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Replication-deficient mutant Herpes Simplex Virus-1 targets professional
antigen presenting cells and induces efficient CD4⁺ T helper responses
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**Replication-deficient mutant Herpes Simplex Virus-1 targets
professional Antigen Presenting cells and induces efficient CD4⁺ T
helper responses**

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Abstract

Both neutralizing antibodies and cytotoxic T-cells are necessary to control a viral infection. However, vigorous T helper responses are essential for their elicitation and maintenance. Here we show that a recombinant replication-deficient *Herpes Simplex Virus* (HSV)-1 vector encoding the *Human Immunodeficiency Virus* (HIV)-1 matrix protein p17 (T0-p17) was capable to infect professional Antigen Presenting Cells (APCs) in vitro and in vivo. The injection of T0-p17 in the mouse dermis generated a strong p17-specific CD4⁺ T helper response preceding both p17-specific humoral and effector T cell responses. Moreover, we show that T0-p17 infection did not interfere with the endogenous processing of the transgene encoded antigen, since infected APCs were able to evoke a strong recall response in vitro. Our results demonstrate that replication-deficient HSV vectors can be appealing candidates for the development of vaccines able to trigger T helper responses.

KEY WORDS: HELPER T-CELLS; HIV-1; GENETIC VECTORS; HSV-1

RUNNING TITLE: T helper immunity induced by replication-deficient HSV-1

1. Introduction

T helper (Th) responses play a crucial role in the promotion and sustenance of effector responses. A consistent body of experimental evidences are accumulating on the capability of antigen specific CD4⁺ T-cells to influence the outcome of disease in viral infections. Persistent viral infections are characterized by a deficient CD4⁺ T cell function, whereas the presence of a strong Th response defines successful viral clearance. These findings have important implication in designing vaccination strategies aimed to trigger and sustain antigen specific CD4⁺ in addition to CD8⁺ effector immune responses [1,2].

CD4⁺ T cell responses are more and more important in immunity to HIV-1 [3]. Infected humans that control HIV-1 replication and remain disease free for years after infection display a potent Th response [4]. In HIV-1 vaccine preclinical studies, although CD8⁺ T cell and neutralizing antibody responses have demonstrated to be critical in protecting animals from infection and/or disease, they are not sufficient to provide solid protective anti-HIV-1 immunity [5] unless the appearance of a strong virus-specific CD4⁺ T cell response, characterized by proliferating elements [3,6,7]. Thus, the induction of CD4⁺ T cell responses is a fundamental characteristic of an ultimate anti-HIV-1 vaccine candidate.

Professional APCs play a key role in the development of primary T cell immune response [8]. Dendritic cells (DCs) and macrophages, are permissive to HSV-1 infection [9-11]. Using a recombinant HSV-1 capable of a single replicative cycle, Salio *et al.* [12] showed that synthesis of MHC class II antigens is up-regulated or unaffected in infected immature and mature DCs, respectively.

In this study, we show that a recombinant replication-deficient (rd) HSV vector encoding the cytosolic HIV-1 matrix protein p17 (T0-p17), a protein that is suggested to play a role in

AIDS pathogenesis [13] and reported as a possible candidate for an AIDS vaccine, is capable to efficiently infect professional APCs in vitro and in vivo. Immunization of mice with T0-p17 generates a T helper response able to trigger both p17-specific humoral and effector responses.

2. Materials and Methods

2.1. Generation of the p17-expressing rdHSV

A p17-expressing rdHSV vector was produced as previously described [14]. A recombination HSV-plasmid containing the HIV-p17 was constructed by cloning the Rev-independent coding sequence of p17 (kindly provided by G. Pavlakis, Frederick, MD; GenBank Accession number L04602). The genome of a non-replication HSV-1 viral vector expressing the Green Fluorescent Protein (GFP) (T0-GFP) was used to recombine with the p17 plasmid (T0-p17). The T0-p17 vector provides a robust, but very transient, GFP expression while it ensures a long-lasting transgene expression [15]. In all the experiments the vector T0-GFP was used as negative control. Protein expression from the T0-p17 vector was analyzed using anti-p17 mAb MBS-3 as specific reagent [13].

2.2. Cell cultures

Splenocytes, peritoneal macrophages and bone marrow-derived DCs (BMDCs) from BALB/c mice were cultured in RPMI-1640 (Sigma) medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (complete medium).

2.3. Vaccination of mice

BALB/c mice were purchased from Charles River International (Calco, Lecco, Italy). Animals were maintained in vented cages under standard conditions according to our institutional guidelines. Animals were divided in four groups of fifteen mice each, and the

mice were each immunized with 4×10^6 virus particles of T0-p17 or T0-GFP by intradermal (i.d.) or intraperitoneal (i.p.) injection. Five mice in each group were sacrificed at day 6, at day 11 and day 35 after immunization.

2.4. Generation of DCs from bone marrow precursors.

BMDCs were prepared from female six- to nine-week-old mice using a modification of the protocol from Inaba *et al.* [16]. Briefly, after lysis of erythrocytes, 1×10^8 bone marrow cells were plated on 100-mm Petri dishes and, after 2 hours, non-adherent cells were transferred in 6-well-plates at 2×10^6 cells/ml in complete medium supplemented with GM-CSF (10 ng/ml; R&D Systems, Minneapolis, MN). On day 6, cells were 84–88% CD11c⁺.

2.5. Proliferation assays

For proliferation experiments splenocytes were seeded in 96-well U-bottom culture plates at a density of 2×10^5 cells/well in complete medium and grown in the presence or absence (as negative control) of recombinant p17 [13] (10 µg/ml) for 6 days. At day 5 cells were pulsed with 1 µCi of [³H]-thymidine (Amersham Biosciences) and harvested after 18 hours.

2.6. Flow cytometry

Cells were stained for 30 minutes on ice with fluorescein isothiocyanate (FITC)-conjugated anti-mouse mAbs to CD4, CD8 and MHC class II molecules (BD Biosciences, Milan, Italy). The uptake of T0-p17 by BMDCs cells was determined by staining cells with allophycocyanine (APC)-conjugated anti-CD11c and evaluating the percentage of GFP-expressing cells within the CD11c⁺-gated DCs. BMDCs were pre-blocked using anti-mouse

CD32/16 antibody (BD Biosciences) for 15 min at 4°C. In all experiments dead cells were excluded by the addition of propidium iodide (PI). Only cells negative for red (PI) fluorescence were included for analysis.

2.7. Immunohistochemistry

Mice were infected intradermally with 4×10^6 T0-p17 virus particles/mouse. Tissue specimens were taken 12, 24 or 36 hours p.i. through punch biopsy, mounted in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen precooled isopentane. Five-micron sections were air-dried overnight and fixed in acetone for 10 min. Sections were incubated with 10% normal goat serum for 20 minutes, followed by hamster anti-mouse CD11c (BD Biosciences) and rabbit anti-GFP (Santa Cruz Biotechnology); primary antibodies were then revealed with biotin-conjugated goat anti-hamster IgG (Vector, Burlingame, CA) followed by Texas red-conjugated streptavidin (Vector), and with FITC conjugated swine anti-rabbit antibody (Dako, Carpinteria, CA), respectively. Sections were photographed on the Olympus BX60 fluorescence microscope using the DP70 Olympus digital camera.

2.8. Measurement of cytokines and chemokines

Culture supernatants were assayed for the presence of interferon-gamma (IFN- γ), tumour necrosis factor-alpha (TNF- α), interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 by flow cytometry, using the Cytometric Bead Array (BD Biosciences).

2.9. Enzyme-linked Immunospot (ELISPOT) assay

For the evaluation of p17-specific IFN- γ -producing CD8, CD4 were removed from splenocytes using a magnetic bead system (Miltenyi Biotec, Bologna, Italy). CD4-depleted cells (5×10^5 cells/well) were stimulated for 18 hours with 15 amino acids-long p17-based peptides (2 μ g/peptide/ml) (NIH AIDS Research & Reference Reagent Program) as recall antigens, and IFN- γ production was evaluated at a single cell level by ELISPOT (BD Biosciences). Such peptides were representative of the entire length of the p17 molecule and overlapped with a shift of 4 amino acids.

2.10. ELISA for anti-p17 antibody detection

For detection of anti-p17 specific antibodies, sera were pooled and tested by a recombinant p17-based ELISA [17]. Serum pools were obtained by mixing 10 μ l of serum taken from each mouse belonging to the same experimental group.

2.11. Isolation and infection of peritoneal macrophages

Mice were sacrificed and macrophages were obtained by flushing the peritoneal cavity of the animals with sterile PBS. Peritoneal macrophages were purified by adhesion to plastic for 2 hours. Adherent cells were scraped, resuspended (5×10^5 /200 μ l) in RPMI 1640 in the absence of FBS and infected with T0-GFP and T0-p17 at 1 m.o.i.

2.12. Extraction and amplification of nucleic acids

DNA extraction was performed by QIAmp DNA blood mini kit (Qiagen) and detection of p17 amplicons was performed using PCR (forward 5'-AGGGAGCTAGAACGATTCGCAG-3'; reverse 5'-TGATTGCTGTGTCCTGTGTCAG-3'); or a nested-PCR approach when

necessary (forward 5'-CTGTAGACAAATACTGGGACAGC-3'; reverse 5'-TGTCTA AAGCTTCCTTGGTGT C-3'). To evaluate the presence of p17 transcripts, RNA was extracted by RNAeasy minikit (Qiagen) and DNA contamination in RNA preparations was eliminated by digestion with 1500 U of RNase-free DNase (Quiagen). RNA (1 µg) was then reverse-transcribed using MULV reverse transcriptase (Applera, Monza, Italy) and used as a template for PCR. To demonstrate the absence of DNA contamination, an aliquot of RNA sample was amplified without previous retrotranscription.

3. Results

3.1. Expression of p17 in cells infected with T0-p17

Expression of p17 in T0-p17 infected simian (Vero), human (293) and mouse (G16-9) cells was examined by Western blot. The cells were infected with either T0-p17 or T0-GFP vectors at a m.o.i. of 1. In all three types of cells infected with T0-p17 vector we observed, at 24 and 48 hours, a band of 17 kDa corresponding to p17, whereas in cells infected with T0-GFP control vector, there were no signs of detectable bands (Fig. 1).

3.2. Recombinant T0-p17 vector efficiently infects DCs in vitro and in vivo

HSV has been shown to efficiently infect DCs [18]. Little is known, however, about the transduction efficiency of replication-deficient HSV-1 vectors both in vitro and in vivo. For this purpose, bone marrow cells were obtained from BALB/c mice and DCs were expanded by culturing cells in the presence of GM-CSF. After 6 days of culture more than 85% of the viable, – PI- negative – cells were CD11c⁺ (Fig. 2a, upper panels). Transduction in vitro of CD11c⁺ cells by T0-p17 vector did not affect cell viability as compared to uninfected cells, (data not shown), and resulted in a high percentage of GFP-expressing DCs (Fig. 2a, lower panel).

The capability of T0-p17 to transduce intradermal DCs in vivo was confirmed by i.d. injection with 4×10^6 virus particles of T0-p17. As shown in Fig. 2b, sparse CD11c⁺ cells were identified in the dermis of uninfected mice whereas no labelling was found on anti-GFP stained sections. In contrast, as soon as 12 hours after T0-p17 injection, biopsies showed

several GFP⁺ spindle cells in the dermis that, in the majority of the cases, co-expressed CD11c.

3.3. T cell proliferation of splenocytes from T0-p17 vaccinated mice in response to p17 as a recall antigen

To assess the immunogenicity of endogenously produced p17, mice were immunized by i.d. or i.p. injection of T0-p17. As negative vaccination control we employed the T0-GFP vector. Splenocytes obtained from T0-vaccinated mice were stimulated in vitro with p17 and proliferative activity was measured after 6 days of incubation. A p17-specific T cell proliferative response was observed in T0-p17 immunized mice but not in mice immunized with the T0-GFP control vector. As shown in Fig. 3a, p17 specific T cell clones were absent at day 6 after T0-p17 immunization but were generated at day 11 after virus injection. A more pronounced proliferative activity was observed in cells obtained from i.d. injected mice as compared to cells obtained from i.p. injected animals. Flow cytometric analysis showed that most of the expanded cells in response to p17 were CD4⁺ T-cells (Fig. 3b).

3.4. T0-p17 generates p17 specific CD8⁺ and humoral immune responses

At day 6 and 11 after immunization of mice with T0-p17 there was no evidence for the generation of a p17-specific effector response, as IFN- γ was not detected in the supernatant of p17 stimulated cultures (Fig. 4a) and a very low number of IFN- γ -producing CD8⁺ T-cells was evidenced by ELISPOT assay (Fig. 4b). Moreover, antibodies to p17 were not detected in sera of immunized mice (Fig. 4c). Mice immunized with T0-p17 and sacrificed at day 35 showed a p17-dependent T cell proliferation that was superimposable to that observed in

splenocyte cultures of mice sacrificed at day 11 after virus injection (Fig. 3a). However, 35 days after T0-p17 injection, all mice developed also a p17-specific effector response. Splenocytes obtained from these animals contained a great number of p17 specific IFN- γ producing cells as assessed by a peptide-based ELISPOT and ELISA assays, compared to splenocytes obtained from mice immunized with the T0-GFP control vector (Fig. 4b). Concomitantly, a p17 specific humoral response was detected in T0-p17- immunized animals (Fig. 4c). It is worth noting that i.d. delivery of T0-p17 elicited a better effector response than the i.p. delivery route.

3.5. T0-p17 vector efficiently infects peritoneal macrophages

Experiments were performed to test the capability of T0-p17 to infect freshly collected murine peritoneal macrophages in vitro. As shown in Fig. 5a, peritoneal macrophages showed a good susceptibility to T0-p17 infection (m.o.i. of 1), as more than 30% were infected 48 hours after infection. The capability of T0-p17 to infect peritoneal macrophages was also assessed in vivo, by injecting mice i.p. with 4×10^6 T0-p17 virus particles/mouse. Infection of macrophages with T0-p17 was assessed by nested PCR. The p17 transgene was detected in peritoneal macrophages at 2 and 11 days after T0-p17 injection (Fig. 5b). Moreover, following i.p. injection of the T0-p17 vector, the transgene carried by rdHSV was detectable at day 6, 11 and 35 days after viral infection in the spleen of animals, where it persisted up to eight months (Fig. 5c).

3.6. T0-p17 infected APCs promote the long-term expansion of p17 specific CD4⁺ T cell clones in vitro

Further experiments were run to verify if infection of peritoneal macrophages with T0-p17 resulted in the correct MHC class II presentation of the cytosolic p17 antigen and if, at the same time, infected cells retained the capacity to trigger the expansion of p17-specific CD4⁺ T-cells in vitro. Peritoneal macrophages were collected from T0-p17 infected mice at 48 hours after i.p. virus injection and co-cultured — at a cell ratio of 1:20 — with splenocytes of mice vaccinated i.d. with T0-p17 or T0-GFP. A strong expansion of CD4⁺ T-cells was observed when peritoneal macrophages carrying the p17 transgene — but not peritoneal macrophages obtained from naïve mice or in vivo T0-GFP transduced peritoneal macrophages — were co-cultured with splenocytes collected from mice vaccinated i.d. with T0-p17. Co-cultivation of T0-p17 infected macrophages with splenocytes of naïve or T0-GFP immunized mice did not give rise to any T cell expansion in vitro. Surprisingly, mixed cultures consisting of T0-p17 infected peritoneal macrophages and splenocytes obtained from T0-p17 vaccinated mice resulted in a long-lasting co-stimulation. In fact, these cultures were self-maintaining for more than 1 month, leading to a continuous expansion of CD4⁺ T-cells and to the survival of macrophages, that formed a confluent adherent cell monolayer (Fig. 6a). Flow cytometric analysis of cells after one month from the beginning of co-culture showed the presence of both CD4⁺ T-cells and macrophages expressing MHC class II molecules (Fig. 6b). Analysis of cytokines and chemokines in the supernatant of long-term self-maintaining mixed cultures revealed the presence of high amounts of proinflammatory molecules, attesting the strong activation status of cells in the microenvironment. In particular, TNF- α (range from 0.6 to 0.9 ng/ml), IL-6 (range from 1.3 to 2.2 ng/ml) and MCP-1 (range from 0.8 to 1.6 ng/ml) were constantly detected. On the contrary, a self-limiting proliferation of p17 specific T-cells, lasting no more than 7 days, was obtained by

mixing peritoneal macrophages obtained from uninfected mice with splenocytes of T0-p17 immunized mice (1:20 ratio) and using exogenous recombinant p17 as a recall antigen (data not shown). Interestingly, T0-p17 genome and p17 transcripts were detected in adherent APCs at the end of coculture (30 days) by PCR and RT-PCR, respectively (Fig. 6c). These results confirm the long-lasting persistence of the viral vector in the *in vivo* transduced APCs and its capability to allow the long-term expression of the transgene. On the other hand, adherent APCs did not show any p17 protein expression during the coculture period either by immunofluorescence assay, or by western blot (Data not shown), using anti-p17 mAb MBS-3 as specific reagent [13]. The observation that p17 transcripts persisted for all the period of culture, as revealed by RT-PCR, might be due to the greater sensitivity of this assay. Taken together, these results show that professional APCs are suitable targets for HSV-derived viral vectors and that they provide an optimal presentation of endogenously produced HIV-1 p17 in MHC class II molecules.

4. Discussion

rdHSV vectors have been largely restricted to suicide- [19] or cytokine gene [20] therapy against tumors but recently published studies have shown the potential of these viral vectors also for vaccination [21,22]. Despite the authors reported a detailed analysis of humoral and CTL responses following vaccination, no evidence suggested a potential role of rdHSV vectors in eliciting Th responses. Generation of Th responses depends on the in vivo transduction of DCs [8]. Transfection of predominantly non-professional APCs and only small numbers of DCs may hamper proper T cell activation. HSV-1 has a broad cellular tropism that includes APCs [9]. In this study we show that DCs can be efficiently transduced by rdHSV vectors both in vitro and in vivo. In our experiments we found that BMDCs were highly susceptible (>70%) to T0-p17 transduction in vitro. Furthermore, i.d. injection of T0-p17 in BALB/c mice resulted in an efficient transduction of CD11c⁺ elements as soon as 12 hours post infection, strongly supporting the hypothesis that in vivo transgene expression and presentation is likely to occur in primarily infected DCs rather than in DCs ingesting apoptotic/necrotic transduced cells.

The transduction efficiency to APCs under in vivo conditions seems to rely on not only the ability of the gene vector but also the administration route, since APCs are widely distributed in the body and their accessibility depends largely on the site of injection. Our data showed that vaccination of mice by i.d. or i.p. injection of the T0-p17 vector elicited a strong p17 specific CD4⁺ T cell response. Immune responses to p17 after the T0-p17 i.d. administration was higher than that after the i.p. viral vector delivery. This finding may be explained by the

knowledge that the mouse dermis is the richest tissue in DCs, where these cells reach approximately 60% of all dermal cells [23].

The priming of antigen specific CD4⁺ T-cells requires the presentation of antigenic peptides in the context of MHC class II molecules. This is usually achieved by the uptake of extracellular antigens [24]. On the other hand, there are accumulating evidences that endogenously expressed proteins have also access to the MHC class II-restricted antigen presentation pathway [25-26]. Our data suggest a correct MHC class II processing of endogenously produced p17 by professional APCs. The capability of T0-p17 to transduce professional APCs without disturbing the processes leading to a correct endogenous antigen processing and presentation in the MHC class II molecules was confirmed by experiments performed on peritoneal macrophages. These cells were found to be susceptible to T0-p17 both in vitro and in vivo. In particular, in vivo infected peritoneal macrophages were capable to sustain the expansion of p17 specific CD4⁺ T-cells obtained from mice vaccinated i.d. with T0-p17.

It is interesting to note that, differently from processing of exogenous p17, persistence of the p17 transcripts in peritoneal macrophages as well as the correct presentation of p17 peptides within MHC class II molecules led to the development of self-maintaining macrophage/T cell mixed cultures. The limited expansion of specific Th clones following exogenous antigen stimulation is explained by the physiological removal of MHC class II/peptide complexes following T cell dissociation from the immunological synapse that limits their availability to other T-cells over time [27]. On the other hand, the generation of self-maintaining co-cultures following presentation of antigens by rdHSV transduced macrophages finds a possible explanation in the continuous production of endogenous antigens and re-expression of newly synthesized p17 peptides on nascent or recycling MHC class II molecules [28]. This

may ultimately lead to the maintenance of full Th cell activation and consequently, to a suitable microenvironment for macrophage survival [29].

Co-cultivation of T0-p17 infected macrophages with splenocytes of T0-GFP immunized mice did not give rise to any T cell expansion in vitro. This finding may be explained by the knowledge that a strong and detectable Th response mostly depends on the nature of the antigen [30]. Our hypothesis is supported by the recent finding that mice immunized with the same rdHSV vector we used to perform experiments, but expressing the HIV-1 tat transgene, did not trigger the development of any detectable tat-specific Th response, despite the generation of an effective CTL response [14]. However, we can not completely rule out that rdHSV- or GFP-specific CD4⁺ Th responses can be elicited in T0-p17 vaccinated mice, and that they are not detectable under our experimental conditions. In fact, it is not known whether peritoneal macrophages may allow the adequate processing and MHC class II presentation of rdHSV- or GFP-derived peptides leading to the proliferation of specific Th clones. Whatever the reason may be, our data provide evidence that T0-p17 vector is a powerful tool to detect and expand CD4⁺ p17-specific cells. It is worth noting that following i.p. injection of T0-p17 vector, the transgene carried by rdHSV was detectable up to eight months in the spleen of animals. Taken together, these results demonstrate, for the first time, that rdHSV vectors persist in the host suggesting a durable transgene expression which may enhance the magnitude and longevity of immune responses.

In conclusion, our data provide evidence that T0-p17 vector may closely mimic the in vivo processing events, as the p17 transgene is synthesized and presented within the APCs. Because of their capability to infect professional APCs, their long-lasting persistence in such non dividing cells and lack of interference with APCs' functions, rdHSV vectors have to be considered strong candidates as antigen delivery system for vaccine development. This is

particularly important in the context of AIDS vaccines, which require the generation of strong specific CD4⁺ T-cell responses for providing solid protective immunity.

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Figure Legends

Fig. 1. Western blot analysis of p17 protein expressed by T0-p17 infected cells. HIV-p17 protein expression from T0-p17 vector was analysed in 7b Vero (African green monkey kidney cells), G16-9 (mouse glioblastoma cells) and 293 (human embryonic kidney cells) infected with 1 m.o.i. of T0-p17 virus. Cells were analysed for p17 expression at 24 and 48 hours after viral infection by Western blotting, using the anti-p17 mAb MBS3 as specific reagent [13]. Negative control was represented by the same cells infected with 1 m.o.i. of T0-GFP vector. Recombinant p17 protein (rp17) was also loaded as positive control.

Fig. 2. DCs are susceptible to T0-p17 infection both in vitro and in vivo. (a) BM-derived CD11c⁺ cells were enriched by culturing them as described in the “Materials and methods” section. Dot plots represent CD11c⁺ DCs in freshly isolated cells (upper panel, left) and after 6 days of culture (upper panel, right) within the live gate as defined by PI exclusion. BMDCs 5was determined by FACS analysis 24 hours p.i. (lower panel). Histograms represent GFP expression on PI⁻CD11c⁺-gated uninfected (open histogram) or infected (shaded histogram) cells. (b) I.d. T0-p17 injection results in efficient gene delivery to DCs. To evaluate the ability of T0-p17 to transduce DCs in vivo, expression of GFP in the dermis was evaluated by fluorescence microscopy 12 hours after i.d. injection of T0-p17. A double staining of GFP and CD11c was performed as described in the “Materials and methods” section. Expression of CD11c and GFP in the dermis of skin explants. obtained from uninfected (Control) and infected (T0-p17) mice are shown. Superimposition of GFP and CD11c images indicates co-localization of green and red fluorescences. (original magnification 40x).

Fig. 3. Generation of p17-specific CD4⁺ Th cells after i.d. or i.p. injection of T0-p17. (a) Mice immunized i.d. or i.p. with T0-p17 or T0-GFP were sacrificed 6, 11 and 35 days after immunization and spleens removed for antigen proliferation studies. Cells were treated for 6 days with p17 and cell growth was assessed by [³H]thymidine uptake. Bars represent the value obtained by stimulating cells from T0-p17-injected mice subtracted of the value obtained by stimulating cells from T0-GFP-injected mice. Results are the mean of three replicates per assay and SD never exceeded 10%. Statistically significant differences are indicated by the asterisk ($p < 0.01$). (b) Flow cytometric analysis performed on p17-stimulated splenocytes obtained from T0-p17 vaccinated mice 11 days after injection. The majority of living T-cells in these cultures had a CD4⁺ phenotype (shaded histogram). An isotype-matched antibody was used as control (open histogram). The percentage of CD4⁺ cells is indicated.

All data shown are representative of 5 independent experiments conducted in the same manner and with similar results.

Fig. 4. Generation of p17-specific immune responses. Mice immunization was as in the Fig. 3 legend. (a) Production of IFN- γ was evaluated on supernatants of splenocytes stimulated with p17 as recall antigen. (b) Frequency of IFN- γ -producing CD8⁺ T-cells in response to p17-based peptides stimulation was evaluated by ELISPOT using CD4-depleted splenocytes. In a and b, bars represent the value obtained by stimulating cells from T0-p17 injected mice subtracted of the value obtained by stimulating cells from T0-GFP injected mice. Results are the mean of three replicates per assay (SD never exceeded 10%) and are representative of 5

independent experiments with similar results. Statistically significant differences are indicated by the asterisk, representing a $p < 0.01$. (c) The presence of Ab to p17 in sera of T0-p17 immunized mice was evaluated by ELISA. Sera of mice belonging to the same experimental group were pooled and tested at different serial dilutions. Estimated end-point titers are shown.

Fig. 5. Peritoneal macrophages susceptibility to T0-p17 infection and detection of T0-p17 in splenocytes. (a) Cells were collected and infected as described in the “Materials and methods” section. Infection of cells was followed by the expression of GFP and evaluated at 48 hours p.i. by flow cytometry. T0-p17 infected cells (GFP⁺) are shown as an open histogram whereas uninfected cells are shown as a shaded histogram. The percentage of GFP⁺ cells is indicated. (b) Peritoneal macrophages were infected in vivo, by injecting mice i.p. with T0-p17 or T0-GFP and the presence of p17 transgene was evaluated by nested PCR. A p17-specific band was detected in macrophages obtained from T0-p17-infected mice at day 2 (lane 1) and 11 (lane 2) after injection, whereas it was absent in macrophages obtained at day 2 after T0-GFP injection (lane 4). A pcDNA3-p17 vector was used as positive control (lane 3). (c) Detection of T0-p17 in the spleen of i.p. injected animals was performed by amplifying the p17 gene by nested PCR. DNA was extracted from splenocytes obtained at day 6 (lane 1), 11 (lane 2), 35 (lane 3) and 8 months (lane 4) after infection. DNA from splenocytes obtained at day 6 after T0-GFP injection was used as negative control (lane 5).

Fig. 6. Long-term expansion of p17 specific CD4⁺ T cell clones in vitro. (a) The micrograph was taken 30 days after the beginning of mixed cultures, consisting of T0-p17-infected peritoneal macrophages and splenocytes obtained from T0-p17-vaccinated mice 11 days after

i.d. injection. (b) Flow cytometric analysis of long-term cultures showed the presence of both lymphocytes (gate on the left side of the panel) and macrophages (gate on the right side of the panel), as defined by forward and side scatter analysis of living cells. Analysis of the cell phenotype within gated subsets revealed that lymphocytes belonged exclusively to the CD4⁺ T cell subset (open histogram) and that all macrophages expressed MHC class II molecules (open histogram). Isotype matched antibodies were used as control in both assays (closed histograms). (c) Peritoneal macrophages were purified from long-term mixed cultures by CD4 cell depletion and evaluated for the presence of p17 transgene and transcripts by PCR and RT-PCR, respectively. The p17 transgene was detected 30 days after the beginning of co-culture (lane 1). (Lane 2), negative (no template) and (lane 3), positive (pcDNA3-p17 template) control (upper panel). p17-specific transcript were detected in RNA obtained from the same cells as above. Lane 1: amplification of cDNA from T0-p17-infected APC; lane 2: amplification of unretrotranscribed RNA from T0-p17-infected APC (negative control); lane 3: amplification of pcDNA3-p17 plasmid (positive control) (lower panel).