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In Vitro and In Vivo Down-Regulation of Regulatory T Cell Activity with a Peptide Inhibitor of TGF-β1

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Down-regulation of CD4+CD25+ regulatory T (Treg) cell 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 cells remains controversial, it has been postulated that TGF-β mediates their immunosuppressive activity. In this study, 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 an MLR or to a specific Ag. Moreover, administration of P17 to mice immunized with peptide vaccines containing tumor or viral Ags enhanced anti-vaccine immune responses and improved protective immunogenicity against tumor growth or viral infection or replication. When CD4+ T cells purified from OT-II transgenic mice were transferred into C57BL/6 mice bearing s.c. 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 Ags. The Journal of Immunology, 2008, 181: 126–135.

During the last years, CD4+CD25+ regulatory T (Treg) cells have been the subject of intense study. This interest is because their function appears to be critical in the maintenance of peripheral tolerance and regulation of immune responses to non-self Ags. Treg cells 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 cells may hinder the induction of immune responses against cancer and infectious agents. Thus, the presence of Treg cells within tumors may prevent activation of antitumor immune responses favoring tumor growth. This effect 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 (10) cancers as well as hepatocellular carcinoma (11, 12). Moreover, recent findings suggest that Treg cells 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 cells 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 cells to dampen the antiviral response to favor the persistence of the infection.

Although Treg cells require Ag exposure to initiate suppressive activity, the effector phase seems to be mediated by an Ag nonspecific mechanism (22). The mechanism of suppression by Treg cells remains controversial, with differences between in vitro and in vivo experiments in terms of the relative contribution of soluble cytokines with respect to cell-to-cell contact. In many experimental systems, multiple subsets of Treg cells seem to function in vivo by secreting immunosuppressive cytokines such as TGF-β and IL-10 (23–26). It has been suggested that TGF-β produced by Treg cells 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 cells because it may contribute to the generation and proliferation of Treg cells (29). In addition, in the context of TCR stimulation, TGF-β is able to convert peripheral CD4+CD25− naive T cells to CD4+CD25+ Treg cells 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

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clearly holds promise to boost immune responses by down-regulating DO11.10 mice (33). Thus, as we discuss in more detail, P17 Ag-specific oral tolerance induced in vivo after intragastric supplementation of OVA tumors. Moreover P17 efficiently blocked the induction of TGF-β expression of collagen type I mRNA in the liver of mice orally challenged with carbon tetrachloride (32). In the present study we show that P17 is able to inhibit Treg cells in vivo and improve the efficacy of vaccination in vivo. Also, P17 reduces the number of CD4+Foxp3+ T cells and augments proliferation of OT-II-derived CD4+ T cells after their adoptive transfer in mice bearing EG7-OVA tumors. Moreover P17 efficiently blocked the induction of Ag-specific oral tolerance induced in vivo after intragastric supplementation of the OVA secreting Lactococcus lactis (LL-OVA) in DO11.10 mice (33). Thus, as we discuss in more detail, P17 clearly holds promise to boost immune responses by down-regulation of CD4+CD25+ Treg cells, an effect that can be used to enhance the effectiveness of vaccination.

Materials and Methods

Peptides

KRIWFIPRSSWYFER (P17) is a peptide inhibitor of TGF-β developed in our laboratory (32). Peptide SPSYYYHQF (herein 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 (HCV) NS3 protein containing a Tcd presented by HLA-A2.1 class I molecules (35), the H2-Kb-restricted OVA TCd peptide SIINFEKL, and an irrelevant control peptide (AKAVKTHFETDCC) from human CD81 molecule were synthesized manually in a multiple peptide synthesizer using F-moc chemistry as previously described (36). The purity of peptides was >90% as judged by HPLC.

Mice

Female BALB/c and C57BL/6 mice were purchased from IFFA-Credo. A breeding pair of HHD transgenic mice expressing human HLA-A2 molecule was provided by Dr. J. Samson (Vrije Universiteit, Amsterdam, The Netherlands) and bred at the Academic Medical Center (Amsterdam, The Netherlands). Eight- to 10-wk-old DO11.10 mice were used for the experiments. OVA-specific TCR transgenic mice (DO11.10) on a BALB/c background were provided by Dr. J. Samson. Female BALB/c and C57BL/6 mice were purchased from IFFA-Credo. A breeding pair of HHD transgenic mice expressing human HLA-A2 molecule was provided by Dr. J. Samson (Vrije Universiteit, Amsterdam, The Netherlands) and bred at the Academic Medical Center (Amsterdam, The Netherlands). Eight- to 10-wk-old DO11.10 mice were used for the experiments. OVA-specific TCR transgenic mice OT-II and OT-I (C57BL/6 background) were provided by Dr. I. Melero (Centro de Investigación Médica Aplicada, 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 committee.

Cell culture

BSC-1 cells, provided by Dr. J. A. Berzofsky (National Institutes of Health, Bethesda, MD) were used for titration of vaccinia virus in ovaries. T2 and P815 cells from the American Type Culture Collection (ATCC) 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-2b) were purchased from ATCC and used in vivo for tumor protection and treatment experiments. They were cultured in complete medium (RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 50 μM 2-ME). Medium for E.G7-OVA cells also contained 400 μg/ml G418.

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) and the semiautomated system ABI PRISM 6100 Nucleic Acid PrePation (Applied Biosystems). DNase treatment, reverse transcription, and quantitative real-time PCR for CTLA4, glucocorticoid-induced TNFR-related gene, Foxp3, and IL-10 were conducted as described (38). mRNA values were represented by the 2−ΔΔCt formula, where ΔCt indicates the difference in threshold cycle between control (β actin) and target genes.

In vitro assays for murine or human Treg function

Inhibitory activity of murine or human Treg cells 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 human PBMC (10^5) were stimulated in vitro with the following: 0.5 μg/ml of anti-mouse or anti-human CD3 Ab (BD Pharmingen), respectively; bone marrow dendritic cells (10^5) from C57BL/6 mice (prepared as previously described (39)) or human PBMC (10^5) from a different donor (to induce a MLR); or a specific Ag. To study the effect of Treg cells on Ag-specific T cell stimulation, 10^5 OVA-specific CD8+ T cells from TCR transgenic OT-I mice were incubated with 10^5 bone marrow dendritic cells (from C57BL/6 mice) pulsed with SIINFEKEL peptide (1 μg/ml), whereas CD4+CD25− effector T cells (10^5/well) isolated from human PBMC were cultured in the presence of tetanus toxoid Ag (5 unit of flocculation units/ml). All T cell stimulation was conducted in the presence or absence of CD4+CD25+ Treg cells (10^4), 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 × 10^5 cells/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 (Discovery Labware; BD Biosciences), 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-3H]thymidine incorporation. Briefly, at the second day of culture, 0.5 μCi of [methyl-3H]thymidine was added to each well and incubated overnight. Cells were harvested (Filtermate 96 harvester; Packard Instrument), and incorporated radioactivity was measured using a scintillation counter (TopCount; Packard Instrument). IFN-γ secretion to the culture supernatant was measured by ELISA (BD Pharmingen) according to the 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 for human TGF-β1 was 20 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 PBLs 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 Abs and in the presence or absence of the corresponding IgG1 of exogenously added human TGF-β1 or human TGF-β2 (R&D Systems). P17 or control peptide was added to the cultures at the concentrations indicated in each experiment. T cell proliferation was tested after 3 days of culture by measuring [methyl-3H]thymidine incorporation as described.

Biomolecular interaction analysis

Screening of peptide binding to TGF-β1, TGF-β2, and TGF-β3 was performed by surface plasmon resonance using a BIAcore X Biosensor, TGF-β isoforms (R&D Systems) as well as an irrelevant protein were covalently immobilized onto the surface of flow cell 2 of CMS chips (BIAcore) as described (40). Flow cell 1, 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 nM 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 flow cell 1 from that in flow cell 2.
Immunization experiments and measurement of T cell activation
BALB/c or HHD mice were immunized s.c. at day 0 with 50 nmol TCd peptides AH1 or p1073, respectively, which were emulsified in IFA. Peptide P17 (50 nmol/mouse) was administered i.p. on days 6–9 after immunization. At day 10, mice were sacrificed, and 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 (BD Pharmingen) in culture supernatants (50 μl) harvested after 48 h of culture, according to the manufacturer’s instructions.

Effect of P17 in antitumor peptide vaccination
Animals immunized with 50 nmol AH1 peptide emulsified in IFA as previously described (42) were treated with 50 nmol P17 peptide or with saline on days 6–10. 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 Ab 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 single concentration (100 μM) was tested in C and D. Cell proliferation was analyzed by measuring tritiated thymidine incorporation in the harvested cells using a scintillation counter. *p < 0.05 t test comparison between the indicated group and the corresponding control of immunosuppression in the absence of inhibitors. Results are representative of at least three different experiments per each result.

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 × 10^5 E.G7-OVA tumor cells. When tumors reached 12.5 mm^3 in size, mice were adoptively transferred with 3 × 10^6 CFSE-labeled CD4^+ T cells isolated from OT-II transgenic mice. CFSE cell labeling was conducted 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 nmol P17 by i.p. route. Five days after T cell transfer, mice were immunized i.v. with 50 μg of OVA (Sigma-Aldrich) 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 conducted by flow cytometry. Analysis of CD4^+Foxp3^+ cells was conducted using the mouse Treg cell staining kit (eBioscience) according to the manufacturer’s instructions. Tumor size was measured the day of sacrifice as described.

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, were treated i.p. with 50 nmol P17 or with saline at days 6–9 after immunization. They were challenged i.p. at day 10 with 5 × 10^6 PFU of the recombinant vaccinia vHCV1–3011 expressing HCV polyprotein. Three days after vaccinia challenge, mice were sacrificed, and viral titer was measured as described (43).

Effect of P17 in a model of hypersensitivity

The L. lactis MG1363 strain was genetically modified and used throughout this study, as previously described (44, 45). Bacteria were cultured in GM17E medium, i.e., M17 broth (Difco) supplemented with 0.5% glucose and 5 μg/ml erythromycin (Abbott B.V.). Bacteria were diluted 200-fold in GM17E medium, incubated at 30°C overnight, harvested by centrifugation, and concentrated in BM9 medium at 2 × 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 of OVA in 50 μl of a 1:1 CFA (Difco) 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 days 8–12) administrations using a stainless 18-gauge animal feeding needle. Every other day, starting at day 0, mice received 50 nmol P17 peptide each. Eleven days after sensitization, Ag-specific delayed-type hypersensitivity (DTH) responses were assessed. For DTH measurement, mice were challenged with 10 μg of OVA in 10 μl of saline in the auricle of one ear and 10 μl of saline in the other. The increase in ear thickness was measured in a blinded fashion using an engineer’s micrometer (Mitutoyo) at 24 h after challenge. DTH responses were expressed as the difference in increase between the OVA-injected and 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 Ag (Sigma-Aldrich).

For cytokine measurements, 2 × 10^5 cells of splenocytes were cultured in 96-well U-bottom plates in a total volume of 200 μl of complete medium with 100 μg/ml OVA. Cells were cultured at 37°C in a 5% CO₂ humidified incubator. 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).

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 nonparametric (Kruskal-Wallis and Mann-Whitney U test) tests. For all tests, a value for p < 0.05 was considered statistically significant. Descriptive data for continuous variables are reported as mean ± SD. SPSS 9.0 for Windows was used for statistical analysis.

FIGURE 2. P17 inhibits immunosuppression caused by TGF-β or by human Treg cells. A. Inhibition of TGF-β1. Human PBMC were stimulated with anti-CD3 mAbs in the presence or absence of exogenously added recombinant human TGF-β1 (1 pg/ml) and the indicated concentration of P17 or polyclonal anti-TGF-β or the corresponding isotype control Abs (2 μg/ml). B–D, Inhibition of human Treg cells. B. Human PBMC were stimulated with anti-CD3 mAbs in the presence or absence of purified CD4^+CD25^+ Treg cells. C. MLR 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 cells. Different concentrations of P17 (12–100 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 was analyzed by measuring tritiated thymidine incorporation in the harvested cells using a scintillation counter. *p < 0.05 t test comparison between the indicated group and the corresponding control of immunosuppression in the absence of inhibitors. Results are representative of at least three different experiments per each result.
Results

Peptide P17 inhibits Treg cells in vitro

Previous studies have shown that murine CD4\(^+\)CD25\(^+\) Treg cells produce high levels of TGF-\(\beta\) bound to the cell surface or secreted to the medium. Blockade of this TGF-\(\beta\) by anti-TGF-\(\beta\) 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-\(\beta\), inhibited TGF-\(\beta\)-dependent expression of collagen type I mRNA in a model of liver damage (32). In Figs. 1A and 2A we show that P17 is also able to inhibit, in a dose-dependent manner, the immunosuppressive activity of TGF-\(\beta\) exogenously added to murine- or human-derived effector T cells stimulated with anti-CD3, as described in Materials and Methods. Inhibitory activity of P17 was similar to that found when 2 \(\mu\)g/ml neutralizing anti-TGF-\(\beta\) Abs 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 Abs. Purified Treg cells had high mRNA levels for CTLA4, glucocorticoid-induced TNFR-related gene, Foxp3, and IL-10 and expressed TGF-\(\beta\) when stimulated with anti-CD3 mAbs in the presence or absence of exogenously added recombinant human TGF-\(\beta\) (0.25 pg/ml) and 100 \(\mu\)M P17 or control peptide (Pcont). Cell proliferation was analyzed by measuring tritiated thymidine incorporation in the harvested cells using a scintillation counter.

We then tested the effect of P17 in cocultures 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 Ab, respectively (Figs. 1D and 2B). Addition of anti-TGF-\(\beta\)-neutralizing polyclonal Ab 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 cocultured with nonadherent spleen cells from C57BL/6 mice in the presence of Treg cells (Fig. 1E), or when human PBMC from two different donors were mixed in the presence of human purified Treg cells (Fig. 2C). P17 also reverted Treg-mediated inhibition of Ag-specific T cell activation in the case of T cells from OT-I transgenic mice responding to peptide OVA\(_{257-264}\) (SINFEKL) from OVA (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 \(\mu\)M P17 was found to vary between 25% and 100% in different in vitro models. This variation may be related to the role of TGF-\(\beta\) in each particular model. In summary, results from Figs. 1 and 2 indicate that P17 is able to inhibit, at least partially, both murine and human Treg cells in vitro.

Peptide P17 also inhibits the immunosuppressive activity of TGF-\(\beta\)isoform

The forkhead transcription factor Foxp3 is highly expressed in CD4\(^+\)CD25\(^+\) Treg cells and acts as a key player in mediating Treg inhibitory functions. With this role, a recent study has also described that tumor cells can be induced to express functional Foxp3 by TGF-\(\beta\), in such a way that naïve T cell proliferation is inhibited when cocultured with these Foxp3-expressing tumor cells (49). Because TGF-\(\beta\) 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 Fig. 3A, binding of P17 follows the order TGF-\(\beta\)1>TGF-\(\beta\)2>>>TGF-\(\beta\)3.

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-\(\beta\) isoforms (data not shown). Because P17 was able to bind TGF-\(\beta\)2, we tested its ability to inhibit the immunosuppressive activity of this cytokine in vitro. Thus, we added TGF-\(\beta\)2 to effector T cells stimulated with anti-CD3, as described in Materials and Methods. It was found that 100 \(\mu\)M P17 inhibited the immunosuppressive activity of TGF-\(\beta\)2 by around 50% (Fig. 3B).

Peptide P17 improves immunogenicity of AH1 peptide vaccination leading to protection against CT26 tumor challenge

Down-regulation of Treg suppressor activity in vivo might be beneficial to enhance vaccine immunogenicity against tumor Ags. 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 coimmunization of AH1 and an adequate Th cell determinant capable of inducing a competent Th cell response (42). We have also shown that depletion of CD25\(^+\) Treg cells with anti-CD25 Abs before immunization with peptide AH1 permits the induction of a long-lasting antitumoral immune response (2). All these observations lead us to speculate that in vivo inhibition of Treg cells 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 the AH1 peptide emulsified in IFA at day 0 and treated with saline or immunized with 50 nmol P17 per mouse on days 5–9 after immunization. As shown in Fig. 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. Moreover, when mice were challenged s.c. with $5 \times 10^5$ CT26 tumor cells, eight of six immunized mice with AH1 and treated with peptide P17 remained tumor-free, whereas all mice immunized with AH1 only or left unimmunized developed tumors (Fig. 4B). These results indicate that TGF-β inhibition by P17 improves immunogenicity of AH1 peptide vaccination and concomitantly protects 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 hosts (50, 51). TGF-β1 is important in maintaining functional Foxp3$^+$ CD4$^+$CD25$^+$ Treg cells and can also induce Foxp3 expression in naive 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 cells from OT-II transgenic mice to animals bearing s.c. 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 $5 \times 10^7$ E.G7-OVA tumor cells, and once the tumors reached 12.5 mm$^3$, mice were adoptively transferred with $3 \times 10^7$ CFSE-labeled CD4$^+$ OT-II-derived T cells. After adoptive transfer, mice were treated daily with PBS or with 50 nmol P17 per mouse. Ten days 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 eight mice from both groups of AH1-immunized mice (the one treated with AH1 only and the other treated with AH1 plus P17) as well as mice injected with saline were challenged s.c. with $5 \times 10^5$ CT26 tumor cells. Tumor size was measured as described in Materials and Methods. Mice were sacrificed when tumor size reached a volume $>$4 cm$^3$. The ratio shown indicates the number of mice of the eight treated with AH1 plus P17 that remained tumor free (B). Results are representative of two different experiments.

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

As described earlier, Treg cells may hamper the induction of protective cellular immune responses in several viral infections. In particular, it has been recently reported that patients with chronic HCV have a higher number of peripheral Treg cells, 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 nmol P17 each or treated with saline. Ten days after immunization, animals were sacrificed and spleen cells were cultured with p1073 for 5 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 (Fig. 6A). The ability of P17 treatment to

of the total number of CD4$^+$Foxp3$^+$ T cells (compare Fig. 5D, P17-treated vs 5C, PBS-treated cells). 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 ($p < 0.05$) (Fig. 5E).

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 5–9 after immunization, whereas the remaining 10 mice were treated with 50 nmol P17 per mouse. 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 eight mice from both groups of AH1-immunized mice (the one treated with AH1 only and the other treated with AH1 plus P17) as well as mice injected with saline were challenged s.c. with $5 \times 10^5$ CT26 tumor cells. Tumor size was measured as described in Materials and Methods. Mice were sacrificed when tumor size reached a volume $>$4 cm$^3$. The ratio shown indicates the number of mice of the eight treated with AH1 plus P17 that remained tumor free (B). Results are representative of two different experiments.

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 (A and C) or with peptide P17 (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 (A and B) or the number of splenic CD4$^+$Foxp3$^+$ T cells (C and D) by flow cytometry. E, Tumor size was measured the day of sacrifice as described in Materials and Methods. Results are representative of two different experiments.
Immunization, mice were challenged with 5. 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. After 5 days of culture, CTL activity against peptide-loaded T2 target cells was measured in a conventional chromium release assay.

A

FIGURE 6. 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 peptide P17 (50 μg/mice) at days 5–9 after immunization. Ten days after immunization, mice were sacrificed and spleen cells were cultured in the presence or absence of 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 × 10^6 PFU of the recombinant vaccinia vvHCV 3011 virus 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 indicated. Data represent the mean average ± SEM of virus titers from six mice. Results are representative of two different experiments.

B

FIGURE 7. In vivo injection of P17 inhibits the induction of oral tolerance by LL-OVA and increases the IFN-γ production of bulk splenocytes. DO11.10 mice were sensitized by s.c. injection of 100 μg of 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 of OVA in 10 μl of saline in the auricle of one ear and 10 μl of saline in the other. A, DTH responses are expressed as the mean differences in the increase in ear thickness between the OVA-injected and saline-injected mice, following subtraction of ear thickness before OVA challenge. All groups consisted of six mice. B, On day 12, bulk splenocytes were isolated and tested for IFN-γ production after 72 h of ex vivo stimulation with 100 μg/ml OVA.

Discussion

Naturally occurring Treg cells inhibit T cell proliferation in vitro, a mechanism that plays the beneficial role of controlling T cell responses to self-Ags. This effect 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 response, 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 cells 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 cells 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, dominant negative TGF-β receptor type II transgenic mice develop a lymphoproliferative syndrome and autoimmunity (58–60), probably because their T cells escape control by Treg cells (58). Moreover, a study has described TGF-β produced by Treg cells or bound to the cell membrane may mediate suppression of T cells, (24). Also, recent data have shown that dendritic cells may inhibit T cell activation via the secretion of TGF-β (61) or by surface expression of TGF-β bound by LAP (62). TGF-β is also important in the homeostasis of Treg cells because 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 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 HCV, 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 the mice from each group were treated i.p. with P17. Ten days after immunization, mice were challenged with 5 × 10^6 PFU of the recombinant vaccinia vvHCV 3011 virus, and 3 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 (Fig. 6B).

Breakdown of immunotolerance by P17

We have recently demonstrated that mucosal delivery of OVA by genetically modified 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 cells that critically depend on TGF-β (33). To further validate the ability of P17 in this model, mice were sensitized by s.c. injection of 100 μg of 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 12 LL-OVA-treated mice received 50 nmol P17 peptide in PBS by i.p. route at alternate days until the end of the experiment. LL-OVA-treated mice were significantly tolerized compared with the control mice (1.4 vs 15.8 × 10^−2 mm). Coinjection of P17 blocked the induction of Ag-specific oral tolerance measured by a significant increase in ear thickness compared with the LL-OVA-treated mice (11 vs 1.4 × 10^−2 mm) (Fig. 7A). Immediately after DTH measurements, spleens were isolated and ex vivo stimulated with OVA for 72 h. In agreement with this finding, it was found that P17 treatment restored IFN-γ production by splenocytes in response to OVA Ag, a response that 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 Ag-specific immunotolerance.
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 (32), 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 cells. Similarly, P17 restored, at least partially, T cell proliferation in an MLR inhibited by Treg cells. P17 also inhibited Treg activity over specific T cells stimulated by an Ag. 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 of Treg depletion, was also able to enhance immunogenicity of AH1 peptide vaccination, and protected mice from CT26 tumor challenge. Down-regulation 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 of the immunization of peptide p1073, which encompasses a HLA-A2.1 restricted epitope from HCV NS3 protein, with the outcome being a reduction of recombinant vaccinia vHCV 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 cells (2–5, 64). 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 cells (65). When we compared the effect of anti-TGF-β Abs 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 Abs (five administrations of 100 μg of anti-TGF-β1 per mouse from day 5 to 9 after AH1 immunization). Similarly, in vivo administration of anti-TGF-β1 Abs reverted immunotolerance induced by LL-OVA administration (data not shown). 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 use would reduce the potential toxic effects of long-term inhibition of the cytokine.

Tumors produce factors such as prostaglandins, IL-10, vascular endothelial growth factor, 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 cells accumulate in tumors and secondary lymphoid organs (6–12). This increase in the number of Treg cells may be favored by a recruitment of naturally occurring Treg cells, as well as by a conversion of CD4+ effector Th cells into Treg cells, in this particular TGF-β1-enriched tumor microenvironment (66, 67). 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 cells and Foxp3 expression and by favoring effector T cell proliferation and function. We show in this study 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 number 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 mediation of active suppression against orally administered Ags (reviewed in Ref. 68). Thus, TGF-β knockout mice develop chronic inflammation in many tissues, including the gastrointestinal tract (69). 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 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 Ags.

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 administered 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 Ag-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 Ags.

Disclosures
Javier Dotor is currently an employee of Digna-Biotech, the company holding patent rights on P17.

References


