Kanagawa, Japan) embryonic fibroblasts, as described previously (*12*). The mouse bone-marrow stromal-cell line OP9 was maintained in α-minimum essential medium (Gibco, Rockville, MD) supplemented with 20% fetal calf serum, as previously described (*13*).

For the in vitro differentiation (induction of hematopoietic differentiation), ES cells were seeded onto mitomycin–C-treated confluent OP9 cell layers in culture dishes in Iscove's modified Dulbecco's medium (Gibco) supplemented with 8% horse serum (Gibco), 8% fetal calf serum, 5×10^{-6} M hydrocortisone (Sigma, St. Lois, MO), and multiple cytokines including 20 ng/mL recombinant human (rh) bone morphogenetic protein-4 (BMP-4; R&D, Minneapolis, MN), 20 ng/mL rh stem-cell factor (SCF, Amgen, Thousand Oaks, CA), 20 ng/mL rh interleukin-3 (Research Diagnostics, Flanders, NJ), 20 ng/mL rh interleukin-6 (Ajinomoto, Osaka, Japan), 20 ng/mL rh vascular endothelial growth factor (VEGF, R&D), 20 ng/mL rh granulocyte colony-stimulating factor (Chugai, Tokyo, Japan), 10 ng/mL rh Flt-3 ligand (Research Diagnostics), and 2 IU/mL rh erythropoietin (Chugai). During differentiation, media were changed every 2 to 3 days. After 6 days of culture, cells were dissociated with 0.25% trypsin (Gibco), collected with a cell scraper, washed with Hanks' balanced salt solution (HBSS, Gibco), resuspended in 0.4 mL of 0.1% bovine serum albumin/HBSS, and used for transplantation. Human cord-blood $CD34^+$ cells used in the present study were obtained at Jichi Medical School Hospital with informed consent.

Transplant Procedures

Pregnant Suffolk ewes (Japan Lamb, Hiroshima, Japan) were bred at the Utsunomiya University Farm. Fetal sheep at 55 to 79 days of gestation (full term 147 days) were used. Before transplantation, ewes were sedated with ketamine (10 mg/kg intramuscularly) and received a 0.5% to 1.0% halothane-oxygen mixture by inhalation by way of an endotracheal tube. The uterus was exposed through a midline laparotomy incision. Donor cells were injected into the fetus in the liver through the uterine wall using a 25-gauge needle under ultrasound guidance. After closure of the abdominal wall, penicillin and streptomycin were administered. The fetus was allowed to come to term. After birth, some lambs were intraperitoneally administered rhSCF at a dosage of 60 μg/kg once a day for 18 or 5 consecutive days.

Hematopoietic Progenitor Assay

To assess cynomolgus hematopoiesis in sheep, clonogenic hematopoietic colonies were produced by growing bone-marrow cells in methylcellulose with defined rh cytokines (Methocult GF+ and MegaCult-C, Stem Cell Technologies, Vancouver, Canada). Bone-marrow cells were aspirated from the iliac bone. From harvested bone-marrow cells, a leukocyte cell fraction was obtained after red–blood-cell lysis with ACK buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA; Wako, Osaka, Japan). Cells were plated in triplicate at 1 to 5×10^4 cells per 35-mm plate. After 14 days, individual colonies were plucked into 50 μL of distilled water and digested with 20 μg/mL proteinase K (Takara, Shiga, Japan) at 55°C for 1 hour, followed by 99°C for 10 minutes. Each sample (5 μL) was used for polymerase chain reaction (PCR) amplification to detect cynomolgus-specific β2-microglobulin gene sequences. Nested PCR was performed. The outer primer set was 5'-GTC TGG ATT TCA TCC ATC TG-3' and 5'- GGC TGT GAC AAA GTC ACA TGG-3', and the inner primer set was 5'-GTC TGG ATT TCA TCC ATC TG-3' and 5'-GGT GAA TTC AGT GTA GTA CAA G-3'. Amplification conditions for both outer and inner PCR were 25 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Amplified products (135 bp) were resolved on 2% agarose gel and visualized by ethidium bromide staining.

PCR Southern Blotting

Cellular DNA was extracted from peripheral blood and bone-marrow cells after birth and subjected to the cynomolgus-specific β2-microglobulin PCR as described above. The PCR products were resolved on 2% agarose gel and transferred to Hybond-N+ (Amersham, Cleveland, OH). The membrane was hybridized with a radiolabeled cynomolgus-specific β2-microglobulin probe generated by PCR using the following primers: 5'-GTC TGG ATT TCA TCC ATC TG-3' and 5'-GGT GAA TTC AGT GTA GTA CAA G-3'. Radiolabeling of a probe was performed using a DNA labeling kit (Amersham).

RESULTS

In Vitro Culture and In Utero Transplantation

Cynomolgus ES cells were maintained on mouse embryonic fibroblast feeder cells. They form colonies on feeder cells (Fig. 1a). For induction of hematopoietic differentiation, undifferentiated ES cells were placed on murine stromal OP9 cells (*13*) in the presence of multiple cytokines including human BMP-4 and VEGF (*14*). The cell number increased by 10-fold at day 6, and cobblestone-like cells emerged at this time point (Fig. 1b). These cells were negative or only weakly positive for CD34 (hematopoietic) and CD31 (endothelial) markers as assessed by flow cytometry and had little clonogenic hematopoietic colony forming ability in culture (data not shown). These cells likely represent a differentiated population of mesodermal cells. We then transplanted the day-6 ES derivatives (average 4.8×10^7 cells, Table 1) into fetal sheep (n=4) by way of direct injection into the liver under ultrasound guidance after the first trimester (Fig. 1c).

Hematopoietic Microchimerism after Birth

The animals were delivered at full term. Marrow cells were harvested from the iliac bone and plated in methylcellulose. Clonogenic hematopoietic colonies (colony-forming units [CFU]) were thus produced to examine hematopoietic chimerism in the sheep (Fig. 2). Sheep bone-marrow cells generated colonies of clear hematopoietic morphology in this assay. Cynomolgus and sheep cells were equivalent in their colony-formation ability in this culture, and each colony was derived from a single cynomolgus or sheep hematopoietic progenitor cell. Therefore, the ratio of cynomolgus to sheep colony number is considered a chimeric fraction. To distinguish cynomolgus versus sheep colonies, we tried to immunostain colonies with antihuman class I, and more directly hematopoietic-specific antihuman CD45, but they reacted to sheep counterparts or generated a considerable nonspecific staining. We then conducted PCR for monkey-specific β2-microglobu-

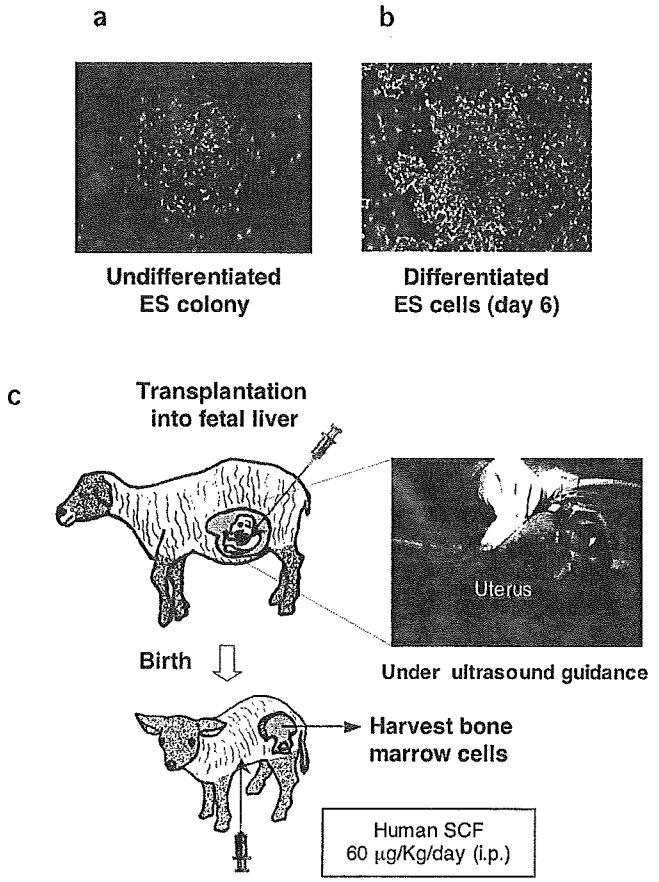

FIGURE 1. In utero transplantation of day 6 cynomolgus embryonic stem (ES)-derived cells. (a) Undifferentiated cynomolgus ES cells were maintained on mouse embryonic fibroblast feeder cells. (b) For hematopoietic induction, undifferentiated ES cells were placed on murine stromal OP9 cells in the presence of multiple cytokines. Cobblestone-like cells emerged at day 6. (c) The day 6 ES derivatives were transplanted directly into the fetal liver after the first trimester. After birth, marrow cells were harvested from the iliac bone and examined for cynomolgus versus sheep hematopoiesis. Some lambs were intraperitoneally (i.p.) administered with human stem-cell factor (SCF) to specifically stimulate cynomolgus hematopoiesis.

lin DNA sequences on DNA isolated from each colony (Fig. 2) (colony PCR). Cynomolgus CFU were clearly detected at a level of 1% to 2% in the bone marrow of all four sheep (Table 1). We repeated colony PCR and confirmed that data were reproducible. We detected both granulocytic and erythroid cynomolgus CFU. Cynomolgus megakaryocytic colonies were not detected, but it was no wonder considering the low frequency of cynomolgus megakaryocytic colony formation in vitro (*15*). The longest follow-up was 17 months after transplantation (14 months after birth), and cynomolgus CFU were still detectable. No lamb developed a teratoma.

Selective Expansion of Cynomolgus Hematopoiesis

To selectively stimulate cynomolgus compared with endogenous hematopoiesis in the sheep, we administered two animals with human SCF, a cytokine that does not cross-react to stimulate sheep hematopoietic progenitors (*16*). A time-course profile of the two animals (No. 141 and No. 182) is shown in Figure 3. In both sheep, in response to the human SCF administration for 18 days, the fractions of cynomolgus CFU increased several-fold (up to 13.2% at day 174 and 4.7% at day 112 posttransplantation, respectively). After cessation of SCF administration, the fractions fell to the original levels. Resumption of human SCF at the same dose but for 5 days produced a similar elevation in the chimeric fractions (up to 4.4% at day 364 and 8.8% at day 187 posttransplantation, respectively). No adverse effects associated with human SCF administration were observed. We did not examine antibody responses to human SCF in the sheep, but this was unlikely to occur because second trials of human SCF administration did work (i.e., increased the chimeric fractions) in both sheep.

Cynomolgus cells were also detected in the circulation, although the fraction was very low (<0.1%), even after human SCF administration as assessed by PCR Southern blotting (Fig. 4). The low levels of cynomolgus cells hampered the lineage analysis by flow cytometry. To detect cynomolgus T lymphocytes in the sheep, we collected the peripheral blood and tried to selectively expand cynomolgus T lymphocytes in the culture with human interleukin-2, antimonkey CD3 (FN-18), and antihuman CD28 (Kolt-2, cross-reacting to monkey CD28) (*17*). However, sheep lymphocytes were also stimulated to expand, and we failed to detect cynomolgus T lymphocytes.

Comparative Study of Repopulating Ability

Next, we transplanted human cord-blood hematopoietic stem cells ($CD34^+$ cells, average 1.8×10^6 cells) into fetal sheep (n=4) instead of day-6 cynomolgus ES derivatives. We used a $CD34^+$ fraction because it was widely used both in sheep in utero transplantation (*18*, *19*) and in clinical transplantation of hematopoietic stem cells (*20*), although some investigators used a human $CD34^-$ fraction for sheep in utero transplantation and obtained hematopoietic chimera as well or even better in serial transplantation experiments (*21*). For the present, however, we considered that the $CD34^+$ fraction was an appropriate standard with which to compare the repopulating ability of day-6 cynomolgus ES-derived cells as well. The chimeric fraction in bone-marrow CFU after in utero transplantation of $CD34^+$ cells was 1% to 9% (average 3.7%). Fractions of donor-derived cells in the peripheral blood were also very low (<0.1%) after the transplantation of $CD34^+$ cells. The average cell numbers necessary to achieve 1% chimerism in CFU after birth were estimated to be 4.3×10^7 and 6.0×10^5 for day-6 cynomolgus ES-derived cells and human cord-blood $CD34^+$ cells, respectively.

DISCUSSION

We have generated long-term hematopoietic microchimerism in sheep derived from ES cells. Achieving hematopoietic reconstitution from ES cells has been an enormous challenge. The difficulty is attributable to the developmental immaturity of ES-derived cells, which most closely resemble primitive embryonic yolk-sac hematopoietic progenitors (*22*). The processes governing embryonic versus adult blood formation are distinct, and mouse ES cells do not contribute

TABLE 1. In utero transplantation and donor-cell engraftment in sheep

Transplanted cells	Animals (sex)	In utero transplantation: Transplanted cell number per fetus	In utero transplantation: Gestational day at transplantation (Full term 147 days)	Donor cell-derived CFU in bone marrow* (months posttransplant): After birth	Donor cell-derived CFU in bone marrow* (months posttransplant): After human SCF administration	Average cell number necessary for 1% chimerism
Day 6 ES-derived cells	No. 57 (male)	5.0×10^7	67	1.1% (1/91) at 3.5 months	ND	4.3×10^7
	No. 55 (female)	5.0×10^7	55	1.1% (1/91) at 5 months	ND	
	No. 141 (male)	7.8×10^7	73	1.1% (1/91) at 3 months	13.2% (12/91) at 6 months	
	No. 182 (male)	1.4×10^7	66	1.6% (1/63) at 3 months	8.8% (8/91) at 6 months	
Human cord blood $CD34^+$ cells	No. 71-1 (male)	2.0×10^6	69	8.8% (8/91) at 1 months	ND	6.0×10^5
	No. 71-2 (female)	2.0×10^6	69	4.4% (4/91) at 3.5 months	ND	
	No. 99-1 (male)	1.5×10^6	79	1.1% (1/91) at 1 months	ND	
	No. 99-2 (female)	1.5×10^6	79	4.4% (4/91) at 2 months	ND	

* Percent cynomolgus CFU was calculated by dividing the number of CFU positive for the cynomolgus-specific β2-microglobulin gene sequence by the total number of CFU analyzed.

CFU, colony-forming unit; ND, not done; ES, embryonic stem.

to hematopoietic reconstitution in irradiated mice, unlike stem cells isolated from adult bone marrow (*23, 24*). An effective approach to this obstacle has recently been reported. Genetically engineering mouse ES cells to express a specific transcription factor (HoxB4) or signaling molecule (STAT5) during a specific developmental window has resulted in hematopoietic reconstitution from ES cells in irradiated mice (*5, 25*). However, the requirement for artificial over-expression of those genes is undesirable for clinical applications. Our method to develop hematopoietic engraftment from primate ES cells does not require genetic manipulation, and it is a combination of two steps: in vitro differentiation into mesodermal cells followed by in vivo development into hematopoietic cells in the proper microenvironment of fetal sheep liver.

To examine whether transplanted day-6 ES-derived cells engraft in other tissues, we did one more in utero transplantation experiment using same cultured cynomolgus ES cells (mesodermal cells at day 6) and delivered a fetus at 1 month after transplantation. Although cynomolgus CFU were detected in fetal liver and cord blood, cynomolgus cells were not detectable in any other tissues by a sensitive PCR after the fetal blood was completely washed out (data not shown). Therefore, our method appears to direct the fate of primate ES cells to the hematopoietic lineage.

We assumed that the initial in vitro culture of ES cells into mesodermal cells is crucial for successful engraftment in the fetal liver. In fact, when we transplanted undifferentiated (day 0) cynomolgus ES cells into fetal sheep (n=2), we failed to detect cynomolgus CFU or other nonhematopoietic cells in any fetal tissue at 1 month posttransplantation as assessed by a sensitive PCR (data not shown). Therefore, undifferentiated ES cells do not appear to engraft in fetal sheep, unlike adult mesenchymal or hematopoietic stem cells (*8, 11*), but day-6 ES derivatives (mesodermal cells) can engraft and are susceptible to hematopoietic specification in the fetal-liver microenvironment. For the successful initial in vitro culture of ES cells, there are some points to be noted. First, we used stromal OP9 cells as a feeder. Second, we included BMP-4 and VEGF in the culture medium. The coculture with OP9 and inclusion of BMP-4 and VEGF promote hematopoietic differentiation of ES cells (*13, 14, 26–28*). Finally, we cultured cells in vitro for a relatively short period (6 days) to avoid over-maturation of cells (*14*). The xenogeneic fetal liver is able to provide such cultured ES cells with an adequate microenvironment for support of hematopoietic development (*29*). Factors present in the fetal liver responsible for the development remain to be elucidated.

On the other hand, several issues remain to be further investigated. Although the chimeric fractions increased by several-fold (up to 13%) after SCF administration, the increase was transient. To stably enhance the ES-derived chimeric fraction, an enrichment of cells responsible for the engraftment will be needed before transplantation, as suggested from Table 1 (see average cell numbers necessary to achieve 1% chimerism). Another issue is a very low level of donor-derived cells in the peripheral blood. Ours is quite different from previous human-to-fetal sheep experiments that demonstrated easily detectable peripheral blood chimerism (*16, 19*). This has hampered the lineage analysis of cynomolgus cells in our sheep to obtain further evidence to support hematopoietic differentiation from ES cells. Low levels of pe-

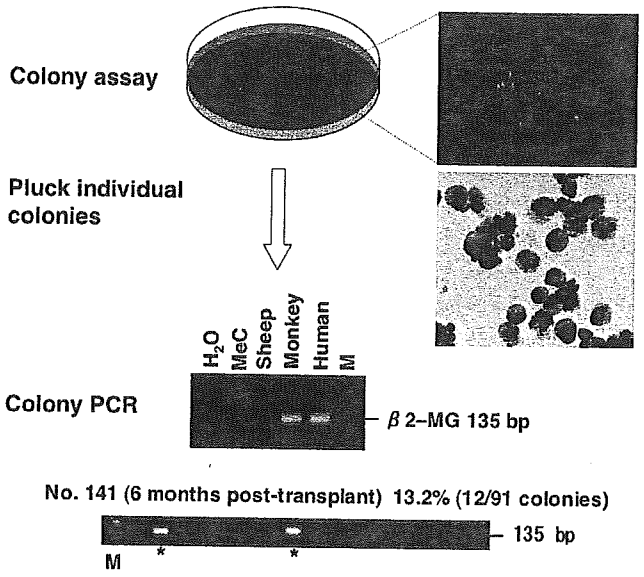

FIGURE 2. Assessment of cynomolgus hematopoiesis in sheep after birth. Bone-marrow cells were harvested from lambs and placed in methylcellulose. Hematopoietic colonies were thus formed. A cytospin specimen (stained with the Wright-Giemsa method) of plucked myeloid colonies demonstrated mature neutrophils. Each colony was derived from a single cynomolgus or sheep hematopoietic progenitor cell. To detect cynomolgus colonies, individual colonies were plucked and examined for cynomolgus-specific β2-microglobulin (β2-MG) sequences by polymerase chain reaction (PCR). PCR products were analyzed on 2% agarose gel. Plucked methylcellulose (MeC) alone (not containing colonies) and sheep colonies generated no bands by the PCR. Colony PCR was repeated at least twice. Representative colony PCR results of sheep No. 141 shown. *Bands positive for the cynomolgus-specific sequence. M, molecular weight marker.

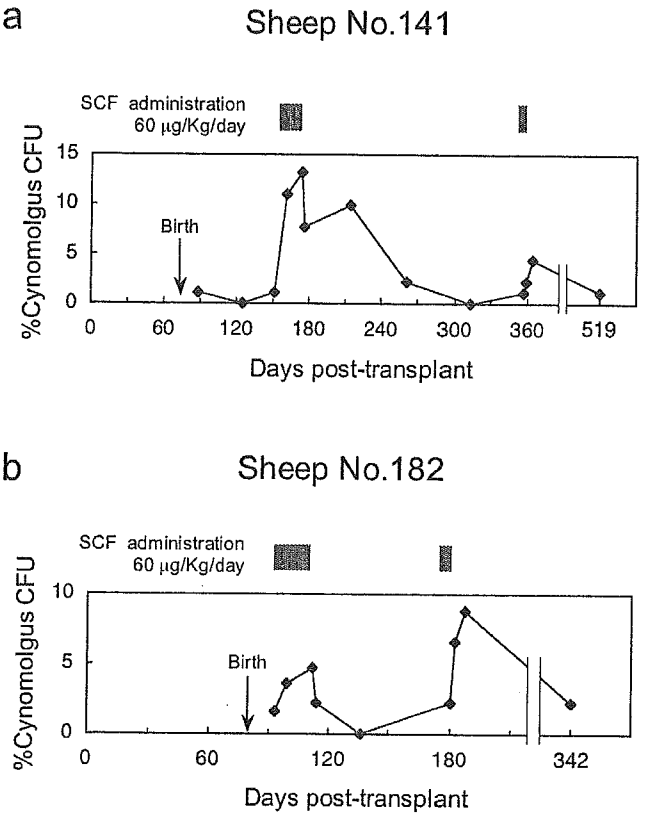

FIGURE 3. Time course of hematopoietic chimerism in the sheep receiving human SCF. (a) In sheep No. 141, human SCF was intraperitoneally administered at 60 μg/kg once a day from day 156 posttransplantation for 18 days. SCF administration was then stopped and tried again from day 352 for 5 days. (b) In sheep No. 182, human SCF was similarly administered from day 94 posttransplantation for 18 days, followed by a second administration at the same dose from day 175 for 5 days. Horizontal axis indicates days after transplantation. Vertical axis shows cynomolgus/sheep chimerism (a ratio of cynomolgus vs. sheep CFU in the bone marrow). Period of human SCF administration (*gray bars*).

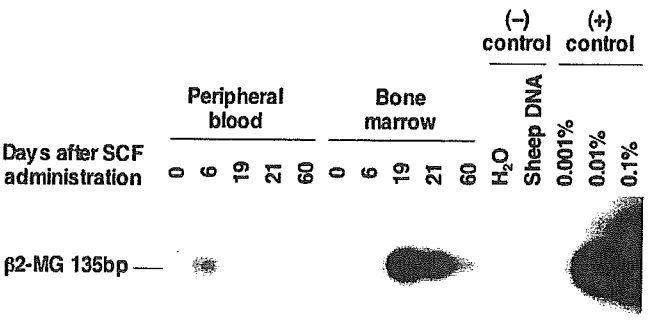

FIGURE 4. Detection of cynomolgus cells in the circulation. DNA was extracted from whole peripheral blood or bone-marrow nucleated cells after birth and subjected to cynomolgus-specific β2-microglobulin (β2-MG) PCR, and Southern blot analysis. Data before and after SCF administration shown. Positive controls show 0.001, 0.01, and 0.1% chimerism (cynomolgus to sheep). Cynomolgus cells were detectable after SCF administration, albeit at low levels (<0.1%).

ripheral chimerism were, however, also found after human $CD34^+$ cells were transplanted in our study. Therefore, the issue is not specific to transplanted ES cells, but is likely to be attributable to the experimental system. A possible explanation may be the different sheep species used in our study (Suffolk versus Dorset Merino). Another possible explanation is immune responses caused by relatively later gestational ages (in the second trimester, day 55–79) at transplantation in our study compared with other recent studies (day 40–45) (*19*). The immune response may have cleared xenogeneic cells from the circulation. The existence of microchimerism does not necessarily guarantee or predict tolerance in other systems (*30, 31*). "The window of opportunity" for successful tolerance induction may be earlier and narrower. To avoid sensitization, transplantation at earlier days may be more efficacious.

In conclusion, long-term hematopoietic microchimerism from primate ES cells is possible after in vitro differentiation to mesodermal cells, followed by in vivo transplantation into the fetal-liver microenvironment. We have used nonhuman primate ES cells in the current study, but if human ES cells are similarly used, human blood cells can be generated in sheep. This procedure should allow for further investigation.

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ORIGINAL ARTICLE

Effect of tolerance induction to immunodominant T-cell epitopes of Sendai virus on gene expression following repeat administration to lung

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Sendai virus (SeV) is able to transfect airway epithelial cells efficiently in vivo. *However, as with other viral vectors, repeated administration leads to reduced gene expression. We have investigated the impact of inducing immunological tolerance to immunodominant T-cell epitopes on gene expression following repeated administration. Immunodominant CD4 and CD8 T-cell peptide epitopes of SeV were administered to C57BL/6 mice intranasally 10 days before the first virus administration with transmission-incompetent F-protein-deleted ΔF/SeV-GFP. At 21 days after the first virus administration, mice were again transfected with ΔF/SeV. To avoid interference of anti-GFP antibodies, the second transfection was carried out with ΔF/SeV-lacZ. At 2 days after the final transfection lung β-galactosidase expression, T-cell proliferation and antibody responses were measured. A state of 'split tolerance' was achieved with reduced T-cell proliferation, but no impact on antiviral antibody production. There was no enhancement of expression on repeat administration; instead, T-cell tolerance was, paradoxically, associated with a more profound extinction of viral expression. Multiple immune mechanisms operate to eradicate viruses from the lung, and these findings indicate that impeding the adaptive T-cell response to the immunodominant viral epitope is not sufficient to prevent the process.*
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Keywords: *lung; Sendai virus; T cells; tolerization*

Introduction

Cystic fibrosis (CF) is a lethal, monogenic disease, caused by mutations in the CF conductance regulator (*CFTR*) gene.[1] CF is an important candidate for gene therapy-based treatments because the median life expectancy is only approximately 30 years and treatment could potentially be administered to the lung noninvasively. CFTR is expressed in the lung in submucosal glands and airway epithelial cells (AECs),[2] the latter likely being the more important target for CF gene therapy. Gene transfer to the airway epithelium via the apical membrane using existing viral and nonviral gene transfer agents (GTAs) is generally inefficient on topical administration. Extracellular barriers such as mucus, mucociliary clearance and glycocalyx proteins limit the access of GTAs to the apical membrane.[3] In addition, the apical membrane of differentiated AECs is relatively devoid of viral receptors, such as those for adenovirus and adeno-associated virus (AAV) type 2. It also has a low basal and stimulated rate of endocytosis, thus discouraging vector entry. Transduction efficiency via the basolateral membrane is generally more efficient, but requires opening of tight junctions, which may not be a clinically viable approach in a disease with a high bacterial load in the lung. Even if the GTA can overcome these extracellular barriers, several intracellular obstacles such as endosomal escape, cytoplasmic trafficking and passage through the nuclear pore complex remain.

We have recently shown that, in contrast to adenovirus and AAV, recombinant Sendai virus (SeV), a murine paramyxovirus, transduces AECs in a variety of animal models efficiently *in vivo*.[4,5] Importantly, SeV carrying the *CFTR* cDNA is able to correct partially the characteristic CFTR-dependent chloride transport defect in the nasal epithelium of CF knockout mice.[6] Several aspects of SeV biology may explain the high gene transfer efficiency into AECs. Importantly, SeV uses cholesterol and sialic acid as receptors. Both are present on the surface of most cell types. Further, SeV requires short contact time with the target cell for internalization, and replicates in the cytoplasm of transduced cells, circumventing the nuclear membrane barrier. Gene expression is transient, with peak expression approximately 48 h after transfection, generally returning to baseline values within 2 weeks of

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transfection.[7] The mouse is a natural host for SeV and wild-type SeV infection causes pneumonia. It is currently unclear why SeV-mediated gene expression is transient. SeV-induced pulmonary inflammation is dose- and time-dependent. At high virus doses accumulation of lymphoid tissue around blood vessels and airways can be observed (J Zhu, personal communication), but elimination of infected cells is unlikely to be the only reason for transient gene expression. If SeV is to be useful in the treatment of a chronic disease such as CF, then sustained or repeated gene expression for the life-time of the patient is required.

Any therapeutic strategy must address the fact that the lung is highly evolved to recognize and eliminate pathogens using a multitude of defense mechanisms.[8] Repeat administration of adenovirus to the lung is not possible, and a variety of strategies to overcome this have been studied.[9–11] However, the efficient adaptive and innate immune responses have thus far prevented successful readministration (reviewed in Ferrari *et al.*[12]). It has been suggested that AAV may be less immunogenic than adenovirus due to the suboptimal ability of the virus to transduce dendritic cells;[13] however, studies have generated conflicting results.[13–17] During SeV replication, viral proteins are presented on the cell surface of AEC, which can act as antigen-presenting cells.[18,19] Strategies involving nonspecific immunosuppression to enable repeated SeV administration are not an option in CF because the lungs of patients are chronically infected with pathogenic bacteria. However, we hypothesized that a strategy involving the induction of specific immunological tolerance to components of the SeV might facilitate repeat administration of SeV while leaving other defenses intact.

Since the early days of cellular immunology when it was noted that administration of antigen at very high or low doses could result in specific unresponsiveness, many parameters of tolerance induction have been characterized.[20] Route of administration is one important factor, oral and intranasal pre-administration of soluble peptide having been shown to induce tolerance in murine models of autoimmunity, transplantation and allergy.[21–24] Induction of tolerance by peptide has also been successfully applied to humans.[25,26] There has been less impetus to investigate the induction of therapeutic tolerance to eliminate antiviral responses, but a number of examples have been described.[27–29] Despite the potential complexity of antiviral T-cell responses, the recognition of virus both in mouse models and in humans is often highly focused on a small number of epitopes, making peptide-induced tolerance a realistic possibility.[30–32] Immunological tolerance may act through a number of different mechanisms, including deletion of reactive clones, anergy through incomplete or altered signalling and regulation by regulatory T cells. Here we evaluate the impact of inducing intranasal tolerance to the CD4 and CD8 immunodominant T-cell epitopes of SeV on gene expression following repeated administration to the airways. This approach is based on the premise that antibody responses (except for rare examples such as the T-cell-independent responses to bacterial polysaccharides) are absolutely dependent on CD4 T-cell help, so that tolerance of T cells should impact also on the neutralizing antibody response.

Results

Repeat administration of ΔF/SeV to the lung leads to reduced gene expression

A schematic diagram illustrating the dosing regime and viral construct used is shown in Figure 1. To assess the effect of repeat administration of ΔF/SeV to the lungs, animals were transduced with ΔF/SeV-GFP on day 0, followed by repeat administration of ΔF/SeV-lacZ on day 21. β-galactosidase (βgal) expression in lung homogenate was measured 2 days after ΔF/SeV-lacZ transduction and compared to animals receiving only one administration of ΔF/SeV-lacZ. Figure 2 shows that although βgal expression levels after repeat administration were still significantly ($P<0.005$) higher than in untreated mice ($n=22$), expression was significantly ($P<0.05$) reduced by 60% when compared to one administration ($n=22$).

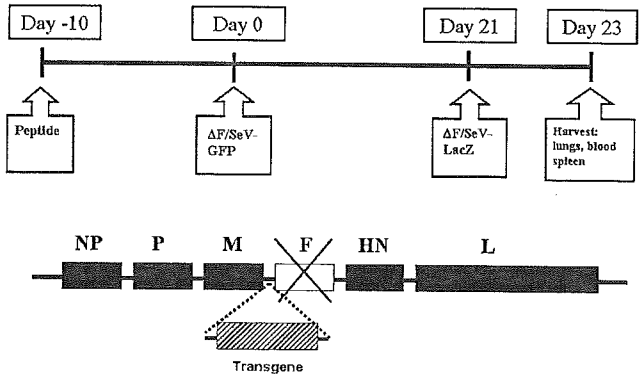

Figure 1 Schematic representation of the protocol for repeat administration of ΔF/SeV to the airways and the viral constructs used. The F protein is deleted from the viral genome and provided *in trans* during vector production. The transgene is inserted between the M and HN proteins.

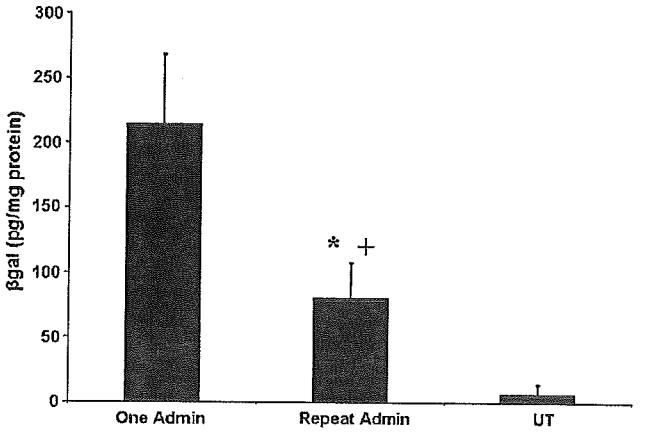

Figure 2 βgal expression after a single and repeat administration of the ΔF/SeV, to the airways. For repeated administration of ΔF/SeV lungs of mice were transduced with ΔF/SeV-GFP (day 0) and ΔF/SeV-lacZ (day 21) using the standard 'sniffing' protocol (repeat admin). The single administration (one admin) group received only the ΔF/SeV-lacZ virus. Lungs were harvested 2 days after ΔF/SeV-lacZ transfection and βgal expression was analyzed. Data are presented as mean ± s.e.m. $^*P<0.005$ when compared to untreated (UT), $^+P<0.05$ when compared to one administration, $n=22$/group.

Intranasal pretreatment with CD4 and CD8 T-cell SeV immunodominant peptide epitopes impacts on T-cell proliferation

In an attempt to improve transfection efficiency after repeat administration of the ΔF/SeV, animals were pretreated intranasally with either the CD4 or CD8 immunodominant T-cell peptide epitopes to SeV at different doses 10 days before the first virus administration (Figure 1). In the C57BL/6 mouse model of lung immunity to SeV infection, the CD8 T-cell response is highly focused on the nucleoprotein (NP) 324–332 epitope and the CD4 T-cell response on hemaglutinin (HN) 419–433.[30,33] One control group of mice received an irrelevant T-cell epitope peptide, ovalbumin (OVA) 326–339, while the other control group of mice received no intranasal peptide pretreatment. The administration of SeV vectors to the mice primes T-cell proliferation to the CD4 (NP 324–332) and CD8 (HN 419–433) immunodominant epitopes. Intranasal pretreatment with a single dose of 1 μg/ml of the CD4 immunodominant peptide epitope resulted in a significant ($P<0.05$) reduction in T-cell proliferation of splenocytes *in vitro*, relative to the no peptide or the irrelevant peptide control groups. This reduced proliferation (equivalent to 2.4±0.7- and 1.5±0.6-fold greater proliferation than in nonantigen-exposed controls after challenge with CD4 and CD8 immunodominant epitopes, respectively) was seen when splenocytes were challenged *in vitro* with 50 μg/ml of either of the CD4 (Figure 3a) or the CD8 (Figure 3b) immunodominant epitopes.

The reduction in proliferation after challenge with the CD8 epitope in the group of mice pretreated with 1 μg/ml of the CD4 peptide epitope reflects the dependence of CD8 responses on CD4 T-cell help. Intranasal pretreatment with 1 μg/ml of the CD4, but not the CD8, immunodominant peptide epitope resulted in the induction of T-cell tolerance.

Intranasal pretreatment with CD4 and CD8 T-cell SeV immunodominant peptide epitopes has no impact on anti-SeV neutralizing antibodies

To obtain a functional readout of neutralizing antibody titers, ΔF/SeV-lacZ virus was pretreated with serum from: (1) untreated mice; (2) mice that had received a single administration of ΔF/SeV-lacZ; (3) mice that had received a repeat administration of ΔF/SeV. Virus were then used to infect HeLa cells. A representative graph is shown in Figure 4a. At low serum dilutions, all the mice that had received two doses of ΔF/SeV produced anti-SeV neutralizing antibodies and showed reduced transfection of HeLa cells. The transduction efficiency was restored by subsequent serum dilutions. In contrast, serum from untreated control mice and mice receiving only one administration of ΔF/SeV (serum collected two days after transduction) did not produce any neutralizing antibodies. In Figure 4b, the serum dilution at which 50% of βgal expression of the untreated control mice was reached is plotted. There was no significant difference in neutralizing antibody measurements between the groups. In contrast to the reduced T-cell proliferation following pretreatment with intranasally-administered peptide, there was no evidence of any reduction in the neutralizing antibody titer indicating 'split tolerance'.

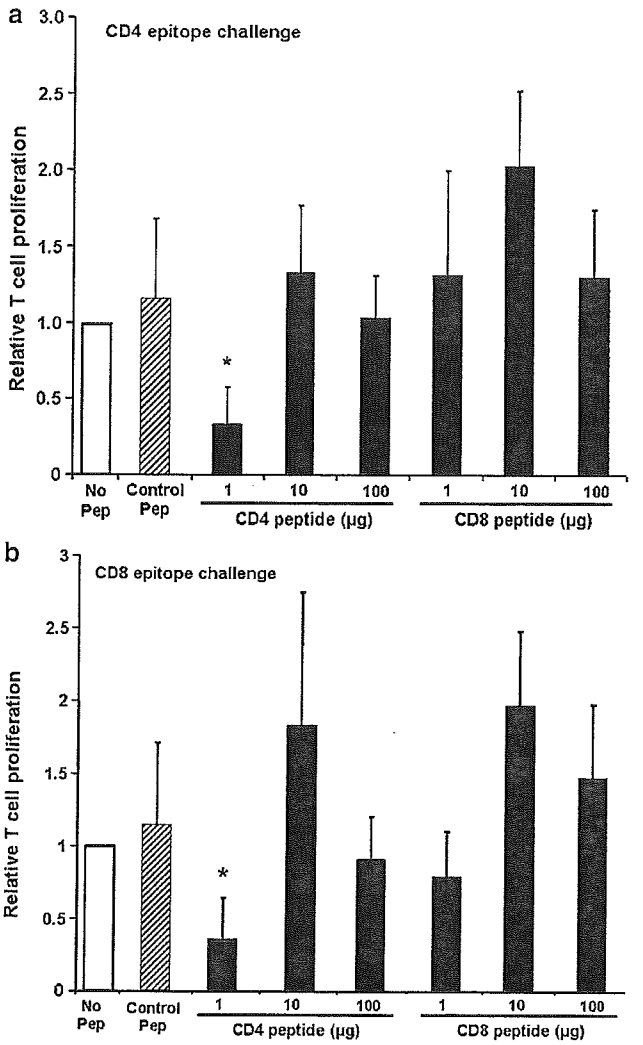

Figure 3 T-cell proliferation against CD4 (a) and CD8 (b) immunodominant T-cell epitopes of SeV following intranasal pretreatment with the CD4 and CD8 epitopes. Splenocytes were incubated in triplicate with peptide at the concentrations indicated. Cultures were harvested at 72 h for counting in a beta-scintillation counter. For the final 12 h of culture, 1 μCi of ^{3}H-thymidine was added to each well. The mean value of the triplicates was calculated and ^{3}H-thymidine incorporation data were standardized by calculating the relative ratio compared to the no peptide control group. The relative proliferation in the presence of 50 μg/ml of the CD4 (a) and CD8 (b) immunodominant epitope to SeV is shown. Groups of mice were administered the concentrations shown of the CD4 or CD8 immunodominant epitopes, control peptide or no peptide to the lungs on day −10 prior to transfection with ΔF/SeV. Data were normalized to the no peptide control group and presented as mean±s.e.m. *$P<0.05$ when compared to no peptide or irrelevant peptide control group ($n=6$–12/group).

Intranasal pretreatment with CD4 and CD8 T-cell SeV immunodominant peptide does not improve levels of gene expression after repeat administration of ΔF/SeV

To assess the effect of tolerization on repeat administration, βgal expression was measured in lung homogenate 2 days after the second ΔF/SeV transfection. Peptide preadministration did not improve gene expression. Indeed, βgal expression was reduced in mice that had a reduction in T-cell proliferation after immunization

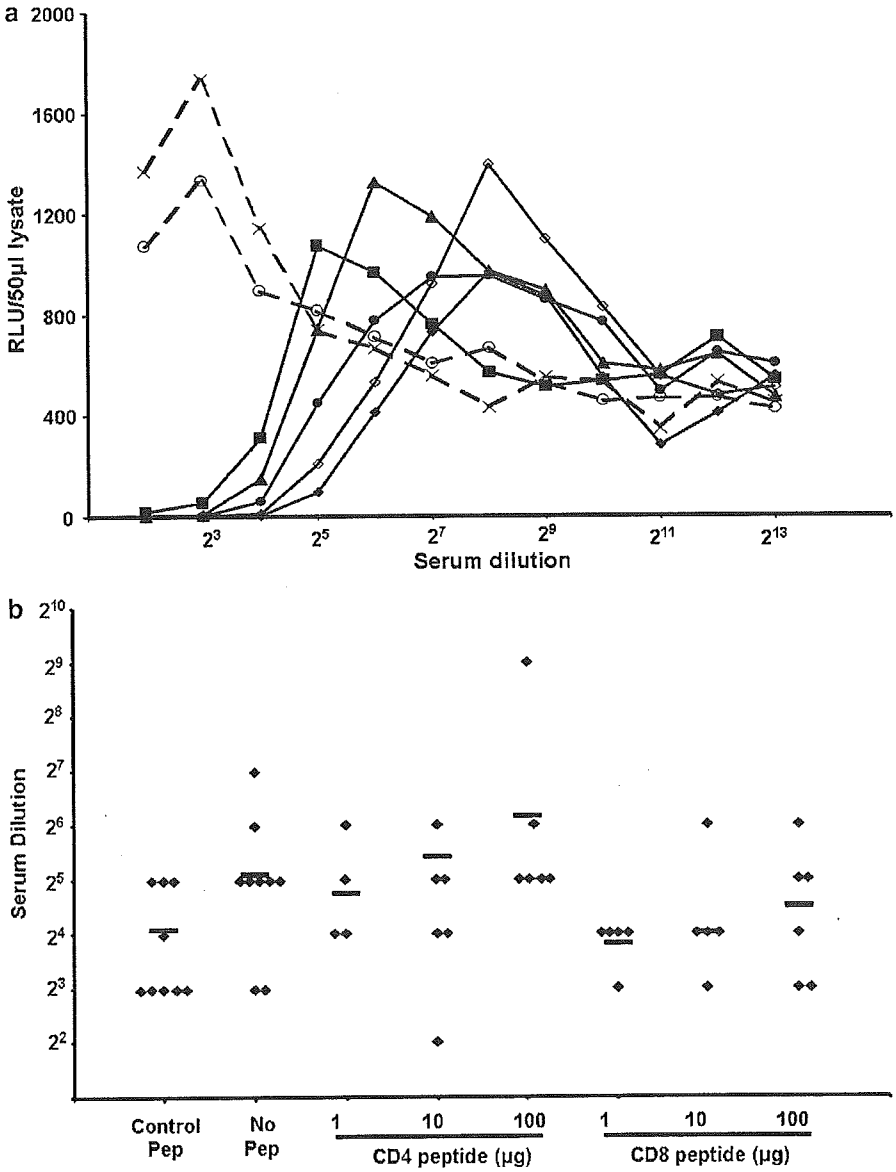

Figure 4 Anti-SeV neutralizing antibodies following pretreatment with CD4 and CD8 immunodominant T-cell epitopes to SeV. Neutralizing serum antibodies were measured by incubating ΔF/SeV-lacZ with serial dilutions of serum from all animals, with subsequent transfection of HeLa cells. A representative example for each group is shown (**a**) (cross = untreated, open circle = one administration, open diamond = no peptide animal, closed circle = control peptide, closed diamond = 1 μg CD4 peptide, closed square = 10 μg CD4 peptide, closed triangle = 100 μg CD4 peptide). The serial dilution at which 50% transfection efficiency of virus incubated with serum from untreated mice was reached was then plotted for each animal (**b**). Each diamond represent one animal and horizontal bars represent the mean.

with 1 μg/ml of CD4 immunodominant peptide compared to control groups (Figure 5).

Discussion

Administration of viral vectors to the lung must overcome the significant hurdle of highly evolved immune mechanisms that exist to recognize and eliminate pathogens.[8] As expected, we show here that βgal expression after repeat administration of recombinant ΔF/SeV-lacZ is comparatively inefficient due to potent immune responses to the vector. We, therefore, assessed the effect of tolerance induction to the immunodominant CD4 and CD8 epitopes from SeV and showed that, despite achieving a reduction in T-cell proliferation via the CD4 epitope, there was no reduction of anti-SeV neutralizing antibodies or increase in gene expression.

Nonviral GTAs are less efficient in transfecting AECs than viral vectors. However, viral GTAs are immunogenic and a significant reduction in gene expression after repeat administration has previously been reported for commonly used viral GTAs such as adenovirus and AAV, and is due to the generation of immune responses against viral proteins.[34]

First-generation recombinant SeV retains all viral genes,[35] and are transmission-competent. Second-generation, attenuated, nontransmissable SeV has been generated by deleting the F protein (ΔF/SeV) required for viral entry into the cell.[36] We have demonstrated that second generation ΔF/SeV is as efficient as first-generation viruses in transfecting AECs.[7] Following transduc-

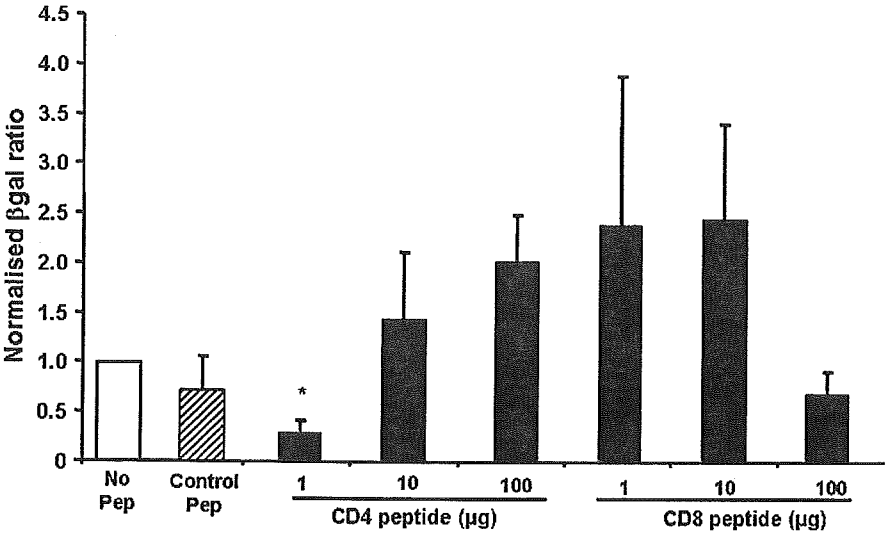

Figure 5 βgal expression after repeated administration of ΔF/SeV following pretreatment using intranasal CD4 and CD8 immunodominant T-cell epitopes to SeV. To assess the effect of peptide tolerization on gene transfer efficiency after repeat administration, peptide was administered to the lungs of mice in a single bolus of 100 μl using the standard 'sniffing' procedure on day −10. Mice were transfected with ΔF/SeV-GFP on day 0 and with ΔF/SeV-lacZ on day 21. Lungs were harvested 2 days after ΔF/SeV-lacZ transfection and βgal expression analyzed. Data were normalized to the no peptide control group and presented as mean ± s.e.m. Please refer to Figure 2 for absolute βgal expression values. The no peptide control group is identical to the repeat administration group in Figure 2. Please refer to $^*P<0.05$ when compared to no peptide control group, $n=12$/group.

tion, ΔF/SeV replicates in the cytoplasm and, although the F protein-deleted second-generation ΔF/SeV is transmission-incompetent, viral proteins are incorporated into the cell membrane of the infected cell and virus-like noninfective particles are released from the cells. Third-generation SeV vectors have been further depleted of the HN and M proteins, but the remaining viral proteins (NP, P, L) cannot be deleted, because they are essential for recombinant protein production.[37] Importantly, however, the complete removal of all viral proteins from adenoviral vectors has not improved their capacity for repeat administration.[38] Alternative strategies to enable repeat administration of SeV clearly need to be developed. Nonspecific immune suppression is not clinically desirable in CF, particularly in the context of SeV gene therapy, which would require prolonged immune suppression for the duration of SeV-mediated gene expression, because of the persistent virus replication. Specific tolerization of CF patients to SeV, which is not a human pathogen, however, might be a clinically feasible option. The induction of specific immunological tolerance is a manipulation that has been successfully applied in a number of other experimental and clinical settings.[26,27]

A wide variety of strategies have previously been investigated for the induction of effective immunological tolerance against viral antigens. While the goal in most systems is to promote antiviral immunity, such responses may be undesirable when they lead to loss of therapeutic gene expression as shown here, or when the response is itself associated with excessive immunopathology.[28,39] Immunological tolerance to viruses has previously been achieved by administration of tolerogenic peptides,[28,40] by oral administration of antigen,[41] by giving virus under the cover of either anti-CD4 or anti-CD40L antibody,[39] or by giving apathogenic virus intravenously.[27,42] While neonatal administration of virus might be expected to induce tolerance, this can alternatively lead in some cases to highly efficient priming of responses. More robust tolerance may be achieved through neonatal expression, which is specifically targeted to the thymus.[29] However, this approach raises concerns about the ease of translation to clinical practice. In light of these considerations, we decided to investigate peptide-induced tolerance, which has a strong track record of translation to the clinic in a range of diseases.[26,43] While there are theoretical concerns that it might not be possible to achieve immunological tolerance to a complex set of viral antigens through administration of a single epitope, analysis of viral immune responses suggests that T cells often focus on a rather small number of immunodominant epitopes.[44] Furthermore, peptides are relatively cheap to prepare, easily administered and well tolerated.

A large body of work by David Woodland and others on protective immunity against SeV indicates a vital role of T cells.[45,46] We, therefore, administered CD4 and CD8-immuno dominant SeV epitopes to the lungs of mice 10 days before ΔF/SeV. While tolerance experiments in mouse models have tended to rely on a form of 'high-dose' tolerance at 100 μg or greater,[23,47] we found, in line with recent findings from clinical studies,[25] that tolerance was actually induced after a single dose of 1 μg peptide, and not at higher doses. Interestingly, a single tolerizing dose of the CD4 epitope largely abrogated not only the response of T cells to this epitope, but also to the immunodominant CD8 class I restricted epitope, presumably because the latter response is dependent on help from CD4T cells. Despite reasonably robust T-cell tolerance, we obtained no evidence for enhanced levels of gene expression from repeat virus administration. The most likely explanation for this is that we generated 'split tolerance' that left neutralizing antibody titers unaltered, and elimination of repeat virus administration was here

achieved through antibody neutralization of input virions. 'Split tolerance', affecting one component of the immune system, but not others, is a frequent observation in viral systems, presumably because a multi-layered immune response against pathogenic viruses has been a strong evolutionary driving force.[27,48] How could an effective neutralizing antibody response develop in the face of a strong reduction in the peptide-specific CD4 helper T-cell response? One possibility is that there is sufficient, residual T-cell effector function available to supply a low level of cytokine support for the B-cell response. Another possibility is that, as a result of peptide-specific tolerance induction, the antiviral response shifts to subdominant epitopes. Analysis of these hypotheses will require further investigation. Even if one could achieve absolute tolerance with respect to the antibody and T-cell limbs of the adaptive immune response, there is still the likelihood of an efficient, or even an enhanced response using other mechanisms, for example, natural killer (NK) 1.1 T cells.[49] Thus, Scaria *et al*.[50] have demonstrated previously that inhibition of cytotoxic T cells (CTLs) through overexpression of ICP47, a protein that prevents MHC class I-mediated antigen presentation to CD8 T cells, leads to an increased activation of NK cells. Indeed, in our study, elimination of βgal viral expression appears enhanced in the presence of split tolerance. This is an important caveat for immunomodulation initiatives: compensatory mechanisms including NK cells that replace the normal, adaptive T-cell response may be even more potent.

In conclusion, induction of tolerance using an immunodominant CD4 peptide epitope does not allow more efficient gene expression following a second administration of a SeV vector.

Materials and methods

Peptide and recombinant SeV preparation

Peptides were synthesized on a 20–40 mg scale (Bio-Synthesis Incorporated, Lewisville, TX, USA) and arrived lyophilized. [(CD4 epitope – SeV HN protein 419–433): NH2-VYIYTRSSGWHSQLQIG-OH, (CD8 epitope – SeV NP protein 324–332): NH2-FAPGNYPAL-OH, control peptide epitope OVA 326–339: NH2-AVHAA-HAEINEAGR-OH]. The peptides were dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored at a concentration of 75 mg/ml at −80°C. Final working concentrations of peptide for experimental use were made up on the day using PBS.

The generation and propagation of the recombinant ΔF/SeV vector carrying a green fluorescent protein (ΔF/SeV-GFP) or a lacZ reporter gene (ΔF/SeV-LacZ) was carried out as described previously.[4] The supernatant of LLC-MK2/F7 cells containing infectious particles was subsequently purified, concentrated and stored at −80°C. Virus titer was determined by infecting LLC-MK2 cells and counting the number of βgal or GFP-expressing cells. For both viruses, the titer was expressed as Cell Infectious Units (CIU) per ml.

Administration of peptide and virus to mouse lung

C57BL/6 mice (female 6–10 weeks) were anesthetized with metophane (Medical Developments Australia Pty Ltd, Springvale, Australia) and the peptide (1, 10 or 100 μg in 100 μl PBS) was placed as a single bolus into the nasal cavity and the solution rapidly 'sniffed' into the lungs. At 10 days after peptide administration, mice were transduced with ΔF/SeV-GFP (10^6 CIU/mouse in 100 μl PBS), and 21 days later with ΔF/SeV-lacZ (10^7 CIU/ mouse in 100 μl PBS) using the 'sniffing' technique. Control groups receiving no virus or one administration of ΔF/SeV-lacZ were included. At 2 days after the last virus administration, all animals were culled. Lungs from individual mice were snap-frozen in liquid nitrogen and stored at −80°C for βgal quantifiaction. Spleens from individual mice were harvested for T-cell proliferation assays. Blood was collected and stored at 37°C for a minimum of 2 h. After clotting, the blood was spun at 4000 r.p.m. for 10 min and serum was frozen at −80°C for anti-SeV neutralizing antibody measurements.

T-cell proliferation

Spleen cells were disaggregated using fine gauge needles, resuspended in HL1 serum-free medium (Hycor Biomedical, Irvine, CA, USA) supplemented with L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), 30 IU penicillin and 30 μg/ml streptomycin, and 4×10^5 cells/well were aliquoted in triplicate in flat-bottom microculture plates and incubated with peptide at different concentrations to measure T-cell proliferation in response to the CD4 and CD8 immunodominant peptide epitopes of SeV. For the final 12 h of culture, 1 μCi of ^{3}H-thymidine was added to each well. Cultures were harvested at 72 h for counting in a beta scintillation counter. The mean values of the triplicates were calculated and ^{3}H-thymidine incorporation data were standardized by calculating the relative ratio compared to the no peptide control group.

Neutralizing antibody assay

HeLa cells (2500 per well in a 96-well plate) were seeded 1 day before transfection. Serum was heat-inactivated for 40 min at 55°C and 1:2 serial dilutions in DMEM (+1% FCS +1% penicillin/streptomycin) prepared (1:2–1:8192). ΔF/SeV-LacZ (5×10^4 CIU) was added to each serum dilution and incubated at 37°C for 1 h in an incubator (5% CO_2). Following the incubation, the medium is removed from the HeLa cells and replaced with the 100 μl of the serum dilutions containing the virus. Cells were incubated for 5 h at 37°C, after which the serum was removed and replaced with normal growth medium (DMEM+10% FCS and 1% penicillin/streptomycin). At 24 h after transfection the medium was removed, cells are washed with PBS and lysed with 120 μl Universal lysis buffer (Roche), freeze/thawed three times, and frozen at −80°C. βgal protein expression was quantified in homogenates using the luminescent βgal Reporter System 3 (Clontech, UK) according to the manufacturer's recommendations.

Reporter gene assay

Lungs were homogenized in 300 μl Universal lysis buffer (Roche), freeze/thawed three times, spun at 10 000 r.p.m. for 10 min and supernatant was frozen. βgal protein expression was quantified in lung homogenates using the luminescent βgal Reporter System 3 (Clontech, UK) according to the manufacturer's recommendations. Total protein was quantified using the DC Protein Assay Kit

(BioRad Herts, UK) according to the manufacturer's recommendations and expressed as pg βgal/mg total protein. Data were then normalized, by calculating the relative ratio compared to the no peptide control group, which was arbitrarily set to one.

Statistical analysis

All values are expressed as mean±s.e.m. for convenience. Individual data points are shown as diamonds, with the mean indicted by a bar. *N* refers to the number of animals used. The independent-sample Student *t*-test was used after confirmation of normal distribution (Figure 2) or Wilcoxon sign-rank test (Figures 3 and 5). The null hypothesis was rejected at $P<0.05$.

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