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A single vaccination with attenuated SIVmac 239 via the tonsillar route confers partial protection against challenge with SIVmac 251 at a distant mucosal site, the rectum

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1. ABSTRACT

Elucidating the mechanisms that protect monkeys previously immunized with attenuated SIV (SIVDeltanef) against challenge infection with pathogenic virus may reveal new strategies for the development of an effective HIV vaccine. Here we show that a single atraumatic application of SIVDeltanef to the tonsils of four rhesus macaques conferred protection against SIVmac251 applied intrarectally 26 weeks later. While this protection was not complete, i.e., challenge virus could be isolated from all immunized animals, it was reflected by significantly lower viral loads in the blood (weeks 2-16 after challenge, $p<0.01$) and considerably lower loads in lymphoid organs, and more stable peripheral CD4 counts in a proportion of the immunized animals as compared to four non-immunized, SIVmac251-infected control monkeys. SIV-specific humoral as well as systemic and mucosal T cell responses were detected in the immunized animals, but there was no correlation between their magnitude of expression and the level of protection. Analyses of leukocyte subsets in these animals at necropsy (24 weeks after challenge) did not reveal a significantly enhanced proportion of gamma/delta T cells in the tissues of protected monkeys. Therefore, tonsillar application of attenuated SIV induces protection in some animals against a superinfection with wild-type SIV distant at a distant mucosal site.

2. INTRODUCTION

Despite more than 20 years of vaccine research in the field of human and simian immunodeficiency virus (HIV and SIV, respectively) infection, still no protective vaccine has been developed in the appropriate animal model, i.e., the infection of Asian macaque species with SIV. In fact, none of the propagated vaccine candidates has allowed the induction of protection against pathogenic wild-type virus to a similar or better degree than live attenuated SIV strains with deletions in the nef gene (SIVΔnef). To gain valuable insights for the development of vaccines with a more promising safety profile, leading researchers recently called to elucidate the underlying mechanisms of protection (1).

While the mucosa of the oral cavity can serve as entry site for pathogenic HIV (2) and experimentally for SIV (3, 4), the oral route has also successfully been used for the delivery of live vaccines, e.g., against poliomyelitis and typhoid fever (5). Likewise, we have previously shown that the application of SIVΔnef to the palatine and lingual tonsils confers protection against challenge with infectious SIVmac251 applied through the same route (6). We were now interested as to whether tonsillar immunization with SIVΔnef would also confer long-term protection against challenge with wild-type SIV at a distant mucosal site. Although a common mucosal immune system (CMIS) has been proposed early (7, 8), antigen-presentation and the induction of immune responses at different mucosal sites lead to an uneven distribution of immune responses at the various mucosal effector sites, thus indicating a compartmentalization within the CMIS (9, 10). In fact, responses induced through the gut-associated lymphoid tissue (GALT) are mainly (but not exclusively) expressed in the gut itself and in milk of lactating females, whereas those induced in the upper respiratory tract are preferentially expressed in nasal secretions, saliva, and the genital tract. While those data indicate that the route of immunization chosen by us, i.e., through the tonsil, may not necessarily result in protective immune responses in the gut, this might be different after immunization with attenuated SIV. Following tonsillar application of SIVΔnef the virus spreads within 2 weeks to the blood and all lymphoid organs including the GALT where it mainly replicates in $CD4^+$ T lymphocytes (11). However, the gut and GALT also provide one of the first niches for replication of pathogenic HIV and SIV independent of intravenous or rectal infection, which results in a rapid depletion of $CD4^+$ T lymphocytes from the lamina propria (12-14). Thus, challenging immunized animals through the rectal route gives the virus direct access to the memory CD4 cells of the gut, its preferred site of replication, which may override weaker effector immune responses. Nevertheless, any promising HIV vaccine candidate most likely needs to interfere with this early viral amplification at the mucosal sites in order to provide a substantial level of protection. In addition, we intended to investigate in the present study whether SIVΔnef-induced humoral or cellular immune responses or both may contribute to the vaccine efficacy after tonsillar application.

Therefore, we immunized 4 rhesus macaques with SIVΔnef via the tonsils, challenged these animals intrarectally with pathogenic SIVmac251 26 weeks later, and then observed the animals for additional 23/24 weeks. The immunized animals showed considerably lower viral loads than control monkeys although challenge virus could be recovered from either animal at least once during the period of observation. We observed in only one immunized animal declining CD4 T cell counts at the same magnitude as in non-immunized controls. Protection did not correlate with titers of binding and neutralizing antibodies or systemic and mucosal cellular immune responses.

3. MATERIALS AND METHODS

3.1. Animals

Young adult rhesus monkeys (*Macaca mulatta*) were imported from China through R.C. Hartelust BV, Tilburg, The Netherlands. Monkeys were of either sex, had a body weight of 4.1 to 5.9 kg, and were antibody negative for simian T-lymphotropic virus type 1, simian D-type retrovirus, and SIV. Viral application, physical examinations, and bleeding were done under ketamine anesthesia. Animal care was in accordance with guidelines of the German Primate Centre.

3.2. Vaccination with attenuated SIVΔnef and challenge with pathogenic wild-type SIVmac

Four macaques were immunized via the tonsils with the SIVmac239 stock attenuated by a 513 bp-deletion in the *nef* gene and the U3 region (15). Application of SIVΔnef at 10^5 $TCID_{50}$ to the palatine and lingual tonsils was performed as described (4, 11). After 26 weeks, all vaccinees were exposed intrarectally to 50 MID_{50} of SIVmac251 (kindly provided by Steve Norley, Robert-Koch-Institute, Berlin, Germany (11)) along with four untreated controls. All animals were sacrificed at weeks 23/24 following intrarectal application of wild-type SIVmac251.

3.3. Determination of viral loads

Cell-associated virus loads were determined in limiting dilution co-culture assays with mononuclear cells from blood and lymphoid organs as described before (11, 16)). Viral RNA in plasma was determined by quantitative real-time PCR (6). Briefly, virus RNA (virus RNA isolation kit, Qiagen, Valencia, CA) in 10 µl per reaction was amplified by a one-tube real-time RT-PCR using a brilliant single-step QRT-PCR core kit (Stratagene, Amsterdam Zuidoost, The Netherlands). PCR was run in 40-µl reaction tubes in duplicates with 4 µl of 10 x core buffer (5 mM $MgCl_2$, 0.8 mM dNTP mix, 200 nM primers, 150 nM fluorogenic TaqMan probe), 1.25 U Stratascript RT, and 0.025 U *Taq*DNA-polymerase. To quantify plasma viral load, standard RNA templates were created from the p239Sp5′ plasmid (kindly provided by R. M. Ruprecht, Dana–Farber Cancer Institute, Boston; (17)) with a detection limit of 25 viral copies per ml of plasma.

3.4. Characterization of viral isolates

C8166 cells infected with isolates recovered from PBMCs or mononuclear cells from lymphoid organs were

lysed in buffer K (50 mM KCl 15 mM Tris HCl, pH 7.5 2.5 mM $MgCl_2$ 0.5% Tween 20 100 g of proteinase K per ml) for 0.5 h or up to an overnight incubation period, followed by heat inactivation at 95°C for 10 min. The lysates were PCR-amplified with two different primer pairs (Sns and Sna, which flank the deletion in nef and the U3 region of SIV *nef*, and Sns primer and the primer Δna, which is complementary to the U3 region deleted in SIV *nef* (6)), which allowed discriminating between SIVΔnef virus and challenge virus. The PCR conditions were: 94°C for 2 min, 40 cycles of 40 sec at 94°C, 1 min at 61°C, and 1 min at 72°C. Isolates were classified as vaccine virus (V) when the Sns+*Sna*PCR resulted in a 163-bp fragment, as challenge virus (C) when the Sns+Δna PCR resulted in a 556-bp fragment, or as vaccine and challenge virus (V+C) when both PCRs gave products of the expected size.

3.5. *In situ* hybridization (ISH)

The *in situ* hybridization was performed on paraffin sections using a ^{35}S-labeled, single-stranded (antisense) RNA probe of SIVmac239 (Lofstrand Labs, Gaithersburg, MD) as described in detail elsewhere (18, 19). Four to 6 sections from each sample were hybridized because in partially protected animals cells expressing viral RNA could be rare. To increase the penetration of the probe half of the sections were boiled in a domestic pressure cooker in citrate buffer (pH 6.0) for 5 min. The other set of sections was treated with proteinase K (0.01 mg/ml) for 8 min at room temperature. The hybridization was performed for overnight at 45^{o}C. The slides were dipped into photo emulsion (NTB2; Kodak, Rochester, NY, USA), exposed for 7 days, developed, counter-stained with hemalaun, and mounted. As a positive control, cytospin preparations of SIV-infected PBMCs were used. As a negative control, sections were hybridized with a ^{35}S labeled sense probe. The sections were examined with a microscope equipped with epiluminescent illumination (Axiophot; Carl Zeiss Inc., Jena, Germany), 3CD color camera, and a PC-based image analysis system (KS 400; Kontron, Esching, Germany). Cells expressing SIV RNA were counted, the area of the section was measured, and the frequency of cells producing viral RNA cells per mm^2 of tissue section calculated.

3.6. Serology

SIV-specific binding antibodies were detected by ELISA. Briefly, flat-bottom plates (Greiner bio-one, Frickenhausen, Germany) were coated overnight with 50 µl/well of 0.4 µg/ml solution of p27 (EVA643, NIBSC, Potters Bar, UK) or gp130 (EVA670, NIBSC) in 0.1 M carbonate buffer (pH 9.6), and a standard ELISA was performed using 1:200 dilutions of the plasma samples. The optical density (OD) was measured using an ELISA reader (Dynatech Laboratories, Guernsey, UK).

To determine neutralizing activity of vaccine-induced antibodies, sequential dilution of heat-inactivated serum samples (ranging from 1:10 to 1:1000) were incubated with SIVmac251 virus (1:300 of a 7 µg/ml of p27 antigen stock solution) together with normal monkey serum as a complement-source (1:10 final dilution) or heat-inactivated (56 °C, 30 min) normal monkey serum. The virus-antiserum mixture was then cultured with 4 x 10^4 human CD4$^+$ CEMx174-SEAP cells (NIH AIDS Research and Reference Reagent Program, Rockville, MD, USA) in a total volume of 200 µl per well. Cell culture supernatants were harvested on day 7 post infection. The relative extent of replication of SIVmac251 incubated in the different sera was subsequently determined in a SIV p27 core protein ELISA. In brief, ELISA plates (Greiner, Kremsmünster, Austria) were coated overnight at 4 °C with the first mAb, clone AG 3.0 (kindly provided by Stephen Norley, Robert Koch-Institut, Berlin, Germany). After blocking (PBS, 2 % skim milk), the lysed samples (in RPMI, 1 % Igepal (Sigma, Vienna, Austria)) were added to the plate. After washing, the second mAb, clone 37G12 (donated by H. Katinger, Polymune, Vienna, Austria) was applied (1:10000 diluted in PBS, 1 % (w/v) BSA) for further 60 min, and plates were washed again. Then, strepavidin-ß-galactosidase (1:10000 diluted in PBS, 1 % (w/v) BSA with 2 mM $MgCl_2$) and, following an additional washing step, the substrate solution, resorufin-ß-D-galactopyranoside (Boehringer Mannheim, Germany) were added. Bound p27 was detected by measuring the optical density at 550 nm and a reference wavelength at 620 nm.

Neutralisation titers were calculated using the ID50 software available from the National Center for Biotechnology Information (National Institute of Health, author J.L. Spouge). The program calculates the sequence Poisson model, descriptive logistic fit or the Spearman-Karber calculation, with omission of error estimation for the Spearman-Karber fit. The assay was performed in duplicates. Means of two independent experiments are given

3.7. Generation of tissue-derived single cell leukocyte suspensions

At necropsy (23/24 weeks post challenge), 5 to 10 lymph nodes (LNs) from distinct locations, approximately 1/4 spleen, 10 cm jejunum, and 2 to 3 cm of the distal rectum were obtained and used for analyses of T cells and cellular immune responses. To isolate leukocytes from jejunal and rectal sections, mucus was first removed by shaking in 150 mg/l Dithiothreitol (DTT, Sigma, Taufkirchen, Germany) in PBS at 37°C for 15 min, followed by washing in PBS. The mucosa was removed with scalpels from submucosal tissue and cut into pieces approximately 1 cm^2 in size, incubated overnight at 37°C under constant shaking in culture medium (RPM1640, 2 mM glutamine, 0.05 mM 2-mercaptoethanol, 10% fetal bovine serum, and antibiotics), additionally supplemented with gentamycin (50 µg/ml), amphotericin B (2.5 µg/ml; both Biochrom), 0.01% collagenase D, 0.01% DNAse I (both Roche, Basle, Switzerland), and 0.01% soybean trypsin inhibitor (Sigma). On the following morning, the digested tissues were passed through steel sieves, washed in culture medium, and purified by centrifugation in a 30% Percoll (Amersham Biosciences, Uppsala, Sweden) solution, followed by washing of the pellet and Ficoll gradient centrifugation. The resulting interphase was washed twice, resuspended in culture medium, and viable cells were counted in trypan blue solution.

To isolate leukocytes from spleen sections and pooled LNs, the tissue was injected with culture medium containing 400 U/ml collagenase D, disintegrated between tweezers, and incubated for 30 min at 37°C. The suspensions were then passed through steel sieves, washed in medium without collagenase, and filtered through a 70 µm cell strainer (BD Falcon, Heidelberg, Germany). Contaminating erythrocytes in spleen cell suspensions were lysed by incubating the cells in 20 ml of distilled water with 8.26 g/l ammonium chloride, 1 g/l potassium hydrogen carbonate, and 0.5 M EDTA (all Sigma) for approximately 5 min on ice, and the reaction was stopped by adding PBS when hemolysis became visible. Cells were washed, purified by Ficoll gradient centrifugation, resuspended in culture medium, and viable cells were counted by trypan blue exclusion.

For virus isolation and ELISPOT assays mononuclear cells were obtained from lymphoid organs as previously described (11).

3.8. Flow cytometry

Isolated PBMCs, splenic, and LN cells as well as leukocytes obtained from the jejunal and rectal mucosa were characterized by flow cytometry using PE- or FITC-conjugated anti-human mAbs with known cross-reactivity with the following rhesus macaque proteins: CD3, CD4, CD8, CD14, CD20 (all BD Pharmingen, Heidelberg, Germany), and TCR γδ chains (Serotec, Düsseldorf, Germany), or the appropriate isotype controls (BD Pharmingen). $5x10^4$ cells in 5% FCS/10 mM sodium azide/PBS were incubated with the mAbs for 20 min at 4°C, washed three times, fixed in 4% formalin, and analyzed on a FACS Calibur® cytometer using CellQuest Pro® software (both BD, Heidelberg, Germany). Counts of peripheral $CD4^+$ T cells were determined as previously described (20).

3.9. ELISPOT assays and intracellular cytokine staining for the detection of IFN-γ secreting cells

ELISPOT assays were performed using commercially available reagents (Mabtech AB, Hamburg, Germany) (20). Briefly, PBMCs or mononuclear cells from lymphoid organs were resuspended in culture medium and seeded at 2 x 10^5 cells/well in 96-well plates (MAIP S4510, Millipore, Schwalbach, Germany), which had been coated with 1 µg/well of anti-human IFN-γ monoclonal antibody overnight at 4°C. For antigen stimulation, a pool of SIVp26 peptides at 5 µg/ml each (ARP714.1-22, NIBSC), 5 µg/ml SIV Gag peptides (EVA7066.1-16, NIBSC), 5 µg/ml Tat peptides (EVA7069.1-10, NIBSC), or 2 µg/ml Nef peptides (EVA777.1-15, NIBSC) were added to the wells in triplicates. Positive and negative controls consisted of cells stimulated by PMA (50 ng/ml, Sigma) plus ionomycin (1 µg/ml, Calbiochem-Novabiochem, Bad Soden, Germany) and cells kept in medium alone. After 20 h of incubation at 37°C in 5% CO_2, cells were removed and biotinylated anti-human IFN-γ detector antibody was added (0.1 µg/well), followed by the addition of streptavidin-alkaline phosphatase conjugate at 1:1000 in PBS/0.1% FBS. Spots were developed with NBT/BCIP solution (25 µg NBT and 15 µg BCIP in 0.1 M Tris–HCl pH 9.5 per well) for 30 min, the wells were washed with distilled water and air-dried, and spots were counted using a BIOSYS2000 ELISPOT reader. The counts were extrapolated to 10^6 PBMCs.

Additionally, PBMCs as well as splenic, LN, and jejunal (the latter from all but animal 11101, which yielded insufficient cell numbers) leukocyte suspensions obtained at necropsy were analyzed for the presence of IFN-γ secreting SIV-specific T cells by intracellular cytokine staining and flow cytometry. The following antigens were used in the assays at the concentrations indicated: SEB (2.5 µg/ml), the chemically (aldrithiol-2, AT-2) inactivated non-infectious SIVmneE11S (AT-2 SIV, 300 ng p27/ml) provided by the AIDS Vaccine Program (SAIC-Frederick, Inc., National Cancer Institute, Frederick, MD, USA) (21), the parental canarypox virus ALVAC (MOI 10, Virogenetics Corp., Troy, NY, USA), and the SIVgag/pol/env containing recombinant canarypox virus construct vCP180 (MOI 10, Virogenetics)(22). Cells were cultured at $1x10^6$/well in 96-well round bottom plates (Nunc, Roskilde, Denmark) in the presence or absence of antigens. All conditions were stimulated with mAbs against human CD28 and CD49d (both BD Pharmingen) at a final concentration of 2 µg/ml. The cells were incubated for 18 h at 37°C, and Brefeldin A (Sigma) was added at a final concentration of 10 µg/ml for the last 5 h. Cells were harvested, washed, resuspended in 5% FCS/10 mM sodium azide/PBS, and stained with FITC-conjugated mAbs against CD4 and CD8, and PE-Cy5-labeled mAbs against CD69 (Caltag Laboratories, Hamburg, Germany). After incubation for 30 min at 4°C the cells were washed again, fixed with 4% paraformaldehyde in PBS at 4°C for 30 min or overnight, and washed. Cells were then permeabilized with 0.1% saponin (Sigma) for 30 min at 4°C, washed, and resuspended in 5% FCS/10 mM sodium azide/PBS for staining with PE-conjugated mAbs against human IFN-γ (both BD Pharmingen) for 30 min at 4°C. The cells were washed, fixed in 4% formalin, and analyzed on a FACS Calibur® cytometer as described above. Results are presented as % IFN-γ secreting cells in stimulated cell suspensions minus background responses, i.e., % vCP180 minus % ALVAC and % AT-2 minus % medium control.

3.10. Proliferation assays

To assess the SIV-specific proliferative response of T cells assays were set up at necropsy as standard proliferation assays by using single cell suspensions of leukocytes obtained from blood, spleen, LNs, and the mucosa of the jejunum and rectum. AT-2 SIV was added at a concentration of 300 ng p27/ml (21) to $1x10^5$ PBMCs, LN cells, spleen cells, or leukocyte suspensions obtained from the mucosa of jejunum or rectum. Cells were cultured in a final volume of 100 µl/well in triplicates in 96-well round-bottomed plates at 37°C for 5 days. To verify the responsiveness of the T cells in each of the cultures, control cultures were set up with cells incubated in the presence of staphylococcal enterotoxin B (SEB, 5 ng/ml; Alexis Corp., Lausen, Switzerland) or Concavalin A (ConA, 5 µg/ml; Sigma) for 3 days. On d 3 or d 5, respectively, 1 µCi/well ^{3}H-thymidine was added, cells were harvested 18 h later

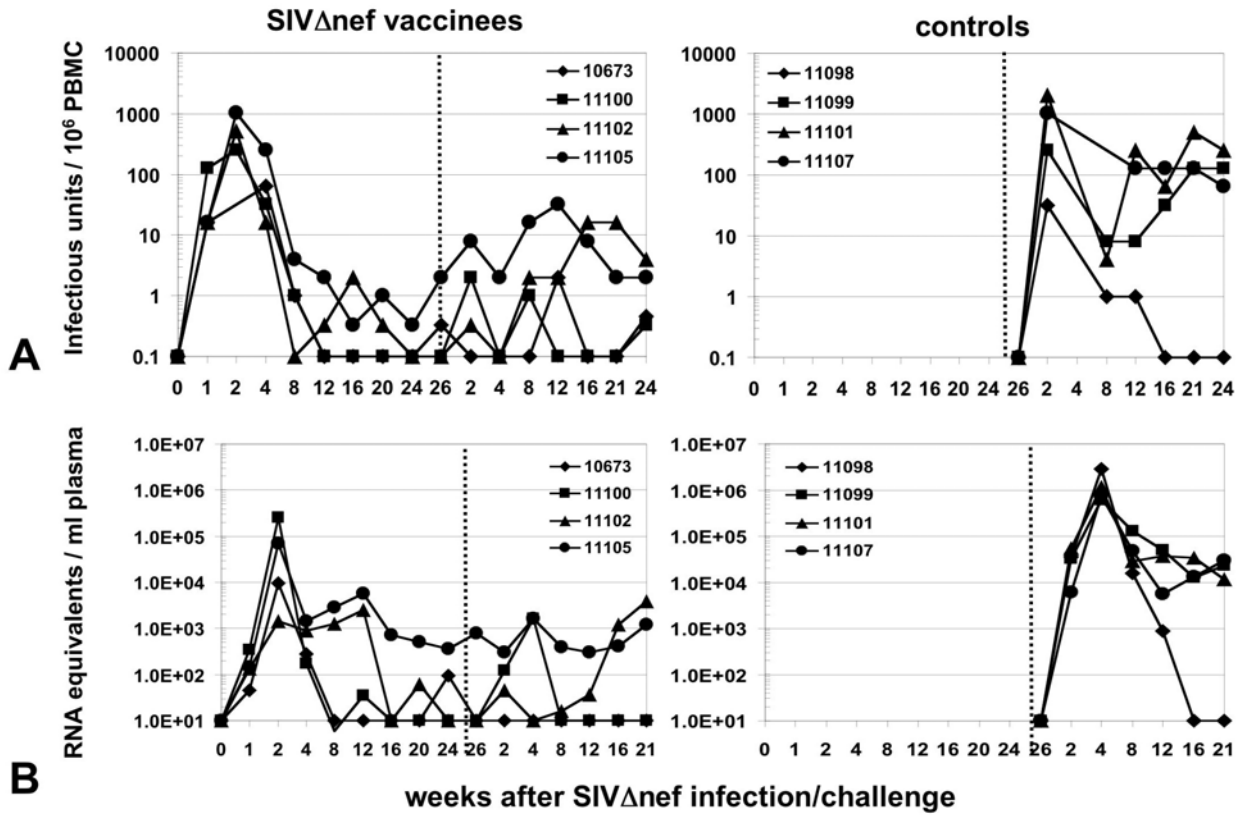

Figure 1. Long-term protection after SIVΔnef vaccination. Monkeys were vaccinated through the tonsils, challenged intrarectally with SIVmac251, and studied for 23 to 24 weeks. (A) Cell-associated viral load (assay threshold: 0.33 infectious units/10^6 PBMCs), and (B) plasma viral RNA (detection limit: 25 viral copies/ml) before and after challenge with SIVmac251.

onto glass fibre filter mats (ICN Biomedicals, Aurora, OH, USA), and incorporated ^{3}H-thymidine was measured in a liquid scintillation counter. Results are represented as mean counts per minute (cpm) of ^{3}H-thymidine uptake of triplicate cultures, or as stimulation indices (SI) by dividing the mean cpm of triplicates of antigen-containing wells by the mean cpm of triplicate wells with unstimulated PBMCs. SIs >5 were considered positive.

3.11. Statistical analyses

Data on numbers of RNA copies and percentage of γ/δ T-cells in blood and tissue were analyzed with the two-tailed student t-test, and a p value < 0.05 was considered significant.

4. RESULTS

4.1. Tonsillar application of SIVΔnef confers long-term protection against pathogenic wild-type SIV applied at the rectal mucosa

Four monkeys (animals 10673, 11100, 11102, 11105) were immunized through atraumatic application of 1 x 10^5 $TCID_{50}$ of SIVΔnef onto the palatine and lingual tonsils, and replication of attenuated virus was monitored at indicated time points thereafter. All four animals became infected with SIVΔnef and showed peak viral loads (both plasma RNA and cell-associated virus) around 2 weeks post immunization (Figure 1A, B). As compared to replication of wild-type virus, the peak levels of attenuated virus were 1 to 2 log steps lower in the immunized animals. While 2 animals (11100, 11105) showed peak levels of around 10^5 RNA copies/ml, we detected 10^4 and 10^3 copies/ml, respectively, in the plasma of animals 10673 and 11102. At week 26, all animals and 4 additional naive monkeys (animals 11098, 11099, 11101, 11107) were infected with wild-type SIVmac251 (50 MID_{50}/animal) by the rectal route and further blood samples were collected to determine the viral load and identify the replicating virus strains. As expected, all control animals became infected and developed peak levels of plasma viremia (around 10^6 RNA copies/ml) 4 weeks after the infection (Figure 1A, B). While we observed declining virus levels in animal 11098, the other 3 control monkeys showed viral set points of $\geq 10^4$ viral copies/ml plasma until the end of the study. In contrast, none of the 4 immunized monkeys showed a substantial increase in cell-associated or plasma viral load after challenge (Figure 1A, B). Differences between control animals and vaccinees regarding their RNA viral loads between week 2 and 16 post challenge were statistically significant ($p<0.01$).

All animals were sacrificed at 23/24 weeks after challenge, and cell-associated viral loads were determined

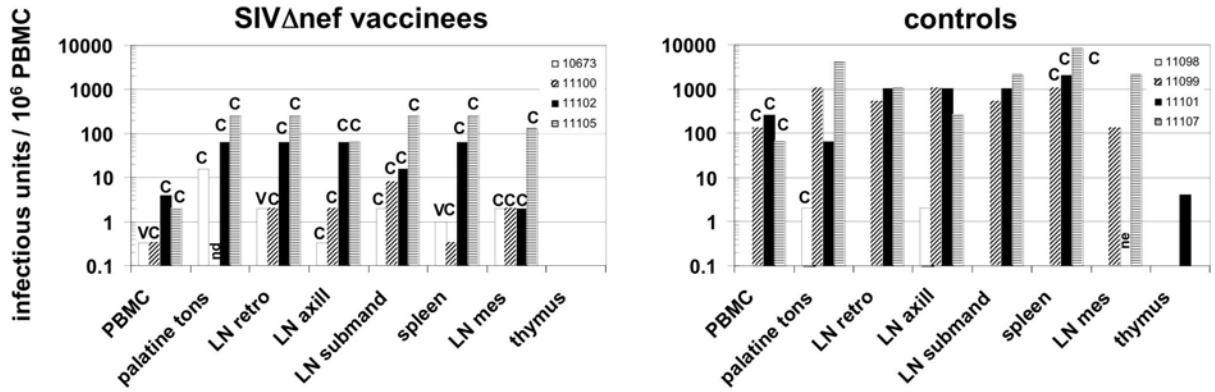

Figure 2. Cell-associated viral loads in blood and lymphoid tissues from SIVΔnef-immunized animals and control animals at necropsy 23 to 24 weeks after challenge with SIVmac251and detection of vaccine and challenge forms of SIV by PCR. (A) SIVΔnef-immunized and (B) naïve control monkeys. Cell-associated viral loads were determined in limiting dilution co-culture assays as described (JGV 1996, (11)). Viral isolates obtained from immunized animals following challenge and selected ones from the control animals were characterized by PCR as described in "Materials and Methods" (V, vaccine virus; C, challenge virus). The absence of a bar means that no virus was detected. nd, not done; ne, not evaluable due to bacterial contamination.

for the site of entry (palatine tonsil), different LNs proximal or distal to the inoculation site, the spleen, the thymus, and PBMCs. In general, viral loads in the controls were 10- to 100-fold higher than in the immunized monkeys (Figure 2). Looking at individual monkeys, animals 10673 and 11100, which showed also the lowest pre-challenge levels of attenuated virus in the plasma (Figure 1), continued to maintain markedly lower viral loads than the animals 11102 and 11105. Coinciding with the declined plasma and cell-associated viral loads in the blood of control animal 11098 (Figure 1), virus could be isolated only at low levels and from few of its organs (Figure 2B). In contrast, high cell-associated viral loads were detected in all cellular compartments except the thymus for the other 3 control animals (Figure 2B).

4.2. Detection of SIV RNA$^+$ cells in lymphoid and mucosal tissue

Two weeks after the tonsillar application of SIVΔnef and 2 weeks before challenge with pathogenic SIV, we removed superficial LNs from all immunized monkeys to enumerate SIV RNA$^+$ cells and study their distribution in the lymphoid tissue. Low numbers of RNA$^+$ cells were found in all immunized monkeys after immunization (data not shown). Following necropsy we had the chance to further investigate a large number of peripheral and mucosal lymphoid tissue specimens as well as samples of the mucosa itself (palatine and lingual tonsil; retropharygeal, cervical, axillary, inguinal, and mesenteric lymph nodes; proximal and distal ileum, colon, and rectum) from both the immunized and control group of macaques. Very low numbers of RNA$^+$ cells were detected in tissues from the control animal 11098, which was also able to clear viremia (Table 1). While numbers for the other animals slightly differed, there were in general more positive cells in the germinal centers than in extrafollicular lymphoid tissue, and this difference seemed to be less in the control monkeys. Germinal centers could rarely be found in the GALT. Thus, the mechanisms of protection against wild-type SIV following tonsillar application of SIVΔnef appears to be expressed to a different extent in the extrafollicular tissues as compared to the germinal centers of the lymphoid tissue.

Only modest trapping of immune complexed virions was detected in the germinal centers of LNs from animals 11102 and 11105 at necropsy in some LNs, while in animals 10673 and 11100 no trapping was observed before or after challenge with SIVmac251. In contrast, trapping of virions was detected 2 to 6 weeks after infection and until necropsy in the control monkeys 11099, 11101, and 11105. Corresponding to the previous virological findings in the control animal 11098, we observed only some fine trapping in LNs from this animal at weeks 2 and 10 after infection but no trapping in any tissue investigated at necropsy (data not shown).

4.3. Characterization of viral isolates obtained from blood and tissue samples of immunized macaques

Virus could be isolated at various time points after challenge from the blood and at necropsy also from various lymphoid tissues from all 4 immunized monkeys (Figures 1 and 2). To discriminate between wild-type and attenuated SIVmac, all viral isolates obtained after challenge from the vaccinees and some isolates from selected organs of the controls were characterized by PCR, recognizing the attenuated virus by its deletion in the U3 region of the nef gene. The immunized animals considerably differed regarding the isolation of the challenge virus from peripheral blood (Table 2). While we detected exclusively SIVΔnef in blood samples from monkey 10673, samples from animal 11105 yielded only challenge virus. Likewise, challenge virus was also frequently detected in blood samples from animal 11102 (Table 1). Characterization of the viral isolates obtained from tissues at necropsy showed that in fact none of the immunized animals had developed sterilizing immunity (Figure 2, left panel), i.e., even in animal 10673 we could isolate challenge virus from lymphoid tissue. In blood and tissues obtained from animal 11105, which had maintained the highest viral loads of SIVΔnef before challenge, we

Table 1. Numbers of SIV RNA-positive cells per mm^2 of whole sections as compared to mm^2 of germinal centers and extrafollicular lymphoid tissue at the time of necropsy

Animals	Tissue	Palatine tonsil	Lingual tonsil	Retrophar. LN	Cervical LN	Axillary LN	
10673	Section	0.59	ND	0.11	0.95	0.16	
	GC[1]	1.48	ND	ND	1.87	ND	
	ELT	0.19	ND	ND	0.83	ND	
11100	Section	0.08	0	0.08	0.15	0.08	
	GC	0.55	0	ND	ND	ND	
	ELT	0	0	ND	ND	ND	
11102	Section	0.2	-	1.42	0.24	0.9	
	GC	ND	-	4.29	5.95	5.01	
	ELT	ND	-	0.11	0.05	0.17	
11105	Section	0.47	-	1.03	1.04	1.5	
	GC	2.81	-	2.84	2.71	4.87	
	ELT	0.32	-	0.58	0.47	0.87	
11098	Section	0.07	-		0.05	0.08	
	GC	ND	-		0.24	0	
	ELT	ND	-		0.03	-	
11099	Section	5.9	3.7		5.09		
	GC	8.13	ND		9.59		
	ELT	5.28	ND		4.38		
11101	Section	6.75	4.4		5.8		
	GC	12.4	8.1		5.54		
	ELT	5.96	3.5		5.88		
11107	Section	4.7	-		3.2	3.1	
	GC	11.9	-		16.2	8.1	
	ELT	3.66	-		1.8	1.6	
Animals	**Tissue**	**Inguinal LN**	**Mesenteric LN**	**Proximal ileum**	**Distal ileum**	**Colon**	**Rectum**
10673	Section	0.27	0.02	-	-	-	-
	GC[1]	0.65	ND	-	-	-	-
	ELT	0.14	ND	-	-	-	-
11100	Section	0.05	0.03	-	0		0
	GC	ND	ND	-	0		0
	ELT	ND	ND	-	0		0
11102	Section	0.47	0.2	0	0.22		
	GC	4.36	ND	0	ND		
	ELT	0.15	ND	0	ND		
11105	Section	0.82		1.25		1.59	
	GC	2.94		ND		ND	
	ELT	0.51		ND		ND	
11098	Section	0.08	0.03	0.12	0	0.06	0.35
	GC	0	0	ND	0	0	0
	ELT	-	-	0	0		
11099	Section	8.03	4.76	4.84	6.74	14.63	4.85
	GC	12.31	ND	No GC	No GC	0	No GC
	ELT	6.41	ND	-	-	-	-
11101	Section	5.86	4	7.2	16.8	4.56	6.2
	GC	4.07	5.25	5.1	5.2	ND	ND
	ELT	8	3.75	8.1	9.3	-	-
11107	Section	3.02	3.2	2.56	5.8		ND
	GC	4.76	6.46	No GC	No GC		ND
	ELT	2.27	1.83	-	-		ND

[1] GC, germinal center; ELT, extrafollicular lymphoid tissue

exclusively detected the challenge SIV strain and at higher concentrations than in the other immunized animals. Expectedly, only challenge virus was detected in tissue from the controls. Thus, while tonsillar application of SIVΔnef conferred protection against infection with pathogenic SIVmac251 in some animals, the mechanisms of protection did not lead to sterile immunity but to considerably lower peak viral loads and viral set points as compared to the control animals.

4.4. Tonsillar application of SIVΔnef provides protection against early loss of peripheral CD4 T cells following intrarectal challenge infection with wild-type SIVmac251

Like HIV infection in humans, SIV infection of rhesus macaques is accompanied by a gradual loss of peripheral $CD4^+$ T lymphocytes slowly leading to simian AIDS, and any promising vaccine candidate must protect against this feature of immune dysfunction. We therefore closely analyzed the numbers of peripheral $CD4^+$ T cells in immunized vs. control animals. All immunized monkeys maintained overall stable $CD4^+$ T cell counts following tonsillar application of SIVΔnef (data not shown). After challenge virus infection the immunized monkey 11105 showed a significant decline of $CD4^+$ lymphocytes starting already at 4 weeks after challenge (Figure 3). While in animal 11100, this process began shortly before necropsy despite relative low viral loads, the immunized animals 10673 and 11102 continued to keep normal peripheral CD4 T cell counts. In contrast, $CD4^+$ T cell numbers declined in all control animals including animal 11098 despite its capability to clear viral load (Figure 3). At necropsy, the

Table 2. Cell-associated viral loads and detection of vaccine and challenge forms of SIV by PCR in blood of SIVΔnef-vaccinated macaques following intrarectal SIVmac251 challenge

	Animals			
wpc[1]	**10673**	**11100**	**11102**	**11105**
2	-	2 [2] (C)	-	8 (C)
4	-	-	-	2 (C)
8	-	1 (V)	2 (C)	16 (C)
12	2 (V)	-	2 (V/C)	32 (C)
16	-	-	16 (C)	8 (C)
21	-	-	16 (C)	2 (C)
24	0.45 (V)	0.33 (C)	4 (C)	2 (C)

[1] wpc, weeks post challenge; -, virus isolation negative; V, vaccine virus; C, challenge virus, [2] Cell-associated viral load expressed as infectious units per 10^6 PBMC. Characterization of re-isolates by PCR is shown in parenthesis

relative loss of $CD4^+$ T lymphocytes in controls was between 40 and 80%. Similarly, we detected only 20% of $CD4^+$ T cells as compared to baseline data in animal 11105. Animal 11100 just went down to around 70%, while animals 10673 and 11102 had maintained their peripheral $CD4^+$ T lymphocyte numbers to 100%.

At necropsy we also analyzed the percentages of $CD4^+$ T lymphocytes of the entire T cell ($CD3^+$) population of the spleen, LNs, and mucosal tissue derived from jejunum and rectum of the animals. Most striking differences were observed for the cell populations obtained from the mucosa (Table 3), while the percentages determined for LNs did not differ considerably between immunized and control monkeys. In fact, data for $CD4^+$ T cells obtained from the jejunum and rectum from the vaccinees 11100 and 11105 were in the same range as those of the control monkeys, while the vaccinees with normal peripheral CD4 cell numbers, i.e., monkeys 10673 and 11102, also yielded considerably higher mucosal CD4 T cell counts. The data for animal 11100 are further supporting the phenomenon that following retroviral infection the CD4 cells in the gut may disappear before major changes in the peripheral cell counts become manifest. Nevertheless, in the vaccinees 10673 and 11102 lower viral loads correlated with stable CD4 T cell counts in the periphery, in lymphoid organs, as well as in the intestinal mucosa.

4.5. Humoral immune responses following tonsillar immunization with SIVΔnef

SIV-specific antibody responses could be detected in all four vaccinated monkeys after tonsillar immunization (Figure 4A, B). Both p27- and gp130-specific antibodies rose slowly reaching their maximum at the time of challenge, i.e. week 26. Notably, at the time of challenge antibody responses were similar in all immunized animals. We also analyzed the neutralizing capacity of the antibodies. Neutralizing antibodies appeared in serum samples from all immunized monkeys between week 4 and 12 post immunization (Figure 4C). Interestingly, the least-protected animal (11105) showed an earlier induction and at the time of challenge a slightly higher titer of neutralizing antibodies than the other 3 immunized animals. Thus, there was no correlation between the degree of protection against wild-type challenge infection in the animals induced through tonsillar application of SIVΔnef and the titers of binding and/or neutralizing antibodies in the serum of the monkeys.

4.6. Cellular immune responses following tonsillar immunization with SIVΔnef

A second aim of the present study was a broad analysis of the cellular immune responses induced through the tonsillar application of SIVΔnef and expressed in the periphery as well as in lymphoid tissue and mucosa. We therefore performed three different forms of immunological assays (ELISPOT assays using overlapping peptide pools for the viral antigens gag including one for the whole p26, rev, and tat; standard proliferation assays with AT-2 inactivated SIV as antigen; intracellular cytokine FACS assays) at various time points during immunization, after challenge infection with SIVmac251, and at necropsy.

Post-imunization/pre-challenge: None of the animals showed substantial SIV-specific responses in blood in the ELISPOT assays before immunization, while incubation of cells in the presence of PMA and ionomycin as positive control yielded high numbers of IFN-γ secreting cells (>10^3 spots/10^6 PBMCs) for all animals throughout the whole study (data not shown). Two to 4 weeks following immunization the animals developed gag-specific responses (Figure 5), although responses in animals 10673 and 11100 were considerably lower than the ones observed in the other 2 immunized monkeys. These early SIV-specific cellular responses coincided with the peak loads of SIVΔnef in the monkeys (Figure 1), consistent with the finding that viremia during SIVΔnef infection is mainly controlled by $CD8^+$ T lymphocytes (23). Numbers of peripheral SIV-specific IFN-γ secreting T cells had dropped by week 8 (Figure 5), and only one of the 3 monkeys with low viremia (11102) maintained higher numbers of gag-specific T lymphocytes in the peripheral blood until challenge (week 26). In animal 11105 small numbers of SIV-specific cells re-appeared at week 26, 18 weeks after the disappearance of cells from the peripheral blood, whereas the other 2 animals did not show considerable numbers of SIV-specific cells by the time of challenge (Figure 5). A similar pattern of ELISPOT data was obtained for 4 additional animals (data not shown), which were identically immunized but went into a different study (6). In total 4 of 8 animals revealed gag-specific T cells at 26 weeks after tonsillar immunization.

Post-challenge: Further ELISPOT data were obtained with PBMCs collected at weeks 2, 4, 8, 12, 16, and 21 after challenge. Out of the immunized animals, both the protected monkey 10673 as well as the animal with eventually declining CD4 counts (monkey 11100) continued to yield low numbers of IFN-γ secreting cells until necropsy. In contrast, in animal 11102 (with repeatedly isolated challenge virus but rather stable CD4 counts), which had also shown the highest numbers of gag-specific T cells before challenge, the infection with SIVmac251 gave rise to very high numbers of IFN-γ secreting CD8 T cells. Increasing numbers of gag-specific

Table 3. CD4 T cells (% of $CD3^+$ cells) in blood and tissues derived from immunized and control animals at necropsy

Tissues	Immunized animals				Control animals			
	10673	**11100**	**11102**	**11105**	**11098**	**11099**	**11101**	**11107**
LNs[1]	54.2	61.1	52.7	45.5	49.0	36.8	50.6	39.9
Spleen	48.0	42.3	40.7	32.2	26.4	22.5	44.0	19.5
Jejunum	46.2	22.3	56.1	26.0	19.3	2.3	11.7	18.9
Rectum	46.5	34.0	50.3	13.5	29.8	13.4	31.2	29.8

[1] LNs, lymph nodes

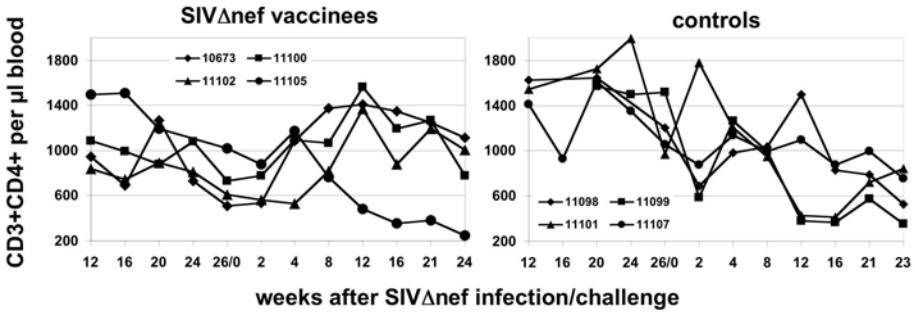

Figure 3. Conservation of peripheral $CD4^+$ cell counts in the majority of monkeys after immunization with SIVΔnef via the tonsils and following intrarectal challenge infection with pathogenic SIVmac251. Absolute numbers of $CD4^+$ T lymphocytes were determined in (A) SIVΔnef-immunized and (B) control animals.

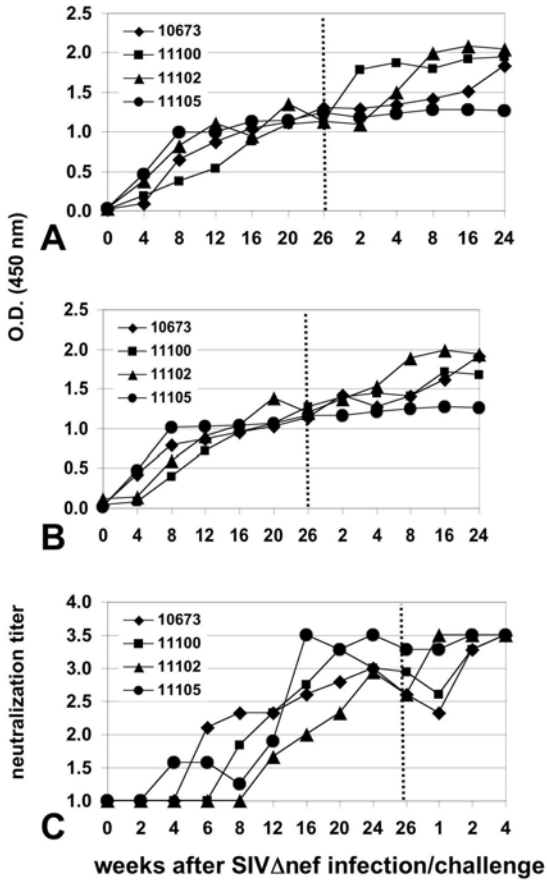

Figure 4. Binding and neutralizing SIV-specific antibody responses in vaccinated macaques before and after rectal challenge with SIVmac 251. Binding antibodies were determined by ELISA using recombinant SIV gp130 (A) and SIV p27 (B) and are shown as optical densities at 450nm using plasma diluted 1:200. Neutralization titers (C) were calculated using sequential dilutions of heat-inactivated serum samples, incubated with SIVmac251 virus together with normal monkey serum or heat-inactivated normal monkey serum. The virus-serum mixtures were then cultured with human $CD4^+$ CEMx174-SEAP cells, supernatants harvested on day 7, and the relative extent of replication of SIVmac251 was determined in a SIVp27 ELISA.

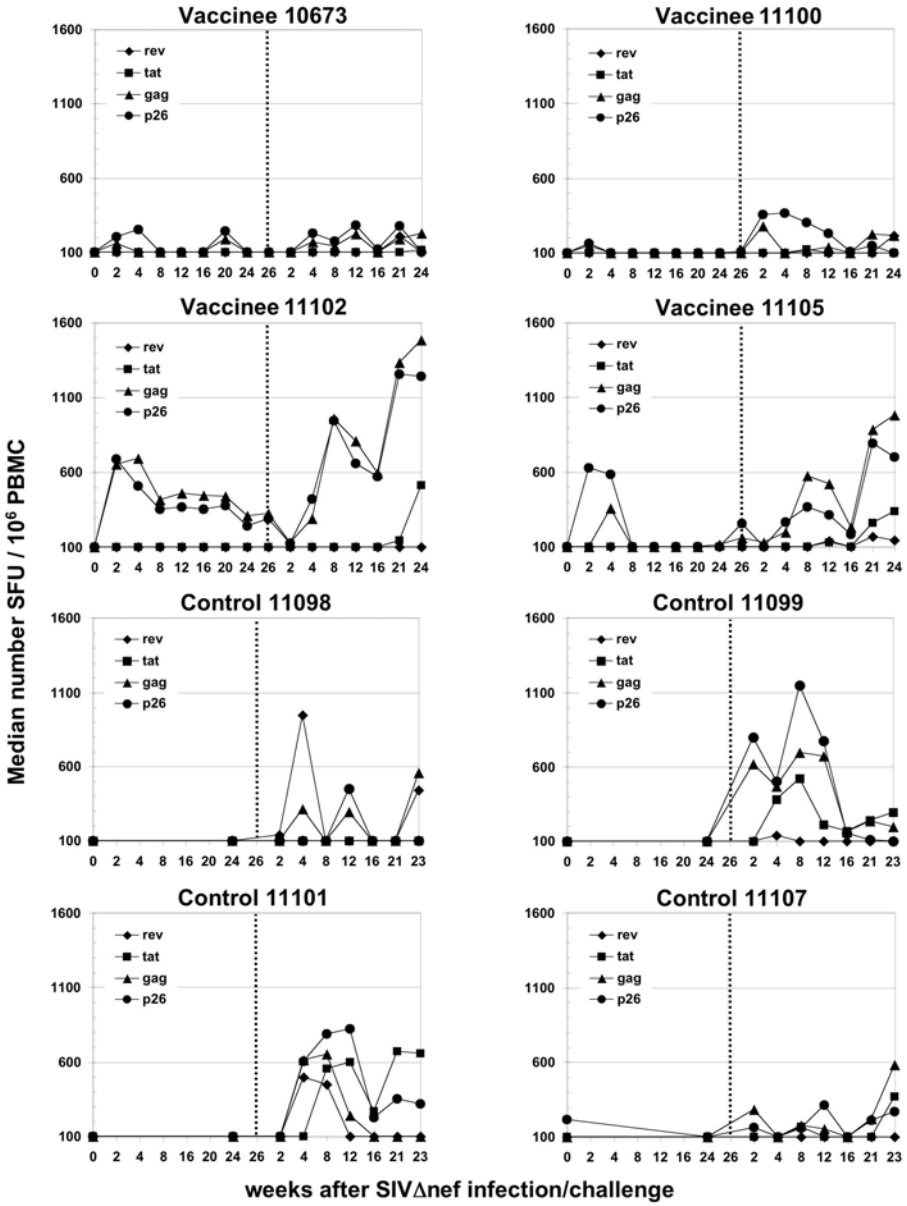

Figure 5. Numbers of SIV-specific IFN-γ secreting T-cells in the peripheral blood after tonsillar SIVΔnef immunization and following intrarectal challenge with pathogenic SIV. Numbers of IFN-γ secreting cells stimulated by SIV-specific peptide pools (for details see Materials and Methods), PMA, or kept in medium were determined by Elispot at regular intervals during immunization and after challenge. The respective vaccinees and the corresponding control animals are indicated. Given are median numbers of spot-forming cells per 10^6 PBMC; positive: >100 spots above and twice the background.

T cells following challenge infection were also observed in the least-protected animal (11105).

IFN-γ secreting cells were also detected in the control animals following intrarectal infection (Figure 5). While the numbers of those cells varied between the animals, there were no particularly strong or persistent responses in monkey 11098, which was able to reduce viremia despite the loss of CD4 cells. In fact, animals 11099 and 11101 showed higher numbers of peripheral IFN-γ secreting cells than monkey 11098, suggesting that the magnitude of T-cell responses might rather correlate with levels of viral replication than with protection.

Time of necropsy (week 23/24 after challenge): Similarly, at necropsy there were no clear patterns of cellular immune responses that could be related to protection (Figures 5 and 6). While monkey 10673 yielded relatively low responses in the ELISPOT assays performed with cells obtained from various organs and the peripheral blood, animal 11102 showed high responses in all organs tested (Figure 6). The other 2 immunized animals also

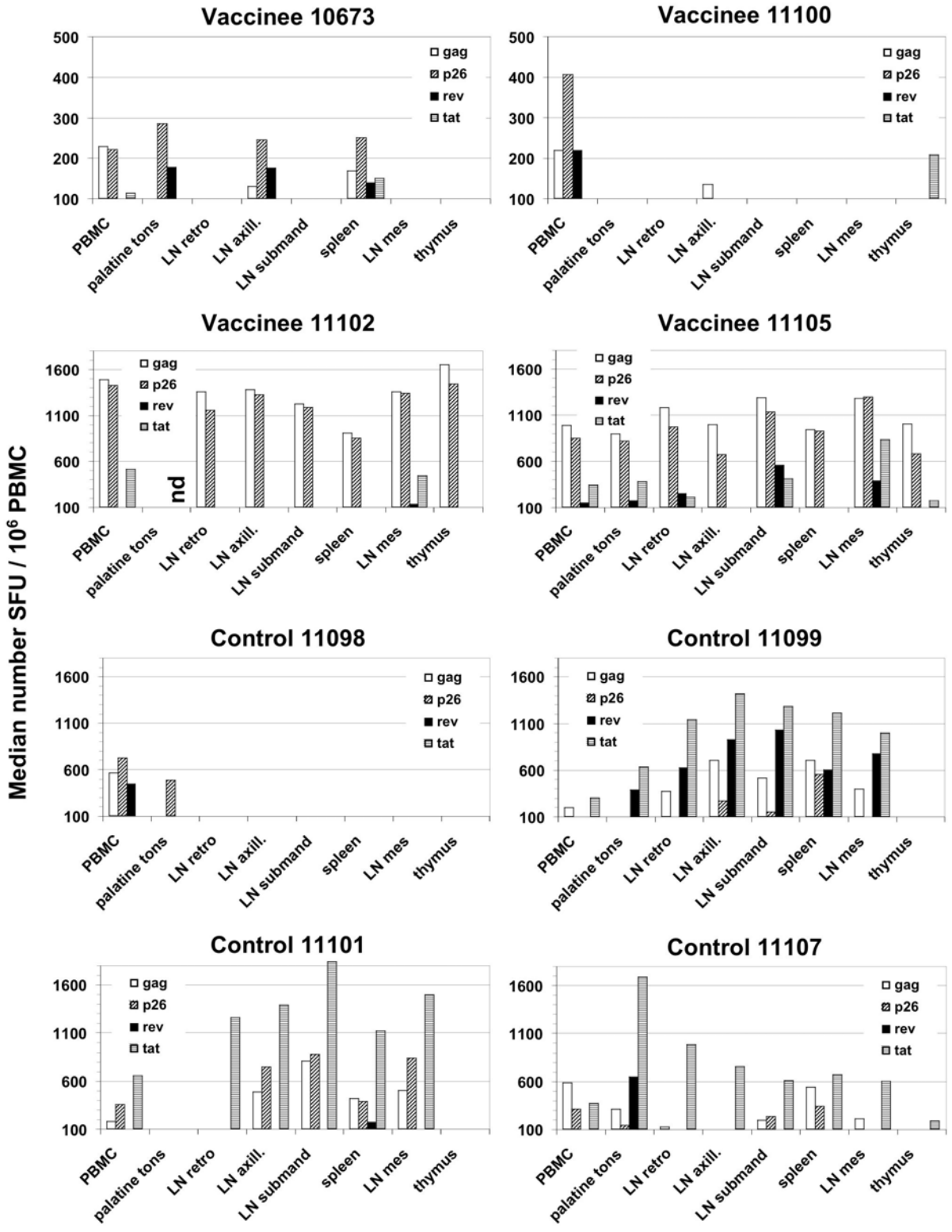

Figure 6. Numbers of SIV-specific IFN-γ secreting tissue-associated and blood-derived T-cells at necropsy. Mononuclear cells were isolated from various lymphoid organs and blood and stimulated by different SIV peptide pools. For identification of animals and definition of positive results see legend of Figure 5. The absence of a bar means that no positive responses were observed.

Table 4. SIV-specific T cell-proliferative responses (cpm) in PBMCs and tissue derived from immunized and control animals at necropsy

Animals	Stimulus	PBMCs	Spleen	LNs[1]	Jejunum	Rectum
10673 (v)[3]	AT-2 SIV	1856	5709	11452	1168	n.d.
	Medium	1263	3188	4057	1297	
11100 (v)	AT-2 SIV	12809	11289	9463	669	13725
	Medium	9516	5398	5469	1566	8082
11102 (v)	AT-2 SIV	15113	4230	10182	3479	7986
	Medium	10847	3564	4479	2238	3794
11105 (v)	AT-2 SIV	**8973**[2]	8480	6164	1242	n.d.
	Medium	**1169**	2977	3398	1637	
11098 (c)	AT-2 SIV	28569	8513	3965	625	6202
	Medium	15182	5770	3773	1173	3739
11099 (c)	AT-2 SIV	**8069**	**10509**	4147	531	13533
	Medium	**1148**	**1718**	4383	877	9653
11101 (c)	AT-2 SIV	1710	1250	1365	939	3321
	Medium	1575	1670	2003	1255	2227
11107 (c)	AT-2 SIV	**13092**	2934	5718	1937	51860
	Medium	**2091**	2811	3581	3170	35797

[1] LNs: lymph nodes, [2] Data in bold, SI > 5., [3] (v), vaccinee; (c), control animals, cpm: counts per minute

considerably differed regarding numbers of IFN-γ secreting cells in the ELISPOT assays, i.e., animal 11105 showed similar responses as animal 11102, whereas in animal 11100 we detected spot-forming cells only in PBMCs but hardly in tissue-derived mononuclear cells. In all vaccinees the detected T-cell responses were predominantly directed against SIVgag.

In contrast to the three control animals with persistent replication of challenge virus (11099, 11101, and 11107), monkey 11098 did not show responses against gag, rev, or tat in most of the tissues. Notably, T-cell responses in the control animals were mainly directed against rev and more obvious against tat, whereas in the vaccinees SIVgag-specific responses were stronger than those directed against the regulatory proteins.

Slightly different responses, but neither correlating with the degree of protection, were found using AT-2 SIV or vCP180 as antigens in intracellular FACS assays. In the immunized animals, we detected low responses in spleen cells in animal 11102 (0.19 and 0.52%, respectively). However, similar responses were also found with AT-2 SIV in spleen cells from animals 11100 (0.21%) and 11105 (0.49%), and animal 11100 showed equivalent responses also in LN cells (AT-2 SIV: 0.16%; vCP180: 0.27%) and PBMCs (AT-2 SIV: 0.26%). Data obtained for monkey 10673 could not be interpreted as the positive controls (cells incubated in the presence of SEB) yielded negative results.

Within the control group, monkeys 11098 and 11099 showed the highest tissue-associated SIV-specific cellular responses. In animal 11098, we detected IFN-γ secreting cells in the jejunum (vCP180: 0.80%, AT-2 SIV: 0.53%) and spleen (AT-2 SIV: 0.66%) in similar quantities as in PBMCs (vCP180: 0.55%; AT-2 SIV: 0.75%). Animal 11099 yielded positive responses in spleen cells (vCP180: 0.21%), LN cells (AT-2 SIV: 0.50%) as well as in PBMCs (vCP180: 0.28%; AT-2 SIV: 0.60%). In monkey 11101 we detected IFN-γ secreting cells in LNs only (vCP180: 0.32%), and in animal 11107 in spleen cells (vCP180: 0.24%) and cells obtained from the jejunal mucosa (AT-2 SIV: 0.71%). Thus, at necropsy, SIV-specific IFN-γ secreting cells could be detected by one or more methods in most of the immunized and control animals in cells from the peripheral blood as well as from various tissues.

Blood- and tissue-associated SIV-specific T cell-proliferative responses were also determined for all animals at necropsy. Leukocytes were isolated from the peripheral blood as well as from lymphoid tissues (spleen and LNs) and mucosa (jejunum and rectum) and cultured in the presence or absence of AT-2 SIV, which we have previously shown to be a valuable antigen for the detection of proliferative responses of T cells isolated from blood and tissues of SIV infected rhesus macaques (21). Only the least-protected animal of the vaccinees (11105) demonstrated low SIV-specific T cell proliferation in PBMCs (SI: 7.7), while the SIs for the other 3 immunized animals were < 2 (Table 4). Interestingly, 2 of the control animals showed also proliferative responses of a similar magnitude as animal 11105 (11099, SI: 7.0; 11107, SI: 6.3). In contrast, the other 2 control animals, including animal 11098 with the spontaneously declining viral load, did not reveal significant responses (SIs < 2). No SIV-specific proliferative responses could be detected in any other compartment investigated in immunized and control animals except in spleen cells obtained from the control monkey 11099 (SI: 6.1). Hence, the protection of SIVΔnef-immunized animals against wild-type SIV infection did not coincide with the expression of SIV-specific T cell proliferative responses, at least at necropsy.

4.7. Detection of γ/δ T cells in blood, lymphoid, and mucosal tissues from immunized and control monkeys

In addition to SIV-specific conventional $CD4^+$ and $CD8^+$ T lymphocytes, which express the α/β T cell receptor, T cells bearing the γ/δ T cell receptor may significantly contribute to anti-retroviral immune responses by the secretion of β-chemokines and IFN-γ (24). We therefore analyzed the percentages of γ/δ T cells among $CD3^+$ T lymphocytes obtained from blood, spleen, LNs, and the mucosa of the jejunum and the rectum of all animals at the time of necropsy. In both PBMCs and spleen cells, the highest percentages of γ/δ T cells were seen in the immunized animals 11102 and 11105, while the other animals did not differ considerably (Table 5). We detected

Table 5. γ/δ T cells (% of $CD3^+$ cells) in blood and tissues derived from immunized and control animals at necropsy

Tissues	Immunized animals				Control animals			
	10673	**11100**	**11102**	**11105**	**11098**	**11099**	**11101**	**11107**
PBMCs	14.5	4.4	28.8	69.2	17.7	14.3	14.4	10.4
LNs[1]	4.6	14.1	21.5	2.9	40.6	50.1	33.4	25.7
Spleen	21.5	28.7	58.0	63.4	19.3	21.3	24.1	16.5
Jejunum	11.5	6.0	13.9	8.1	10.6	24.6	26.0	18.9
Rectum	4.3	4.6	8.9	7.2	7.0	10.1	5.0	23.8

[1] LNs: lymph nodes

more γ/δ T cells in the LNs from control animals than in those from immunized monkeys ($p<0.01$). A similar pattern was observed for the cells obtained from the jejunal mucosa, whereas an elevated percentage of γ/δ T cells in leukocytes from the rectal mucosa was only detected in the control animal 11107. Therefore, protection against wild-type SIV induced by tonsillar application of SIVΔnef did not correlate with considerably higher numbers of γ/δ T cells in blood or tissues of immunized animals at necropsy. In fact, as seen for the LNs of the control monkeys numbers of γ/δ T cells may rather correlate with infection, i.e., ongoing viral replication may attract γ/δ T cells to the lymphoid tissues.

5. DISCUSSION

The main finding of the present study is that a single tonsillar application of attenuated SIVΔnef to rhesus macaques induces protection against challenge infection with pathogenic SIV at a distant mucosal site, i.e., the rectum, and that this protection does not seem to be directly related with the magnitude of SIV-specific humoral or cellular immune responses analyzed.

We did not observe complete protection, as we could isolate challenge virus from all 4 immunized animals at necropsy (around 6 months after challenge with SIVmac251). Additionally, the degree of protection varied between immunized animals. While a protective effect due to the SIVΔnef immunization was evident in all immunized monkeys by lower peak viral loads than in the controls early after challenge, the animals differed regarding their degree of loss of peripheral $CD4^+$ T cells. Data obtained at necropsy of one animal (11100) revealed a substantial loss of mucosal $CD4^+$ T cells as a sign of the onset of progressing immunodeficiency, while the numbers of peripheral $CD4^+$ T lymphocytes were only slightly reduced. In contrast, 2 other vaccinees (10673 and 11102) had high numbers of both peripheral and mucosal CD4 cells at necropsy. The fourth immunized monkey (11105) with a reduced capacity to control the immunizing infection with SIVΔnef showed the highest viral burden following challenge infection accompanied by declining CD4 cells in both the peripheral blood and mucosa. Likewise, Lewis and co-workers observed an accelerated course of infection with SHIV 89.6P in animals that were unable to efficiently control the immunizing SIVΔnef infection (25). In accordance with our data, i.v. immunization of cynomolgus macaques with the attenuated strain SIVmacC8 protected only a proportion of the immunized animals against intrarectal infection with SIVsm (26), while SIVmacC8-immunized rhesus macaques were protected against intrarectal infection with SIVmacJ5 and with an SIV/HIV chimeric virus (SHIV) (27). Similarly, pig-tailed macaques immunized orally with an attenuated SHIV were protected against vaginal challenge with the pathogenic $SHIV_{KU-1}$ (28). Collectively, these and our data demonstrate that both routes of immunization with attenuated SIV or SHIV, i.e., mucosal application and i.v. injection, can confer protection against wild-type SIV or SHIV in a number of animals of different macaque species. Differences in the degree of protection observed in these studies might be due to the distinct SIV strains used for immunization and challenge, and also to the application of different doses of virus.

Interestingly, we observed declining virus levels also in the control animal 11098, and at the moment we do not have an explanation for this course of infection. Previous typing of 44 rhesus monkeys of Chinese origin for Mamu-A^*01 and –B^*17 alleles did not reveal the Mamu-A^*01, while Mamu-B^*17 was only rarely detected in these macaques after DNA sequence analysis of amplified PCR-products (Dr. U. Sauermann, German Primate Center, unpublished observation). Despite extensive typing and DNA-sequence analysis no MHC-I alleles could be linked to an attenuated disease course in Chinese macaques so far. Since data obtained in humans indicate that stronger immune responses are induced at the mucosa of the rectum after rectal immunization as compared to oral immunization (29), it would be interesting to subsequently compare in our model the protective effects of intrarectal versus tonsillar immunization. But do SIV-specific immune responses contribute at all to the observed protection of macaques infected with attenuated SIV? As we also observed, humoral immune responses most likely do not, since immunized animals are also protected against immunodeficiency viruses with divergent envelope proteins (27, 30, 31) and several other groups did neither detect a correlation between titers of binding and/or neutralizing antibodies and protection (26, 32-35).

We were particularly interested in to what extent cellular immune responses would be induced following a tonsillar application of SIVΔnef, whether these responses might be expressed both mucosally and systemically, and how they would correlate with protection. We therefore employed three different experimental methods for the detection of SIV-specific cellular immune responses, which might be additive in their results, since they relied on distinct antigen sources, thereby most likely leading to the presentation of different antigenic epitopes. For instance animal 11098 that showed higher responses in intracellular

FACS than in ELISPOT assays may have developed immune responses predominantly against env, which was not included as antigen in the ELISPOT assays. In fact, we detected stronger SIV-specific cellular immune responses in animal 11102 than in most other animals of the study and this monkey was protected against a progressive CD4 cell loss despite a continuous low-level replication of wild-type virus after challenge. Nevertheless, the analyses and the comparison of the data obtained for all immunized and control monkeys did not reveal a clear correlation of the level of protection induced by tonsillar application of SIVΔnef with the magnitude of cellular immune responses. While Joag *et al.* (28) have previously observed a strong correlation of suppression of SHIV replication with robust cell-mediated immune responses in orally SHIV-immunized pig-tailed macaques, recent data from others support the concept that functions of antigen-specific $CD8^+$ cytotoxic T cells are not the main mechanism of protection conferred by an immunizing infection with attenuated SIV (36-39). This applies also to the acute phase of SIVΔnef infection when cytotoxic T cells substantially contribute to the drop of viral loads. Depletion of these cells, however, did not abrogate the protection against a superinfection with wild-type virus, which was evident as early as 20 days after immunization (40, 41). Similarly, in monkeys immunized through tonsillar application of SIVΔnef we did not observe an influx of perforin-positive cells into the local draining tissues early after tonsillar challenge with wild-type SIV (6).

T cells expressing the γ/δ T cell receptor may mediate protective effects against SIV infection possibly through the secretion of chemokines (24). Previously, we documented an influx of γ/δ T cells into lymphoid tissue shortly after tonsillar application of wild-type SIV, while vaccinated and protected animals showed greater numbers of γ/δ T cells before and after challenge than naïve animals (6). In the present study, we were able to analyze the percentages of γ/δ T cells of all T cells derived from various types of tissue (including mucosa) at the time of necropsy. We detected substantially higher proportions of γ/δ T cells in PBMCs and spleen cells from 2 of the immunized animals including the least-protected monkey (11105). Interestingly, these were also the 2 vaccinees with the highest viral burden in plasma and tissues at necropsy. In the control animals, increased percentages of γ/δ T cells were seen in LN cells and T cells derived from the jejunum. Whereas the loss of CD4 T cells could account for a relative increase of other T cells including γ/δ T cells in the jejunum, this explanation does not hold true for the LNs where the $CD4^+$ T cell populations were not significantly decreased in the controls as compared to the immunized macaques. Therefore, chronic wild-type SIV infection may result in increased numbers of γ/δ T cells in the LNs where most of the viral replication takes place. Notably, the well-protected macaque 10673 did not show higher numbers of γ/δ T cells than most of the other animals in any tissue investigated. Lehner *et al.* (24) found significantly increased percentages of γ/δ T cells in the rectal mucosa of immunized and protected monkeys, but those animals had been immunized with recombinant SIV gp120 and p27 in alum through targeted iliac lymph node injection, which could have a different impact on the expansion and migration of γ/δ T cells than the tonsillar immunization with attenuated SIV. Together, these data indicate that γ/δ T cells might have a dual role in the defense of both attenuated and wild-type immunodeficiency viruses: Immediately after viral entry they pose a first line of defense in the mucosa itself and in tissues adjacent to the mucosa as observed by Lehner *et al.* (24) and us (6). However, after the virus has spread systemically, γ/δ T cells may then be involved in immune responses at sites of enhanced viral replication, such as lymph nodes and the jejunum, as demonstrated in the present study.

Notably, the protection after tonsillar application of SIVΔnef did most likely not depend exclusively on mechanisms in the mucosal tissue. Although the peak of wild-type viral replication, which is usually observed around 2 to 4 weeks after infection, was lacking in all immunized macaques, the challenge virus spread systemically after intrarectal infection, and it could be isolated from all immunized animals from distant peripheral lymphoid tissue. Thus, the "failure of protection" that we observed in a proportion of monkeys may not be due to a reduced protection at the site of the mucosal challenge itself, but rather to differences in the immune responses expressed in other compartments of the body. The local mechanisms of innate immunity, such as the increase in γ/δ T cells and also mature dendritic cells in the draining lymphoid tissues (6), probably interfere with an early replication of the challenge virus thereby prohibiting the viral peak in plasma. However, they do not prevent the establishment of a low-level systemic infection with wild-type virus, which leads to $CD4^+$ T cell loss in some but not all animals.

We cannot explain at the moment why monkey 11100 obviously progressed to a stage of advanced immunosuppression indicated by declining peripheral and low mucosal $CD4^+$ T cell counts, although its viral load throughout the whole time after challenge was low as compared to other immunized animals. If replication of the vaccine virus is mandatory for a blockade of the same receptor/co-receptor system, which would also be required by the following challenge virus, one could speculate that this mechanism was not working here, since viral loads in monkey 11100 dropped below the detection limit at week 12, and virus could not be isolated from all subsequent samples before challenge. The data obtained for animal 11105 would also argue against this kind of explanation, as this monkey maintained the highest viral burden after immunization but was the least-protected one of all 4 vaccinees. Another explanation for the immunosupression in monkey 11100 could be an enhanced immune activation in this animal, thereby leading to progressive T cell loss through activation-induced cell death and limited T cell renewal rather than through direct virus-mediated effects (42). Therefore, depending on thus far unknown host factors, wild-type SIV infection may remain at a persistently low level in animals previously immunized through infection with attenuated SIV, as we observed in animal 10673, or show an increasingly progressive

development as detected in monkeys 11000 and particularly 11105. These factors are most likely systemically active and therefore independent of the entry site of the challenge virus. Interestingly, while the resistance to challenge gradually increases over time in monkeys infected with attenuated SIV (34), the degree of protection inversely correlates with the level of attenuation (35). Hence, further studies are needed to elucidate how deletions of the various viral genes may influence different elements of the innate and adaptive immune response.

6. ACKNOWLEDGEMENTS

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