-M2). Epitope-specific CD8⁺ T cells from spleen or peritoneum were sought by using peptide-specific IFN-γ production, demonstrated by intracellular cytokine staining (ICCS). Representative FACS plots of peritoneal cells of a mouse from each group stimulated with the M282-90 peptide (Fig. 1a, upper panel) or left unstimulated (Fig. 1a, lower panel) are shown. The antigen-specific CD8⁺ T cells were CD44high, consistent with a ‘primed’ phenotype. A distinct population of peritoneal, M282-90-specific (IFN-γ⁺) CD8⁺ T cells was detected in M2/β₂m DNA-vaccinated mice and in some M2 DNA-vaccinated mice. By contrast, virtually no M282-90-specific CD8⁺ T cells could be detected in control-vaccinated mice. The percentages of M282-90-specific, IFN-γ⁺-producing CD44high CD8⁺ T cells (upper right quadrant of the FACS plot) for individual mice are indicated in Fig. 1(b). Similar differences between M2/β₂m DNA-vaccinated and control-vaccinated mice could be detected when a control DNA vaccine encoding an irrelevant, MHC class I-restricted peptide (the immunodominant NP118-126 of LCMV) linked to human β₂m (NP/β₂m) was used (data not shown). The frequency of splenic M282-90-specific CD8⁺ T cells bordered on the detection limit in
DNA vaccination accelerates antiviral CD8\(^+\) T-cell responses on RSV infection

To examine the recall response in DNA-vaccinated mice, we next measured the number of M2\(_{2-90}\)-specific CD8\(^+\) T cells in the draining lymph nodes (MLNs) on days 4 and 7 after i.n. challenge with RSV. Mice were immunized with either M2\(\beta_2m\) or M2 DNA vaccines. For controls, mice were either vaccinated with an irrelevant construct (NP\(\beta_2m\)) or left unvaccinated. Three weeks after the last immunization, all mice were i.n. infected with RSV A2. As seen in Fig. 2, significantly more M2\(_{2-90}\)-specific CD8\(^+\) T cells were observed in the draining lymph nodes from M2\(\beta_2m\) and M2 DNA-immunized mice on day 4 p.i. than from NP\(\beta_2m\) control-vaccinated and unvaccinated mice. Furthermore, there was a tendency towards lower frequencies in M2 DNA-vaccinated mice, compared with M2\(\beta_2m\) DNA-vaccinated mice. By day 7 p.i., there was no longer a difference in the number of virus-specific cells among the groups. The same kinetic pattern was seen when analysing pulmonary cells recovered from lung mash (data not shown). The induction of T-cell recognition was specific, in that M2\(\beta_2m\) and M2 DNA vaccination did not induce CD8\(^+\) T cells that produced IFN-\(\gamma\) on stimulation with NP\(_{118-126}\) synthetic peptide (data not shown). In addition, an increased NP\(_{118-126}\)-specific response was not seen in NP\(\beta_2m\) DNA-vaccinated mice infected with RSV, thus excluding the possibility of cross-reactivity between NP\(_{118-126}\)-specific CD8\(^+\) T cells and RSV (data not shown).

DNA vaccination induces long-lived CD8\(^+\) T-cell responses

To examine the longevity of DNA vaccine-induced CD8\(^+\) T-cell responses, groups of mice were infected 3 months after the last DNA immunization and the frequency of CD8\(^+\) T cells in MLNs was measured 4 days later (Fig. 3). Four out of five M2\(\beta_2m\)-immunized mice had increased levels of M2\(_{2-90}\)-specific CD8\(^+\) T cells, compared with unvaccinated mice. Moreover, the frequencies were comparable with those seen in similarly vaccinated mice infected 3 weeks after the last immunization (see Fig. 2, upper panel). The same longevity of T-cell responses was seen in M2 DNA-vaccinated mice, but the responses were weaker than those seen in M2\(\beta_2m\) DNA-vaccinated mice. Thus, the DNA vaccine-induced CD8\(^+\) T-cell responses were long-lived in the majority of mice and the inclusion of \(\beta_2m\)再次促进了体内的CD8\(^+\)T细胞反应。

DNA vaccine-elicited CD8\(^+\) T cells mediate partial resistance to RSV infection

An essential measure of a good vaccine is its ability to protect against or clear infection. As rapid virus clearance from
internal organs is known to be CD8$^+$ T-cell-mediated (Ahmed et al., 1988; Openshaw et al., 1990), we studied virus elimination from the lungs as a parameter of the in vivo efficiency of the vaccine-induced CD8$^+$ T-cell memory response. Mice were vaccinated as described previously with M2$\beta_2$m, M2, pcDNA control vaccine or rVV-M2. Virus levels in the lungs were assayed 4 days after i.n. challenge with RSV A2; this is the time point at which lung virus titres usually peak during a primary RSV infection (Taylor et al., 1984). As can be seen in Fig. 4, M2$\beta_2$m DNA-vaccinated mice had significantly lower virus titres than control-vaccinated mice. By contrast, M2 DNA-vaccinated mice did not show reduced virus titres compared to controls.

**Fig. 2.** Frequency of MLN-derived, M2$\beta_2$m-specific CD8$^+$ T cells with an activated phenotype (CD44$^{hi}$, IFN-$\gamma$+) in DNA-vaccinated, control-vaccinated and unvaccinated BALB/c mice 4 and 7 days after i.n. RSV challenge. DNA-immunized mice were vaccinated on days 0 and 21 with the M2$\beta_2$m vaccine, M2 vaccine or NP$\beta_2$m control vaccine. On day 42, mice were infected with $1 \times 10^6$ p.f.u. RSV A2. Mice were killed either 4 or 7 days p.i. and MLNs were harvested. Single-cell suspensions were incubated for 5 h with M2$\beta_2$m peptide. Following incubation, cells were surface-stained, permeabilized and stained for IFN-$\gamma$. NP$\beta_2$m or unstimulated (unvacc) cells served as controls; in these cases, the frequency of IFN-$\gamma$-producing, activated CD8$^+$ T cells was <0.3% (data not shown). Results are shown as mean±SD for groups of four to ten mice. *, $P<0.05$ (Mann–Whitney rank sum test) relative to NP$\beta_2$m-vaccinated and unvaccinated mice.

**Fig. 3.** DNA vaccine-primed CD8$^+$ T cells are long-lived in the majority of vaccinated mice. M2$\beta_2$m-vaccinated and M2-vaccinated mice were immunized twice, 3 weeks apart. Three months after the last immunization, DNA-vaccinated as well as unvaccinated control mice were infected i.n. with $1 \times 10^6$ p.f.u. RSV A2. The data depict the frequency of MLN-derived M2$\beta_2$m-specific CD8$^+$ T cells with an activated phenotype (CD44$^{hi}$, IFN-$\gamma$+) that were recovered 4 days after infection. Points represent individual mice.

**Fig. 4.** Lung virus titres in DNA-vaccinated, control-vaccinated and rVV-M2-vaccinated BALB/c mice 4 days after RSV infection. DNA-immunized mice were vaccinated on days 0 and 21 with M2$\beta_2$m, M2 or pcDNA control vaccine. Mice that were vaccinated with rVV-M2 were scarified once on the rump with $3 \times 10^6$ p.f.u. rVV-M2 on day 21. On day 42, mice were infected i.n. with $1 \times 10^6$ p.f.u. RSV A2. The data depict p.f.u. RSV recovered from lungs of individual mice 4 days after virus challenge. $P$ values (Mann–Whitney rank sum test) relative to pcDNA control-vaccinated mice are depicted when $<0.05$. 

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not show a significant reduction in virus load in the lungs, indicating that inclusion of $\beta_2$m augmented the efficiency of the vaccine. Similar, statistically significant differences in virus titres were also observed between M2$\beta_2$m DNA-vaccinated mice and control-vaccinated mice (using the NP$\beta_2$m vaccine as control; data not shown). In rVV-M2-immunized mice, virus was eliminated completely from the lungs in eight out of nine mice (Fig. 4, and data not shown). Thus, M2$\beta_2$m DNA vaccination resulted in accelerated virus clearance on RSV infection, but when compared with the rVV-M2 vaccine, the protection achieved by DNA vaccination was only partial.

**DNA vaccination aggravates RSV-induced pneumonitis**

Vaccine-enhanced disease is a common phenomenon in the mouse model of RSV. In addition to the vaccine-enhanced, T$_{H2}$-mediated immunopathological responses, various CD8$^+$ T-cell-inducing vaccines (e.g. scarification with rVV-M2 or i.n. peptide) lead to enhanced disease following RSV challenge (Openshaw et al., 1992; Simmons et al., 2001). As our DNA vaccine induced a weaker CD8$^+$ T-cell response, we deemed it likely that such vaccine-induced disease would be less severe. To study this prediction, the severity of clinical symptoms in DNA-vaccinated and rVV-M2-vaccinated mice infected with RSV was scored.

DNA vaccination induced signs of enhanced disease (ruffled fur and a hunched posture), as well as weight loss (Fig. 5) following RSV infection. We were surprised to find that disease severity in M2$\beta_2$m DNA-vaccinated mice was similar to that in rVV-M2-scarified mice, despite reduced CD8$^+$ T-cell responses. Similar disease severity was seen in M2 DNA-vaccinated, RSV-infected mice (data not shown). CD8$^+$ T-cell depletion significantly reduced the severity of illness and weight loss in M2$\beta_2$m DNA-immunized mice, but these mice still suffered more severe weight loss than non-immunized, RSV-infected mice. Even more surprisingly, NP$\beta_2$m control-vaccinated mice suffered weight loss that was almost as severe as that suffered by M2$\beta_2$m DNA- and rVV-M2-immunized mice, although it was delayed by 2 days.

Giemsa staining of BAL cells recovered from M2$\beta_2$m DNA-immunized, control-vaccinated, rVV-M2-scarified and unimmunized, RSV-infected mice revealed an increase in lymphocytes and PMNs on day 4 p.i. in M2$\beta_2$m DNA-immunized mice and M2$\beta_2$m-scarified mice (Fig. 6), which is indicative of a T$_{H1}$/T$_{C1}$-like immune response (Alwan & Openshaw, 1993). The elevated levels of PMNs declined with time. Furthermore, there was no longer a difference in lymphocyte levels by day 7 p.i. (Fig. 6). The increased lymphocyte numbers in BAL on day 4 p.i. in M2$\beta_2$m DNA-immunized mice correlated well with elevated M2$\beta_2$-specific responses in the draining lymph nodes and lung mash at these time points (Fig. 2, and data not shown). Notably, CD8-depleted, M2$\beta_2$m DNA-immunized mice had increased numbers of eosinophils 7 days after RSV infection, confirming previous findings that CD8$^+$ T cells prevent an eosinophilic infiltrate from developing (Hussell et al., 1997; Srikitakhachorn & Braciale, 1997).

The latter observation led us to investigate whether DNA vaccine-primed CD8$^+$ T cells could decrease such an infiltrate in a model where high numbers of eosinophils are induced (Openshaw et al., 1992). We therefore pre-sensitized DNA-vaccinated mice to the RSV G protein by rVV-G scarification prior to RSV infection. It has previously been found that strong priming of RSV-specific CD8$^+$ T cells (e.g. by rVV-M2) before RSV-G sensitization can suppress this disease (Simmons et al., 2001). However, lung eosinophilia was not reduced in M2$\beta_2$m DNA-vaccinated, G-primed mice, compared with control-vaccinated and unvaccinated, G-primed mice 7 days after RSV infection (data not shown), indicating that the DNA vaccine-primed CD8$^+$ T-cell response is not strong enough to have an immunoregulatory effect in this model.

**DISCUSSION**

The development of an RSV vaccine that confers better protection than natural immunity remains a challenge. In addition, the unwanted immunopathogenic effects that are associated with RSV immunity remain a barrier to vaccine development. Consequently, we decided to investigate whether DNA vaccination could provide enhanced protection against RSV infection, and if not, what the underlying mechanisms are. In this study, we investigated whether DNA vaccination with M2 could enhance RSV immunity, and if so, whether this immunity was enhanced at the cost of increased disease.

**Fig. 5.** Severity of RSV infection in DNA-vaccinated, control-vaccinated, rVV-M2-vaccinated and unvaccinated BALB/c mice. Mice were DNA-immunized with M2$\beta_2$m vaccine or NP$\beta_2$m control vaccine on days 0 and 21. rVV-M2-vaccinated mice were scarified once on the rump on day 21 with $3 \times 10^6$ p.f.u. rVV-M2. All mice were infected i.n. with $1 \times 10^6$ p.f.u. RSV A2 on day 42. Some of the M2$\beta_2$m-vaccinated mice were treated with anti-CD8 antibodies on days 41, 42, 44 and 47 (M2$\beta_2$m + αCD8). The data depict the mean body weight of five to eight mice, expressed as percentage of the pre-challenge weights. For clarity, the SD at each time point is not shown. Instead, * denotes P < 0.05 (Mann–Whitney rank sum test) relative to M2$\beta_2$m- and rVV-M2-vaccinated mice.
development. Thus, vaccines that induce a TH2-biased immune response cause pulmonary lesions that are characterized by an extensive eosinophilic infiltrate upon RSV infection (Openshaw et al., 1992), whereas vaccines that induce a strong TH1/TC1 response lead to peribronchiolar and perivascular infiltrations of PMNs and lymphocytes upon infection and, subsequently, cell damage due to cytotoxic T lymphocyte (CTL)-mediated killing of infected cells and production of inflammatory cytokines such as tumour necrosis factor alpha and IFN-γ (Alwan & Openshaw, 1993; Simmons et al., 2001). Based on theoretical considerations, an ideal RSV vaccine should therefore probably either induce a balanced and controlled TH1/TH2 response or a weaker TH1/TC1-biased response that induces minimal cell damage, but still manages to control the infection.

In this study, we investigated the protective capacity of a DNA vaccine-induced CD8+ T-cell response specific for the immunodominant H-2Kd-restricted epitope from the second matrix protein of RSV (M282–90). This immune response was elicited by gene-gun DNA immunization using a construct encoding the M282–90 epitope, linked covalently to human β2m and a similar construct without human β2m for comparison. DNA vaccination with the M2β2m construct induced an antigen-specific CD8+ T-cell memory response that was detected in the peritoneum by ICCS for IFN-γ. The M2 DNA vaccine only occasionally induced vaccine-specific cells to the same extent. Splenic M282–90-specific CD8+ T cells were below the detection limit when analysed by ICCS for IFN-γ. However, restimulation of splenocytes with the M282–90 peptide did give rise to in vitro CTL responses (data not shown). The fact that higher frequencies could be detected in the peritoneum than in the spleen is in agreement with the theory that primed cells are enriched in tertiary tissues (Masopust et al., 2001).

Infection of M2β2m and M2 DNA-vaccinated mice with RSV gave rise to accelerated M282–90-specific CD8+ T-cell responses when compared with control-vaccinated and unvaccinated mice. Thus, vaccine-primed cells were able to expand rapidly on encountering virus. Similarly accelerated responses were observed in the majority of mice that were infected 3 months after the last immunization, demonstrating that the vaccine-induced responses were long-lived. M2β2m DNA-vaccinated mice, but not M2 DNA-vaccinated mice, had significantly lower virus titres in the lungs 4 days after RSV infection, compared with control-vaccinated mice. This indicated that inclusion of human β2m augmented priming, consistent with findings in the LCMV model (Bartholdy et al., 2003), and also showed that DNA vaccine-primed CD8+ T cells are capable of functioning in vivo. Thus, gene-gun immunization with very low doses of DNA managed to mediate significant control of RSV infection. The DNA vaccine-elicited, M282–90-specific CD8+ T-cell response was weaker than that induced by rVV-M2 scarification. This was revealed by several observations. Firstly, lower frequencies of M282–90-specific CD8+ T cells were found prior to infection in the spleen and the peritoneum by ICCS for IFN-γ. The M2 DNA vaccine only occasionally induced vaccine-specific cells to the same extent. Splenic M282–90-specific CD8+ T cells were below the detection limit when analysed by ICCS for IFN-γ. However, restimulation of splenocytes with the M282–90 peptide did give rise to in vitro CTL responses (data not shown). The fact that higher frequencies could be detected in the peritoneum than in the spleen is in agreement with the theory that primed cells are enriched in tertiary tissues (Masopust et al., 2001).
mice, in contrast to previous findings in rVV-M2-vaccinated mice (Simmons et al., 2001).

As a weaker CD8+ T-cell response was found after DNA vaccination than after rVV-M2 scarification, we expected to find less severe disease in DNA-vaccinated mice on subsequent RSV infection. However, similar weight loss was seen in M2β2m and M2 DNA- and rVV-M2-immunized mice that were infected with RSV. CD8+ T cells were only partly responsible for disease in M2β2m DNA-vaccinated mice, as anti-CD8 antibody treatment did not completely abrogate weight loss and clinical symptoms. More surprisingly, mice that were vaccinated with a DNA vaccine encoding the immunodominant H-2Ld-restricted LCMV epitope NP118–126 linked to human β2m had disease symptoms that were almost as severe as those suffered by M2β2m DNA- and rVV-M2-immunized mice, although clinical symptoms and weight loss were delayed by approximately 2 days.

Analysis of the cell infiltrate in BAL revealed that M2β2m DNA-vaccinated mice had few eosinophils after RSV infection. Instead, lymphocytes and PMNs were present, which is indicative of a Th2-biased response. However, in the absence of CD8+ T cells, an influx of eosinophils was detected in M2β2m DNA-vaccinated mice, supporting a role for CD8+ T cells in preventing eosinophilia. The increased number of eosinophils in these mice may also account for some of the weight loss.

Notably, no early influx of lymphocytes and PMNs and no eosinophils could be detected in NPβ2m DNA-vaccinated, RSV-infected mice. This eliminated the theoretical possibility that the vector encoded an unknown immunogen that stimulated cross-reactive, RSV-specific cells.

It has recently been reported that memory T cells to one virus can become activated during infection with an unrelated heterologous virus and may play a role in antiviral immunity and immunopathology (Chen et al., 2001; Welsh & Selin, 2002). However, the lack of NP118–126-specific CD8+ T-cell responses on days 4 and 7 post-RSV infection and the lack of accelerated virus clearance in NPβ2m DNA-vaccinated mice suggested strongly that this was not the case in our studies.

Interestingly, and contrary to the above-described hypothesis, it was recently shown that in LCMV-immune BALB/c mice challenged with RSV, bystander recruitment of memory T cells actually impaired virus clearance and enhanced immunopathology (Ostler et al., 2003). The authors suggested that the presence of irrelevant, heterologous memory T cells in some way competed with RSV-specific cells for recruitment to the lungs. However, the frequency of irrelevant memory cells induced by our DNA vaccine was very low (0.05–0.12% of epitope-specific CD8+ T cells) (Bartholdy et al., 2003) and we did not see any difference in either the number of M2β2m-specific CD8+ T cells and the recruitment of lymphocytes to the lungs or virus clearance in NPβ2m DNA-vaccinated, RSV-infected mice, compared with unvaccinated, RSV-infected mice (Figs 2 and 6, and data not shown). Together, this indicated that unknown, CD8-independent factors account for disease in NPβ2m DNA-vaccinated mice during RSV infection.

It has been observed in several studies that i.d. DNA vaccination favours the development of an immune response that is biased towards Th2 cytokine production (Pertmer et al., 1996; Feltquate et al., 1997; Li et al., 1998), which in the RSV model leads to severe immunopathology. Bembridge et al. (2000) showed that gene-gun-vaccinated mice that were immunized with empty DNA vector had a Th12-biased immune response with increased numbers of eosinophils in the lungs on RSV infection, a phenomenon that was not seen in unvaccinated, RSV-infected mice.

M2β2m DNA vaccination, however, resulted in a Th12-biased response, despite the fact that the vaccine was given intradermally, and this was consistent with previous findings from the LCMV model (Bartholdy et al., 2003). Thus, in contrast to the observations made by Bembridge et al. (2000), disease in NPβ2m DNA-vaccinated, RSV-infected mice did not seem to be the result of Th12-mediated immunopathology that may be induced non-specifically during gene-gun immunization. Rather, non-specific components in the vector prime mice systemically, e.g. to produce more inflammatory cytokines on RSV infection. CpG motifs contained in the vector may be the cause of this difference, due to their non-specific immunostimulatory effects. Co-delivery of CpG motif-containing oligodeoxynucleotides has been shown to be able to shift Th12 immunity, primed by gene-gun DNA vaccination, towards Th11 immunity (Zhou et al., 2003). We suggest that our DNA constructs encoded sufficient CpG motifs to shift the immune response towards Th11 on infection. In addition to the pcDNA 3.1 vector itself, the human β2m gene might be a good source of CpG. The priming of NP118–126-specific CD8+ T cells might also contribute to biasing antiviral immunity towards a Th11 response. It should be noted that the non-specific immunopathology that we observed is not a general phenomenon, as mice that are similarly DNA-vaccinated do not develop enhanced disease on systemic LCMV infection (unpublished observations).

In conclusion, several different factors seem to be able to cause pathology during RSV infection of DNA-vaccinated mice: firstly, RSV may induce some pulmonary cell damage by itself; secondly, vaccine-specific CD8+ T cells cause immunopathology by the killing of infected cells and/or production of proinflammatory cytokines; thirdly, in the absence of CD8+ T cells, a Th2 response is not suppressed and eosinophil recruitment to the lungs is enhanced; and, lastly, unknown factors associated with gene-gun DNA immunization may prime for RSV-induced disease. Thus, even though low doses of DNA are able to induce long-lived CD8+ T-cell memory responses that reasonably mediate control of the infection, improved constructs with fewer
pathogenic effects are needed for this DNA vaccine approach to be beneficial for the host.

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