Antigen-specific oral tolerance for the treatment of inflammatory and allergic diseases
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In vitro and in vivo down-regulation of regulatory T cell activity with a peptide inhibitor of Transforming Growth Factor β-1


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Chapter 4

Abstract
Downregulation of CD4+CD25+ T regulatory cell (Treg) function might be beneficial to enhance the immunogenicity of viral and tumor vaccines or to induce breakdown of immunotolerance. Although the mechanism of suppression used by Treg remains controversial, it has been postulated that TGF-β1 mediates their immunosuppressive activity. Here we show that P17, a short synthetic peptide that inhibits TGF-β1 and TGF-β2, developed in our laboratory, is able to inhibit Treg activity in vitro and in vivo. In vitro studies demonstrate that P17 inhibits murine and human Treg-induced unresponsiveness of effector T cells to anti-CD3 stimulation, in a mixed leukocyte reaction or to a specific antigen. Moreover, administration of P17 to mice immunized with peptide vaccines containing tumor or viral antigens, enhanced anti-vaccine immune responses and improved protective immunogenicity against tumor growth or viral infection/replication. When CD4+ T cells purified from OT-II transgenic mice were transferred into C57BL/6 mice bearing subcutaneous EG.7-OVA tumors, administration of P17 improved their proliferation, reduced the number of CD4+Foxp3+ T cells and inhibited tumor growth. Also, P17 prevented development of immunotolerance induced by oral administration of OVA by genetically modified Lactococcus lactis in DO11.10 transgenic mice sensitized by s.c. injection of OVA. These findings demonstrate that peptide inhibitors of TGF-β may be a valuable tool to enhance vaccination efficacy and to break tolerance against pathogens or tumor antigens.

Introduction
During the last years, CD4+CD25+ regulatory T cells (Treg) have been the subject of intense study. This is because their function appears to be critical in the maintenance of peripheral tolerance and regulation of immune responses to non-self antigens. Treg can inhibit activation of other T cells (1) and are needed for protection against autoimmune diseases and prevention of rejection of allogeneic transplants. However, immunoregulatory function of Treg may hinder the induction of immune responses against cancer and infectious agents. Thus, the presence of Treg within tumors may prevent activation of antitumor immune responses favoring tumor growth. This suggests that counteracting Treg activity could evoke effective antitumor immunity (2-5). Treg cells capable of suppressing the in vitro function of tumor-reactive T cells have been found in humans in tumors such as melanoma (6, 7), lung (8), ovary (8, 9), pancreas and breast cancers (10) as well as hepatocellular carcinoma.
Moreover, recent findings suggest that Treg infiltrating neoplastic tissues might be associated with a higher death hazard and reduced survival (7, 9, 12). In infectious diseases, the control exerted by Treg may limit the magnitude of effector T cell responses and may result in failure to control infection. Indeed, it has been shown that some viruses, such as hepatitis B (13), hepatitis C (14-17) and HIV (18-21), may exploit Treg to dampen the antiviral response to favor the persistence of the infection.

Although Treg require antigen exposure to initiate suppressive activity, the effector phase seems to be mediated by an antigen non-specific mechanism (22). The mechanism of suppression by Treg remains controversial, with differences between in vitro and in vivo experiments in terms of the relative contribution of soluble cytokinines respect to cell-to-cell contact. In many experimental systems, multiple subsets of Treg seem to function in vivo by secreting immunosuppressive cytokines such as transforming growth factor-β (TGF-β) and IL-10 (23-26). It has been suggested that TGF-β produced by Treg and/or bound to the cell membrane, may mediate suppression of T-cells (24). Moreover, it has been described that CD4+CD25+ cell-mediated suppression of autoimmune or antitumor CD8+ cells requires an intact TGF-β receptor II on the CD8+ cells (27, 28). TGF-β is also important in the homeostasis of Treg since it may contribute to the generation and proliferation of Treg cells (29). In addition, in the context of T-cell receptor (TCR) stimulation, TGF-β is able to convert peripheral CD4+CD25- naïve T cells to CD4+CD25+ Treg via induction of transcription factor Foxp3 (30, 31). These data suggest that inhibition of TGF-β, in particular by small molecules that might penetrate the interface between contacting T cells, would be a valuable tool to inhibit Treg activity and concomitantly foster antiviral or antitumor immunotherapies.

Using a phage-displayed random 15-mer peptide library we have identified a peptide inhibitor of TGF-β1, named P17. This peptide blocks the inhibition activity of TGF-β1 on the growth of Mv-1-Lu cells in vitro and also inhibits TGF-β1-dependent expression of collagen type I mRNA in the liver of mice orally challenged with CCl₄ (32). In the present study we show that P17 is able to inhibit Treg in vitro and improve the efficacy of vaccination in vivo. Also, P17 reduces the number of CD4+Foxp3+ T cells and augments proliferation of OTII-derived CD4+T cells after their adoptive transfer in mice bearing EG.7-OVA tumors. Moreover P17 efficiently blocked the induction of antigen-specific oral tolerance induced in vivo after intra-gastric supplementation of the OVA secreting Lactococcus lactis (LL-OVA) in DO11.10 mice (33). Thus, as we discuss in more detail below, P17
clearly holds promise to boost immune responses by downregulation of CD4^+CD25^+ Treg, an effect that can be used to enhance the effectiveness of vaccination.

**Materials and Methods**

**Peptides**

KRIWFIPRESSWYERA (P17) is a peptide inhibitor of TGF-β developed in our laboratory (32), peptide SPSYVYHQF (from now on AH1) is a cytotoxic T cell determinant (TCd) expressed by CT26 cells and presented by H-2Ld MHC-class I molecules (34), peptide p1073 (CVNGVCWTV) from hepatitis C virus NS3 protein containing a TCd presented by HLA.A2.1 class I molecules (35), the H2-K^b^-restricted OVA TCd peptide SIINFEKL, and an irrelevant control peptide (AKAVVKTFHETLDCC) from human CD81 molecule were synthesized manually in a multiple peptide synthesizer using Fmoc chemistry as previously described (36). The purity of peptides was above 90% as judged by HPLC.

**Mice**

Female BALB/c and C57BL/6 mice were purchased from IFFA Credo (Barcelona, Spain). A breeding pair of HHD transgenic mice expressing human HLA.A2 molecule was kindly provided by Dr F. Lemonnier (France), and were bred and maintained in pathogen-free conditions. OVA-specific TCR transgenic mice (DO11.10) on a BALB/c background were kindly provided by Dr. J.N. Samson (Vrije Universiteit, Amsterdam, The Netherlands) and bred at the Academic Medical Center, Amsterdam. Eight to ten week old DO11.10 mice were used for the experiments. OVA-specific TCR transgenic mice OT-II and OT-I (C57BL/6 background), were kindly provided by Dr I. Melero (CIMA, Pamplona, Spain). All mice were housed in a conventional animal facility under routine laboratory conditions. All experiments performed followed institutional guidelines and were approved by the institutional ethical committees.

**Cell Culture**

BSC-1 cells (kindly provided by Dr. J.A. Berzofsky, NIH Bethesda, MD) were used for titration of vaccinia virus in ovaries. T2 and P815 cells American Type Culture Collection ATCC (Manassas, VA) were used as target cells in chromium release assays with CTL from HHD or BALB/c mice respectively.
OVA-transfected E.G7-OVA cells (H-2b)(37) and CT26 tumor cells (H-2d) were purchased from ATCC and used in vivo for tumor protection and treatment experiments. They were cultured in complete medium (RPMI 1640 containing 10% fetal calf serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 2 mM glutamine and 50 µM 2-ME. Medium for E.G7-OVA cells also contained 400 µg/ml of G418.

**Purification of regulatory T cells**

Isolation of murine and human CD4\(^+\), CD4\(^+\)CD25\(^+\), and CD4\(^+\)CD25\(^-\) T cells was performed from murine spleen cells or from human peripheral blood mononuclear cells by using murine and human regulatory T cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) respectively and according to manufacturer’s instructions. The purity of the resulting T cell populations was confirmed to be >95% by flow cytometry. Expression of membrane-associated TGF-β1 on the surface of CD4\(^+\)CD25\(^+\) T cells was measured by flow cytometry using anti-LAP(TGF-β1) antibodies (R&D Biosystems).

**RNA extraction and Real Time RT-PCR**

Total RNA extraction from CD4\(^+\)CD25\(^-\) or from CD4\(^+\)CD25\(^+\) T cells was performed using the Nucleic Acid Purification Lysis Solution (Applied BioSystems, Foster City, CA) and the semi-automated system ABI PRISM 6100 Nucleic Acid PrepStation (Applied BioSystems). DNase treatment, reverse transcription and quantitative real time PCR for CTLA4, GITR, Foxp3 and IL-10 were carried out as described (38). mRNA values were represented by the formula: 2\(^{-\Delta Ct}\), where \(\Delta Ct\) indicates the difference in threshold cycle between control (β actin) and target genes.

**In vitro assays for murine or human T regulatory cell function**

Inhibitory activity of murine or human Treg was measured in three different *in vitro* assays of T cell stimulation. CD25 depleted spleen cells (10\(^5\) cells/well) from BALB/c mice or 10\(^5\) human PBMC were stimulated *in vitro* with: (i) 0,5 µg/ml of anti-mouse or anti-human CD3 antibody (Pharmingen) respectively, (ii) with 10\(^3\) bone marrow dendritic cells from C57BL/6 mice (prepared as described previously (39)) or with 10\(^5\) human PBMC (from a different donor, to induce a MLR), or (iii) with a specific antigen. To study the effect of Treg on antigen-specific T cell stimulation, 10\(^5\) ovalbumin-specific CD8\(^+\) T cells from T-cell receptor (TCR) transgenic OT-1 mice, were incubated with 10\(^3\) bone marrow dendritic cells (from
C57BL/6 mice) pulsed with SIINFEKL peptide (1µg/ml), whereas $10^5$ CD4$^+$CD25$^-$ effector T cells/well isolated from human PBMC were cultured in the presence or absence of tetanus toxoid antigen (5 limit of flocculation (Lf)/ml). All T cell stimulations were carried out in the presence or absence of $10^4$ CD4$^+$CD25$^+$ Treg and the indicated concentrations of peptide P17 or control peptide were added to the cultures. To assess whether CD4$^+$CD25$^+$ T cells exert their regulatory function through direct cell contact or through release of soluble factors, we performed a series of transwell experiments. Once purified, CD4$^+$CD25$^+$ T cells were added at a ratio of 1:10 to autologous CD4$^+$CD25$^-$ T cells seeded at $5 \times 10^5$/well in the lower chamber of a 24-well plate. CD4$^+$CD25$^+$ T cells were either cultured in the lower chamber directly in contact with the target cells or in the upper chamber separated from the target cells by a 0.4-µm pore membrane (BD Biosciences Discovery Labware), which allows diffusion of small molecules, such as cytokines, but not of cells. T cell proliferation was tested after 3 days of culture by measuring [methyl-$^3$H]thymidine incorporation. Briefly, the second day of culture 0.5 µCi [methyl-$^3$H] thymidine were added to each well and incubated overnight. Cells were harvested (Filtermate 96 harvester; Packard Instrument, Meriden, CT) and incorporated radioactivity was measured using a scintillation counter (Topcount; Packard Instrument). IFN-γ secretion to the culture supernatant was measured by ELISA (Pharmingen, San Diego, CA) according to manufacturer’s instructions.

**In vitro assays of T cell proliferation in the presence of TGF-β1 or TGF-β2**

Recombinant human TGF-β1 or TGF-β2 inhibit proliferation of murine or human derived effector T cells stimulated with anti-CD3. The IC50 (inhibitory concentration 50%) for human TGF-β1 was 20 pg/ml and 1 pg/ml when using murine and human derived effector T cells respectively. For TGF-β2, the IC50 was 0.25 pg/ml when using murine splenocytes. Splenocytes from C57BL/6 female mice, or peripheral blood lymphocytes from human blood donors, were cultured (10^5 cells/well) in the presence or absence of 0.5 µg/ml anti-mouse or anti-human CD3 antibodies and in the presence or absence of the corresponding IC50 of exogenously added human TGF-β1 or human TGF-β2 (R&D Biosystems). P17 or control peptide were added to the cultures at the concentrations indicated in figures. T cell proliferation was tested after 3 days of culture by measuring [methyl-$^3$H] thymidine incorporation as described above.
**Biomolecular interaction analysis**

Screening of peptide binding to TGF-β1, TGF-β2 and TGF-β3 was performed by surface plasmon resonance (SPR) using a BIAcore X Biosensor (BIAcore, AB, Uppsala, Sweden). TGF-β isoforms (R&D Systems, USA) as well as an irrelevant protein were covalently immobilized onto the surface of flow cell 2 (FC2) of CM5 chips (BIAcore) as described (40). Flow cell 1 (FC1), which does not contain immobilized TGF-β, was used as the reference flow cell. Individual peptide solutions (5 µM) were injected three times in 10 mM Hepes, 150 mM NaCl, 0.005 % (v/v) Tween-20, 0.1 mg/ml BSA, pH 7.4, at a flow of 30 µl/min. Mass transport limitation was excluded. Curves were processed by subtracting the response in FC1 from that in FC2.

**Immunization experiments and measurement of T cell activation**

BALB/c or HHD mice were immunized subcutaneously at day 0 with 50 nanomoles of TCd peptides AH1 or p1073 respectively, emulsified in IFA. Fifty nanomoles/mouse of peptide P17 was administered intraperitoneally (i.p.) at days 6, 7, 8 and 9 after immunization. At day 10, mice were sacrificed, spleens were removed, homogenized and 8 × 10^5 cells cultured in 96-well plates in complete medium in the presence or absence of 10 nM of the corresponding TCd peptide. Peptide-specific CTL responses were measured using a conventional cytotoxicity assay as previously described (41). IFN-γ produced in response to the TCd was measured by ELISA (Pharmingen) in culture supernatants (50 µl) harvested after 48 hours of culture, according to manufacturer’s instructions.

**Effect of P17 in anti-tumor peptide vaccination**

Animals immunized with 50 nanomoles of peptide AH1 emulsified in IFA as previously described (42), were treated with 50 nanomoles of peptide P17 or with saline at days 6, 7, 8, 9 and 10. Ten days after immunization, mice were challenged by s.c. injection with 5 × 10^5 CT26 tumor cells. In an independent experiment, 100 µg per dose of neutralizing anti-TGF-β polyclonal antibody from rabbit, or the corresponding isotype control (R&D Systems) were administered i.p. to mice using the same schedule as for P17. Tumor size was monitored twice a week with a caliper and it was expressed according to the formula \( V = \frac{(\text{length} \times \text{width}^2)}{2} \). Mice were sacrificed when tumor size reached a volume greater than 4 cm^3.
**Effect of P17 in "in vivo" T cell transfer experiments in E.G7-OVA tumor bearing mice**

Groups of five C57BL/6 mice were challenged with $5 \times 10^5$ E.G7-OVA tumor cells. When tumors reached 12.5 mm$^3$, mice were adoptively transferred with $3 \times 10^6$ CFSE-labeled CD4$^+$ T cells isolated from OT-II transgenic mice. CFSE cell labeling was carried out by incubation with 1 µM CFSE for 10 min at room temperature followed by three washes with PBS. After T cell transfer, a group of mice was treated daily with 50 nanomoles of peptide P17 by i.p. route. Five days after T cell transfer, mice were immunized i.v. with 50 µg of OVA (Sigma, St Louis, MO) in PBS and sacrificed 3 days after immunization. Splenocytes were isolated and the analysis of CFSE-labeled CD4$^+$ T cell proliferation and CD4$^+$Foxp3$^+$ cell staining was carried out by flow cytometry. Analysis of CD4$^+$Foxp3$^+$ cells was carried out using the mouse regulatory T cells staining kit (from eBioscience. San Diego, CA) according to manufacturer’s instructions. Tumor size was measured the day of sacrifice as described above.

**Effect of P17 in in vivo protection against infection with a recombinant vaccinia virus expressing HCV proteins**

HHD mice immunized with peptide p1073 as described above, were treated i.p. with 50 nanomoles of peptide P17 or with saline at days 6, 7, 8 and 9 after immunization. They were challenged i.p. at day 10 with $5 \times 10^6$ pfu of the recombinant vaccinia vHCV1-3011 expressing HCV polyprotein. Three days after vaccinia challenge, mice were sacrificed and viral titre measured as described (43).

**Effect of P17 in a model of hypersensitivity**

The *Lactococcus lactis* MG1363 (LL) strain was genetically modified and used throughout this study, as described before (44, 45). Bacteria were cultured in GM17E medium i.e., M17broth (Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose and 5 µg/ml erythromycin (Abbott B.V., Hoofddorp, The Netherlands). Bacteria were diluted 200-fold in GM17E medium, incubated at 30 °C overnight and harvested by centrifugation and concentrated in BM9 medium at $2 \times 10^9$ bacteria/100 µl. Treated mice, received 100 µl of this suspension daily by intragastric catheter (46). DO11.10 mice were sensitized by s.c. injection of 100 µg OVA in 50 µl of a 1:1 CFA (Difco, BD, Alphen aan de Rijn, The Netherlands) saline solution in the tail base at day 1 (47). Mice were fed BM9 as a control or LL-OVA
(both at days 1-5 and 8-12) administrations using a stainless 18-gauge animal feeding needle. Every other day, starting at day 0, mice received 50 nanomoles/mice of P17 peptide. Eleven days after sensitization, antigen-specific DTH responses were assessed. For DTH measurement, mice were challenged with 10 μg OVA in 10 μl saline in the auricle of one ear and 10μl saline in the other. The increase in ear thickness was measured in a blinded fashion using an engineer’s micrometer (Mitutoyo, Tokyo, Japan) at 24 h after challenge. DTH responses were expressed as the difference in increase between the OVA injected and the saline injected ear thickness, following subtraction of ear-thickness before the challenge (DTH response=OVA-saline-baseline). Intact, LPS-free OVA grade V protein was used as antigen (Sigma Aldrich). For cytokine measurements, 2 x 10^5 cells of splenocytes were cultured in 96-well U-bottom plates in a total volume of 200 μl complete medium with 100 μg/ml OVA. Cells were cultured at 37°C in a 5% CO2 humidified incubator and after 72 h, culture supernatants were collected and frozen at −20°C until cytokine analysis was performed. Cytokine production was quantified using the Mouse Inflammation Cytometric Bead Assay (BD Biosciences, Mountain View, CA, USA).

**Statistical analysis**

Normality was assessed with Shapiro-Wilk W test. Statistical analyses were performed using parametric (Student’s t test and one-way ANOVA) and non-parametric (Kruskal-Wallis and Mann-Whitney U) tests. For all tests a p value < 0.05 was considered statistically significant. Descriptive data for continuous variables are reported as means±SD. SPSS 9.0 for Windows was used for statistical analysis.

**Results**

**Peptide P17 inhibits Treg in vitro**

Previous studies have shown that murine CD4^+CD25^+ regulatory T cells produce high levels of TGF-β1 bound to the cell surface and/or secreted to the medium. Blockade of this TGF-β1 by anti-TGF-β may limit the ability of these cells to suppress CD25^- T cell proliferation (24, 48). In a previous work, we showed that P17, a peptide inhibitor of TGF-β1, inhibited TGF-β1-dependent expression of collagen type I mRNA in a model of liver damage (32). In Figure 1A and 2A we show that P17 is also able to inhibit, in a dose dependent manner, the immunosuppressive activity of
TGF-β1 exogenously added to murine or human derived effector T cells stimulated with anti-CD3 (see methods). Inhibitory activity of P17 was similar to that found when 2 μg/ml of neutralizing anti-TGF-β1 antibodies were added to the cultures. To study the capacity of P17 to inhibit the
suppressor activity of Treg cells in vitro, we purified CD4+CD25+ T cells from murine splenocytes and studied their immunosuppressive activity over effector T cells stimulated with anti-CD3 antibodies. Purified Treg had high mRNA levels for CTLA4, GITR, Foxp3 and IL-10 and expressed TGF-β1 bound to latency-associated peptide (LAP) on their membrane (Figure 1B). To assess whether CD4+CD25+ T cells exert their regulatory function through direct cell contact, or through release of soluble factors, we separated effector T cells from Tregs using Transwell assays. It was found that purified Tregs exerted their inhibitory activity only when in direct contact with effector T cells (Figure 1C).

We then tested the effect of P17 in co-cultures of effector T cells and Treg cells isolated either from mouse spleen cells or from human PBMC. In these assays P17 was able to inhibit Treg suppressive function, restoring the proliferation of murine effector T cells or human PBMC in response to soluble anti mouse CD3 or anti-human CD3 antibody respectively (Figures 1D and 2B). Addition of anti-TGF-β neutralizing polyclonal antibody was also able to significantly inhibit Treg activity. P17 was also able to restore proliferation of effector T cells in MLR, when bone marrow derived dendritic cells from C57BL/6 were co-cultured with non-adherent spleen cells from BALB/c mice in the presence of Treg (Fig 1E), or when human PBMC from two different donors were mixed in the presence of human purified Treg (Fig 2C). P17 also reverted Treg mediated inhibition of

Figure 1. P17 inhibits immunosuppression caused by TGF-β or by murine T regulatory cells. (A) Inhibition of TGF-β1: Spleen cells from BALB/c mice were stimulated with anti-CD3 monoclonal antibodies in the presence or absence of exogenously added recombinant human TGF-β1 (20pg/ml) and the indicated concentration of P17, control peptide (Pcont) or polyclonal anti-TGF-β or the corresponding isotype control antibodies (2 µg/ml). (B) Characterization of purified CD4+CD25+ T cells: Expression of TGF-β1 bound to latency-associated peptide (LAP) on their membrane was measured by flow cytometry, and mRNA expression of Treg associated genes was measured by real time PCR. (C) Immunosuppression caused by purified CD4+CD25+ T cells is contact-dependent: effector T cells were stimulated with anti-CD3 in the presence of purified CD4+CD25+ T cells either in direct contact or separated by a 0.4-µm pore membrane. (D, E and F) Inhibition of murine T regulatory cells by P17: (D) spleen cells from BALB/c mice were stimulated with anti-CD3 monoclonal antibodies in the presence or absence of purified CD4+CD25+ T cells; (E) Mixed leukocyte reaction using spleen cells from BALB/c mice (10^{5} cells/well) and bone marrow derived dendritic cells from C57BL/6 mice (10^{3} cells/well); (F). T cells from OT-1 mice were incubated with DC pulsed with SIINFEKL peptide, with or without of CD4+CD25+ Treg cells. Different concentrations of P17 (12.5 to 100 µM) were tested in (D) to measure inhibition of Treg activity, whereas a single concentration (100 µM) was tested in (E and F). A concentration of 2µg/ml of polyclonal anti-TGF-β or the corresponding isotype control antibodies (2 µg/ml) was used in (A and D). Cell proliferation (A, C, D, E and F) was analyzed by measuring ³H trititated thymidine incorporation in the harvested cells using a scintillation counter. IFN-γ released to the culture was quantified by ELISA (E). * t test *, p<0.05 (comparison between the indicated group (*) and the corresponding control of immunosuppression in the absence of inhibitors). The results are representative of at least three different experiments per each panel.
antigen-specific T cell activation in the case of T cells from OT-I transgenic mice responding to peptide OVA (257-264) (SIINFEKL) from ovalbumin (Fig 1F), or in the case of human PBMC responding to tetanus toxoid (Fig 2D). The percentage of inhibition of the Treg effect by 100 µM of P17 was found to vary between 25% and 100% in different in vitro models. This variation may be related to the role of TGF-β in each particular model. In summary, results from Fig 1 and 2 indicate that P17 is able to inhibit, at least partially, both murine and human Treg in vitro.

**Figure 2.** P17 inhibits immunosuppression caused by TGF-β or by human T regulatory cells. (A) Inhibition of TGF-β1: Human PBMC were stimulated with anti-CD3 monoclonal antibodies in the presence or absence of exogenously added recombinant human TGF-β1 (1pg/ml) and the indicated concentration of P17 or polyclonal anti-TGF-β or the corresponding isotype control antibodies (2 µg/ml). (B, C and D) Inhibition of human Tregs: (B) Human PBMC were stimulated with anti-CD3 monoclonal antibodies in the presence or absence of purified CD4+CD25+ Treg cells; (C) Mixed leukocyte reaction using PBMC isolated from two donors in the presence or absence of CD4+CD25+ Treg cells isolated from one of the human donors; (D) Human PBMC were cultured with or without tetanus toxoid in the presence or absence of human CD4+CD25+ Treg. Different concentrations of P17 (12 to 100 µM) were tested in (B) to measure inhibition of Treg activity, whereas a single concentration (100 µM) was tested in (C and D). Cell proliferation (A to D) was analysed by measuring 3H tritiated thymidine incorporation in the harvested cells using a scintillation counter. t test *, p<0.05 (comparison between the indicated group (*) and the corresponding control of immunosupression in the absence of inhibitors). The results are representative of at least three different experiments per each panel.
**Peptide P17 also inhibits the immunosuppressive activity of TGF-β2 isoform**

The forkhead transcription factor Foxp3 is highly expressed in CD4⁺CD25⁺ Tregs and act as a key player in mediating their inhibitory functions. But also, a recent paper has described that tumor cells can be induced to express functional Foxp3 by TGF-β2, in such a way that naïve T-cell proliferation is inhibited when cocultured with these Foxp3-expressing tumor cells (49). Since TGF-β isoforms have a high homology (70-80%), we investigated the capacity of P17 peptide to bind to these isoforms by Surface Plasmon Resonance. As shown in Figure 3A, binding of P17 follows the order TGF-β1>TGF-β2>>>TGF-β3. As expected, P17 was unable to bind to an irrelevant protein (Fig 3A) and a control peptide from human CD81 molecule did not bind to any of the TGF-β isoforms (data not shown). Since P17 was able to bind TGF-β2, we tested its ability to inhibit the immunosuppressive activity of this cytokine *in vitro*. Thus, we added TGF-β2 to effector T cells stimulated with anti-CD3 (see methods). It was found that 100 µM of P17 inhibited the immunosuppressive activity of TGF-β2 by around 50% (Fig 3B).

**Figure 3.** *P17 binds to TGF-β2 and inhibits its immunosuppressive activity.*

(A) Binding assays: TGF-β1, TGF-β2, TGF-β3 or an irrelevant protein were immobilized covalently on the flow cell FC2 of a CM5 chip via a standard amine coupling procedure. P17 solution (5 µM) was injected three times over the protein bound surfaces. Sensograms of P17 binding capacity to the different TGF-β isoforms or to the irrelevant protein are plotted. (B) Inhibition of TGF-β2 activity: Spleen cells from BALB/c mice were stimulated with anti-CD3 monoclonal antibodies in the presence or absence of exogenously added recombinant human TGF-β2 (0.25pg/ml) and 100 µM of P17 or control peptide (Pcont). Cell proliferation was analyzed by measuring 

[^3H] tritiated thymidine incorporation in the harvested cells using a scintillation counter. t test *, p<0.05 (when compared T cell proliferation in the presence versus in the absence of P17). The results are representative of two independent experiments.
Peptide P17 improves immunogenicity of AH1 peptide vaccination leading to protection against CT26 tumor challenge

Downregulation of Treg suppressor activity in vivo might be beneficial to enhance vaccine immunogenicity against tumor antigens. Immunization of BALB/c mice only with peptide AH1 (a TCd, expressed by CT26 colon cancer cells) is unable to induce a protective CTL response against challenge with CT26 tumor cells (34). However, this result could be overcome by co-immunization of AH1 and an adequate T helper cell determinant capable of inducing a competent T helper response (42). We have also shown that depletion of CD25+ Treg cells with anti-CD25 antibodies before immunization with peptide AH1, permits the induction of a long lasting anti-tumoral immune response (2). All these observations lead us to speculate that in vivo inhibition of Treg by peptide P17 (instead of Treg depletion) in combination with vaccination with AH1, might allow the control of tumor growth. Thus, BALB/c mice were immunized with peptide AH1 emulsified in IFA at day 0 and treated with saline or with 50 nanomoles/mice of peptide P17 at days 5, 6, 7, 8 and 9 after immunization. As shown in Figure 4A, immunization with AH1 does not induce IFN-γ producing cells specific for AH1 peptide. However, in vivo treatment of AH1 immunized mice with P17 strongly augments AH1 immunogenicity.

**Figure 4.** Peptide P17 improves immunogenicity of AH1 peptide vaccination leading to protection against CT26 tumor challenge. Groups of BALB/c mice were immunized with saline (n=10) or, with peptide AH1 emulsified in IFA (n=20). Ten mice immunized with AH1 were treated i.p. with saline at days 5, 6, 7, 8 and 9 after immunization, whereas the remaining 10 mice were treated with 50 nanomoles/mice of peptide P17 at days 5, 6, 7, 8 and 9 after immunization. At day 10 after immunization, two mice from AH1 immunized groups were sacrificed and spleen cells were cultured in the presence or absence of peptide AH1. After 48 hours of culture, supernatants were removed and IFN-γ released to the culture was quantified by ELISA (A). The remaining 8 mice from both groups of AH1-immunized mice (the one treated with AH1 only and the other treated with AH1 + P17) as well as mice injected with saline were challenged s.c with 5 x 10⁵ CT26 tumor cells. Tumor size was measured as described in Methods. Mice were sacrificed when tumor size reached a volume greater than 4 cm³. The ratio 6/8 indicates that 6 mice (out of the 8 treated with AH1 + P17) remained tumor free (B). The results are representative of two different experiments.
Moreover, when mice were challenged s.c. with $5 \times 10^5$ CT26 tumor cells, six out of eight mice immunized with AH1 and treated with peptide P17, remained tumor-free, whereas all mice immunized with AH1 only or left unimmunized developed tumors (Figure 4B). These results indicate that TGF-β inhibition by P17 improves immunogenicity of AH1 peptide vaccination and concomitantly protection against tumor growth.

**Peptide P17 reduces the number of CD4+Foxp3+ cells and improves T cell proliferation of OT-II derived CD4+ T cells adoptively transferred to mice bearing E.G7-OVA tumors**

A number of tumor cells have been shown to produce TGF-β both *in vitro* and *in vivo*, which may mediate immunosuppression in the hosts (50, 51). TGF-β1 is important in maintaining functional Foxp3+ CD4+CD25+ Treg cells and can also induce Foxp3 expression in naïve T cells (30, 31, 52). We therefore wanted to test the *in vivo* effect of P17 on the number of CD4+Foxp3+ T cells after the adoptive transfer of CFSE-labeled CD4+ T cell.  

![Image](image_url)

**Figure 5.** Peptide P17 reduces the number of CD4+Foxp3+ cells and improves T cell proliferation of OT-II derived CD4+ T cells adoptively transferred to mice bearing E.G7-OVA tumors. C57BL/6 mice bearing E.G7-OVA tumor were adoptively transferred with $3 \times 10^6$ CFSE-labeled CD4+ T cells purified from OT-II mice. At day 10 after adoptive transfer, mice were treated daily with PBS (panels A and C) or with peptide P17 (panels B and D) ($n=5$ mice per group). All mice were immunized with OVA protein at day 5 after transfer and sacrificed 3 days later to evaluate proliferation of transferred CD4+ T cells in the spleen (panels A and B) or the numbers of splenic CD4+Foxp3+ T cells (panels C and D) by flow cytometry. Tumor size was measured the day of sacrifice as described in Methods. Results are representative of two different experiments.
cells from OT-II transgenic mice to animals bearing subcutaneous E.G7-OVA tumors. E.G7-OVA cells are derived from EL-4 thymoma cell line, which produces high amounts of TGF-β in vitro and in vivo ((53), and data not shown). Thus, 16 mice were injected with 5 x 10^5 E.G7-OVA tumor cells, and once the tumors reached 12.5 mm³, mice were adoptively transferred with 3 x 10^6 CFSE-labeled CD4⁺ OT-II derived T cells. After adoptive transfer, mice were treated daily with PBS or with 50 nanomoles of peptide P17 (n= 8 mice per group). All mice were immunized with OVA protein at day 5 after adoptive transfer, and sacrificed 3 days thereafter to evaluate proliferation of the transferred CD4⁺ T cells. As shown in Figure 5, P17 treatment improves proliferation of adoptively transferred CD4⁺ T cells (16.5% of undivided OVA specific CD4⁺ T cells (panel B) vs 38.7 % in PBS treated mice (panel A)). This improved T cell proliferation in P17-treated mice, was accompanied by a reduction of the total numbers of CD4⁺Foxp3⁺ T cells (compare panels D (P17 treated) and panel C (PBS-treated)). Improved proliferation of CD4⁺ transferred T cells and reduction in the number of CD4⁺Foxp3⁺ cells was associated with a diminution in tumor size at the day of sacrifice (p<0.05, Figure 5E).

**Peptide P17 improves immunogenicity of p1073 peptide vaccination and protects mice against challenge with recombinant vaccinia vvHCV 3011 virus**

As described above, Treg may hamper the induction of protective cellular immune responses in several viral infections. In particular, it has been recently reported that patients with chronic hepatitis C have a higher number of peripheral Treg, suggesting that these cells might play a role in the chronicity of infection (14-17). We tested the capacity of P17 administration to improve immunogenicity of an HCV-NS3 derived peptide. HHD transgenic mice were immunized s.c with peptide p1073, encompassing a HLA.A2.1 restricted epitope from HCV NS3 protein, emulsified in IFA. At days 5-9 after immunization, mice were treated i.p. with 50 nanomoles/mice of P17 or with saline. Ten days after immunization, animals were sacrificed and spleen cells were cultured with p1073 for five days. Lytic activity was measured in a conventional chromium release assay using T2 cells pulsed with peptide 1073. It was found that P17 treatment of immunized animals was able to prime a cytotoxic T cell response specific for peptide p1073, which was not elicited in animals immunized with p1073 and treated with saline instead of P17 (Figure 6A). The ability of P17 treatment to improve immunogenicity of p1073 was also measured in vivo, based on its capacity to protect mice against challenge with a recombinant vaccinia virus expressing the whole polyprotein of
peptide P17 improves immunogenicity of p1073 peptide vaccination and protects mice against challenge with recombinant vaccinia vvHCV 3011 virus expressing HCV polyprotein. (A) HHD transgenic mice were immunized with peptide p1073 from HCV NS3 protein emulsified in IFA and treated i.p. with saline or with fifty µg/mice of peptide P17 at days 5, 6, 7, 8 and 9 after immunization. Ten days after immunization, mice were sacrificed and spleen cells were cultured in the presence or absence of peptide p1073. After 5 days of culture, CTL activity against peptide-loaded T2 target cells was measured in a conventional Chromium release assay. (B) Groups of HHD transgenic mice were immunized with saline (n=12) or with peptide p1073 from HCV NS3 protein emulsified in IFA (n=12). Six mice per group were treated with P17 as described in (A). Mice were challenged i.p. with 5 x 10^5 pfu of the recombinant vaccinia vvHCV 3011 expressing the HCV polyprotein. Three days after challenge, ovaries were harvested and vaccinia titer was measured by plating on BSC-1 cells. Viral load is expressed as pfu per mg of ovary. Bars represent the mean average of virus titers from 6 mice ±SEM. Results are representative of two different experiments.

Figure 6. Peptide P17 improves immunogenicity of p1073 peptide vaccination and protects mice against challenge with recombinant vaccinia vvHCV 3011 virus expressing HCV polyprotein.

Breakdown of immunotolerance by P17

We have recently demonstrated that mucosal delivery of OVA by genetically modified Lactococcus lactis (LL-OVA) induces suppression of local and systemic OVA specific T-cell response in DO11.10 mice that is mediated by the induction of OVA-specific CD4+CD25- Treg which are critically depend on TGF-β (33). In order to further validate the ability of P17 in this model, mice were sensitized by s.c. injection of 100 µg of hepatitis C virus, as a surrogate of HCV infection. Thus, HHD transgenic mice were immunized s.c. with saline (n=12) or with p1073 emulsified in IFA (n=12). At days 5-9 after immunization half of the mice from each group were treated i.p. with P17. Ten days after immunization, mice were challenged with 5 x 10^6 pfu of the recombinant vaccinia vvHCV 3011 and three days later viral load was measured in the ovaries. It was found that p1073 immunization alone showed a similar replication than PBS treated mice, whereas treatment of p1073 immunized mice with P17 inhibited viral replication by four logs (Figure 6B).
OVA in 50 μl of a 1:1 CFA saline solution in the base of the tail, and oral tolerance was induced with a 10-day oral administration of LL-OVA. Six of the 12 LL-OVA treated mice received 50 nanomoles of peptide P17 in PBS by i.p. route at alternate days until the end of the experiment. LL-OVA treated mice were significantly tolerized compared to the control mice (1.4 x 10^{-2} mm vs. 15.8 x 10^{-2} mm). Co-injection of P17 blocked the induction of antigen specific oral tolerance measured by a significant increase in ear-thickness compared to the LL-OVA treated mice (11 x 10^{-2} mm vs. 1.4 x 10^{-2} mm) (Fig 7A). Immediately after DTH measurements, spleens were isolated and ex vivo stimulated with OVA for 72 hours. In agreement with this finding, it was found that P17 treatment restored IFN-γ production by splenocytes in response to OVA antigen, a response which was totally inhibited by LL-OVA administration (214.4 vs 15.1 pg/ml, respectively, Fig 7B). These results indicate that in this model, administration of P17 effectively interferes with the development of antigen-specific immunotolerance.

**Figure 7.** In vivo injection of P17 inhibits the induction of oral tolerance by OVA secreting Lactococcus lactis and increases the IFN-γ production of bulk splenocytes. DO11.10 mice were sensitized by s.c. injection of 100 μg OVA in CFA at day 1. Mice were orally treated with BM9 (control) or with LL-OVA at days 1-5 and 8-12. Every other day one LL-OVA treated group received i.p. injection of P17. At day 11, mice were challenged with 10 μg OVA in 10 μl saline in the auricle of one ear and 10 μl saline in the other. DTH responses are expressed as the mean differences in ear thickness increase between the OVA injected and saline injected mice, following subtraction of ear-thickness before OVA challenge (A). All groups consisted of 6 mice. On day 12, bulk splenocytes were isolated and tested for IFN-γ production after 72-hour ex vivo stimulation with 100μg/mL OVA (B).
Discussion

Naturally occurring Treg cells inhibit T-cell proliferation *in vitro*, a mechanism which plays the beneficial role of controlling T-cell responses to self-antigens. This prevents the development of autoimmune diseases (54, 55) as well as the induction of harmful immune responses after organ transplants (55). However, Treg cells may also limit the magnitude of effector responses, which although under certain circumstances may reduce collateral tissue damage caused by vigorous antimicrobial immune responses, may result in failure to adequately control infections (56). Moreover, Treg might hinder the induction of immune responses against cancer (57). All these observations suggest that adequate control of Treg activity may have important implications in medicine.

Accumulating evidences support the role played by TGF-β as a mediator of Treg *in vitro* and *in vivo* (54). Thus, TGF-β directly inhibits proliferation and acquisition of effector function of naïve T cells. In the absence of TGF-β signaling in T cells, the dominant negative TGF-β receptor type II (dnTβRII) transgenic mice develop a lymphoproliferative syndrome and autoimmunity (58-60), probably because their T cells escape control by Treg (58). Moreover, it has been described that TGF-β produced by Treg and/or bound to the cell membrane may mediate suppression of T-cells (24). Also, recent data shows that dendritic cells may inhibit T cell activation via the secretion of TGF-β (61) or by surface expression of TGF-β bound by latency-associated peptide LAP (62). TGF-β is also important in the homeostasis of Treg since it may contribute to their generation and proliferation (29-31, 63). All these data suggest that inhibition of TGF-β, in particular by small molecules that might penetrate the interface between contacting T cells, might be useful to potentiate antiviral or antitumor immunotherapies.

We have shown that P17, a TGF-β inhibitor peptide developed in our laboratory (64), is able to inhibit murine or human derived Treg activity *in vitro* in three different experimental settings. Thus, P17 was able to restore murine or human T cell proliferation in response to anti-CD3 stimulation, which was inhibited by the addition of Treg. Similarly, P17 restored, at least partially, T cell proliferation in a MLR inhibited by Treg. P17 also inhibited Treg cells activity over specific T cells stimulated by an antigen. These results prompted us to test P17 *in vivo*. In a previous work we showed that in vivo CD25+ T cell depletion improved immunogenicity of AH1 peptide in vaccination and protected mice against tumor challenge (2). We found that in vivo P17 administration, instead Treg depletion,
was also able to enhance immunogenicity of AH1 peptide vaccination, and protected mice from CT26 tumor challenge. Downregulation of Treg suppressor activity in vivo may be beneficial to enhance immunogenicity of a vaccine (2). Similarly, P17 administration improved the immunogenicity of a peptide vaccine consisting in the immunization of peptide p1073, which encompasses a HLA.A2.1 restricted epitope from HCV NS3 protein, the outcome being a reduction of recombinant vaccinia vvHCV 3011 virus replication after vaccination with p1073. These results are in agreement with previous reports showing an enhancement of immunogenicity of a vaccine by the depletion of Treg (2-5, 65). However, we believe that in vivo inhibition of Treg activity by P17, instead of Treg depletion, might allow a better control of Treg function, reducing the risk of autoimmune diseases that may be favored in the absence of Treg (66). When we compared the effect of anti-TGF-β antibodies with P17 in vivo, both molecules were effective. Indeed, AH1-immunized mice remained protected from CT26 tumor challenge if they were treated with anti-TGF-β1 polyclonal antibodies (5 administrations of 100 µg anti-TGF-β1 per mice from day 5 to 9 after AH1 immunization). Similarly, in vivo administration of anti-TGF-β1 antibodies reverted immunotolerance induced by LL-OVA administration (data not show). Although both molecules are efficient TGF-β inhibitors, the use of peptides might have advantages. Thus, the relative short life of peptides would allow a finer control on the inhibition of TGF-β during the required period in vivo. This would reduce the potential toxic effects of long term inhibition of the cytokine.

Tumors produce factors such as prostaglandins, IL-10, VEGF and TGF-β which may create an immunosuppressive microenvironment and may hamper immunotherapy. This microenvironment, and in particular TGF-β1, might favor Treg development. Indeed, it has been widely described that during tumor progression in humans, Treg accumulate in tumors and secondary lymphoid organs (6-12). This increase in Treg number may be favored by a recruitment of naturally occurring Treg cells, as well as by a conversion of CD4+ effector Th cells into Treg, in this particular TGF-β-enriched tumor microenvironment (67, 68). In addition, it has been recently described that the TGF-β2 isoform may induce Foxp3 expression in pancreatic carcinoma cells, enabling these tumor cells to suppress T cell proliferation (49). Thus, inhibition of TGF-β1 and TGF-β2 isoforms would have an impact in this adverse environment by reducing the number of Treg, Foxp3 expression and favoring effector T cell proliferation and function. We show in this work that P17 is able to inhibit the immunosuppressive activity of TGF-β1 as well as TGF-β2 in vitro. P17 administration, after adoptive transfer of CFSE-labeled
CD4+ T cells from OT-II transgenic mice to C57BL/6 mice bearing EG.7-OVA tumors, improved proliferation of transferred T cells and reduced the numbers of CD4+Foxp3+ T cells. Moreover, P17 treatment after the adoptive transfer of CD4+ T cells inhibited tumor growth, suggesting that this TGF-β inhibitor might be very useful for the development of anti-cancer therapies.

TGF-β plays a central role in oral tolerance. This takes place via regulation of mucosal inflammation and mediating active suppression against orally administered antigens, (reviewed in (69)). Thus, TGF-β knockout mice develop chronic inflammation in many tissues, including the gastrointestinal tract (70). In addition, recent findings suggest that TGF-β may be a primary link between distinct populations of Treg cells that are induced by feeding. We have recently shown that mucosal delivery of OVA by genetically modified L.lactis (LL-OVA) induces OVA-specific CD4+CD25- Treg cells which in turn, suppress OVA specific T-cell responses in DO11.10 mice, in a process critically dependent on TGF-β (33). We have found that P17 administration is able to inhibit induction of oral tolerance in this model, suggesting that P17 may have important applications to enhance the immunogenicity of orally administered antigens.

In summary, we have shown that inhibition of TGF-β by P17 is able to inhibit the immunosuppressive activity of murine and human-derived Treg cells in vitro. Also, and most importantly, in vivo experiments using P17 show that this peptide fosters the immunogenicity of peptide vaccination when administrated 5 days after vaccination. Moreover, P17 was able to improve proliferation of adoptively transferred T cells, and reduce the number of CD4+Foxp3+ T cells in vivo in mice bearing a TGF-β producing tumor. In addition, P17 was also able to inhibit Treg function in an antigen-specific model of oral induced tolerance. In summary, our results demonstrate that inhibition of TGF-β1 and TGF-β2 with a small synthetic peptide can be a useful therapeutic strategy to enhance the immunogenicity of vaccines or to break tolerance against pathogens or tumor antigens.

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References


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