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A Peptide Inhibitor of FOXP3 Impairs Regulatory T Cell Activity and Improves Vaccine Efficacy in Mice

Noelia Casares,* Francesc Rudilla,* Laura Arribillaga,* José Ignacio Riezubojo,* Teresa Lozano,* Jacinto López-Sagaseta,† Laura Guembe,‡,§ Pablo Sarobe,* Jesús Prieto,*‡,§ Francisco Borrás-Cuesta,* and Juan José Lasarte*

Immunosuppressive activity of regulatory T cells (Treg) may contribute to the progression of cancer or infectious diseases by preventing the induction of specific immune responses. Using a phage-displayed random peptide library, we identified a 15-mer synthetic peptide, P60, able to bind to forkhead/winged helix transcription factor 3 (FOXP3), a factor required for development and function of Treg. P60 enters the cells, inhibits FOXP3 nuclear translocation, and reduces its ability to suppress the transcription factors NF-κB and NFAT. In vitro, P60 inhibited murine and human-derived Treg and improved effector T cell stimulation. P60 administration to newborn mice induced a lymphoproliferative autoimmune syndrome resembling the reported pathology in scurfy mice lacking functional Foxp3. However, P60 did not cause toxic effects in adult mice and, when given to BALB/c mice immunized with the cytotoxic T cell epitope AH1 from CT26 tumor cells, it induced protection against tumor implantation. Similarly, P60 improved the antiviral efficacy of a recombinant adenovirus expressing NS3 protein from hepatitis C virus. Functional inhibition of Treg by the FOXP3-inhibitory peptide P60 constitutes a strategy to enhance antitumor and antiviral immunotherapies. The Journal of Immunology, 2010, 185: 5150–5159.

Regulatory T cells (Tregs) are a distinct lymphocyte lineage endowed with inhibitory properties that affect the activation of the immune system. Tregs are characterized by the expression of CD25 and the Treg-specific forkhead/winged helix transcription factor 3 (FOXP3), which is required for their development and function (1, 2). These cells can inhibit activation of other T cells (3) and are needed for protection against autoimmune diseases and prevent rejection of allogeneic transplants. In humans, mutations in FOXP3 result in an autoimmune syndrome termed IPEX (immunodysregulation, polyendocrinopathy enteropathy, X-linked syndrome), an X-linked immunodeficiency syndrome characterized by insulin-dependent diabetes, thyroiditis, x-linked hypothyroidism, X-linked agammaglobulinemia, and growth retardation. FOXP3 deficiency has also been linked to the development of many types of tumors (9–13) and have been associated with a high death hazard and reduced survival (9, 11). It has been shown that counteracting Treg activity can evoke effective antitumor immunity (14–16), and it is acknowledged that inhibition of Treg function in patients with cancer is an essential step to improve the efficacy of antitumoral therapies, especially those based in immunotherapeutic approaches (reviewed in Ref. 17).

Several strategies have been proposed to neutralize Treg activity. Thus, it has been shown that administration of low doses of cyclophosphamide is able to deplete Treg and favors antitumor therapies (18–20). Also, targeting the α-subunit of the IL-2R by using a fusion protein between the IL-2 and the diphtheria toxin has been tested in clinical trials with different results (21, 22). However, these strategies lack of high specificity and might eliminate both effector T cells and Tregs. In addition, depletion of Tregs by these strategies also raises the possibility of autoimmunity (23). An alternative way to control Tregs is their functional inactivation rather than their depletion. Because sustained FOXP3 expression in mature Tregs is necessary for maintenance of the Treg phenotype and suppressor function, and ablation of FOXP3 or reducing its expression results in the loss of their suppressive function (1, 2), we decided to search for peptide inhibitors of FOXP3 using a phage-displayed random peptide library. Using this strategy, we identified peptide P60, which is able to inhibit Treg functions in vitro and in vivo.

Materials and Methods
Preparation of the recombinant FOXP3 protein

Plasmid pDEST15-FOXP3 (provided by Dr. Casal, Centro Nacional de Investigaciones Oncolóxicas, Madrid, Spain) encoding the fusion protein GST-FOXP3 (GST fused to the isoform A from human FOXP3 gene), was transformed into BL21 (DE3; Novagen, Schwalbach, Germany) cells for the expression of the recombinant protein. GST-FOXP3 protein was purified from the soluble fraction of cell extracts, by affinity chromatography (GSTRap; Amersham, Piscataway, NJ) using a fast protein liquid chromatography platform (AKTA; Amersham). Purified recombinant protein was analyzed by SDS-PAGE using Coomassie blue (Bio-Rad, Hercules, CA) and by Western blot using anti-FOXP3

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Abs (clone 236A/E7, provided by Dr. Roncador, Centro Nacional de Investigaciones Oncológicas).

Identification of potential peptide inhibitors of FOXP3 using a phage-displayed random peptide library

Phages from phage library (iUSE5/15-mer library) expressing 15-mer random peptides near the N terminus of phage surface protein pII (a gift of Dr. Smith, University of Missouri-Columbia, Columbia, MO) were allowed to interact with GST-FOXP3. Bound phages were then eluted and amplified in Escherichia coli K91Kan. After three rounds of elution-amplification at decreasing concentrations of FOXP3, best binder phages were selected and sequenced for their corresponding 15-mer peptide sequence as previously described (24) with minor modifications. Binding capacity of the amplified phages was tested by ELISA using GST-FOXP3–coated plates and an anti-M13 Ab-HRP conjugated (Amersham Pharmacia Biotech, Uppsala, Sweden). Phages were selected when the OD ratio between GST-FOXP3 and BSA was >2. Fourteen phages were selected and the corresponding 15-mer peptides were synthesized as described later.

Peptide synthesis

Peptides were synthesized by the solid phase method of Merrifield using the fluoresmethylthio carbonyl alternative, as previously described (25). To study peptide internalization into the cells, we labeled peptide P60, as well as peptide OVA(235–264), at the N terminus by the addition of 5(6)-carboxyfluorescein N-hydroxysuccinimid ester (Sigma-Aldrich, Steinheim, Germany) at the end of the synthesis. The purity of peptides was >90% as judged by HPLC. Peptide P60 (RDQFQSRKMWFPAF) and peptide AH1 (SPSVYYHQF) containing a cytosotoxic T cell determinant (Tcd) expressed by CT26 cells and presented by H2Ld MHCI molecule (26) were synthesized by Neomps (Strasbourg, France). Peptide P60 (MKMFDFAPQRQSRWF), which corresponds to a scrambled sequence of P60, was also synthesized and used as a control peptide.

Biomolecular interaction analysis

Screening of peptide binding to FOXP3 was performed by surface plasmon resonance using Biacore T100 (GE Healthcare, Piscataway, NJ) and ProteOn XPR36 (Bio-Rad) optical biosensors. When we used the Biacore T100, GST-FOXP3 fusion protein was covalently immobilized onto the surface of flow cell 2 of a CM5 chip (Biacore) as described previously (24). Flow cell 1, in which surface there is no immobilized FOXP3, was used as the reference flow cell. When we used the ProteOn XPR36 system, GST-FOXP3 and GST proteins were covalently immobilized onto the surface of two independent channels of a GLC sensor chips (Bio-Rad) using the coupling reagents N-hydroxysuccinimid ester and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Bio-Rad). After protein immobilization, chip surface was treated with ethanolamine to deactivate the excess of reactive esters. In both systems, individual peptide solutions (5 μM) were injected twice in running buffer (PBS, 0.005% [v/v] Tween 20, pH 7.4) at a flow rate of 30 μL/min. In the experiments carried out in the Biacore T100, curves were processed by subtracting the response in flow cell 1 from that in flow cell 2. In the ProteOn XPR36 system, the signal obtained in the channel immobilized with GST protein was used as reference. Association and dissociation phases were monitored for 60 and 30 s, respectively.

Cell lines and viruses

Kaposi-299 cell line, a human T cell lymphoma that shows characteristics typical of natural Tregs (27), was purchased from DSMZ (Braunschweig, Germany). CT26 murine tumor cells (H-2d) from American Type Culture Collection were used in vivo for tumor protection and treatment experiments. BSC-1 cells (provided by Dr. Berzofsky, National Institutes of Health, Bethesda, MD) were used to titrate vaccinia virus PFU in the ovaries from infected animals. 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). Human embryonic kidney 293 (HEK 293, American Type Culture Collection) cells were transfected with transfection experiments were cultured in DMEM supplemented with 10% FCS and antibiotics. Recombinant adenovirus expressing hepatitis C virus (HCV) NS3 protein (RAdNS3) was generated as described previously (28). Vaccinia virus vHCp3-3011A expressing HCV/ polyprotein was provided by Dr. C.M. Rice (Washington University School of Medicine, St. Louis, MO).

Confocal microscopy

Jurkat cells (106 cells/well) were plated into eight-chamber glass slides (IBIDI GmbH, Munich, Germany). They were allowed to settle before adding carboxyfluorescein diacetate (CF)-P60 or CF-OVA(235–264) peptide to the culture medium. Intracellular distribution of the fluorescence was analyzed at different time points after adding the peptide (10 μM) using a Zeiss confocal microscope (LSM 510 Meta, Munich, Germany) equipped with a NA 1.4 × 63 oil-immersion Plan-Apochromat objective lens. Percentage of the maximum fluorescence intensity (MF); extracellular mean fluorescence units after addition of CF-labeled peptides was calculated using the formula: %MF = 100*(mfiexp – mfinas)/mfinas, where mfiexp is the mean fluorescence intensity per cell and mfinas is the mean fluorescence intensity of the background. Mean fluorescence intensity at each time point was calculated individually in 20 cells per assay using LSM 510 Meta software (Carl Zeiss, Munich, Germany).

Plasmids and transfections

Plasmids used in this work were pNFAT-luciferase (provided by Dr. Revilla, Autonomous University of Madrid, Madrid, Spain), pNF-κB–luciferase (BD Clontech, Palo Alto, CA), pcDNA-FOXP3 (provided by Dr. Ziegler, Seattle, WA), pMig-R1 Foxp3 (provided by Dr. Majer, Max Delbrück Center for Molecular Medicine, Berlin, Germany), pRenilla (provided by Dr. Fortes, Center for Applied Medical Research, Pamplosa, Spain), and pFOXP3-enhanced GFP (EGFP), which was prepared by cloning FOXP3 in pEGFP-N1 (BD Clontech). Transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions and using 1 μg of the indicated plasmids. After 24 h of culture, cells were analyzed by means of the dual luciferase assay (Promega, Madison, WI) with relative normalization for Renilla.

Mice

Female BALB/c mice and imprinting control region (ICR; CD1) mice were purchased by Harlan (Barcelona, Spain) and bred at the animal facility of Center for Applied Medical Research. All experiments performed followed institutional guidelines and were approved by the institutional ethical committees.

Treg purification

Isolation of murine and human CD4+CD25+ and CD4+CD25− T cells was performed from murine spleen cells or from human PBMCs by using murine and human Treg 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.

Immunohistochemistry

Paraffin liver sections (3 μm thick) were cut, dewaxed, and hydrated. Retrieval of antigen was performed from murine spleen cells or from human PBMCs by using murine and human Treg 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.

Cell proliferation assay

Changes of T cell stimulation. CD25-depleted spleen cells (105 cells/well) were stimulated with addition of CF-labeled peptides) was calculated using the formula: %MF = 100*(mfiexp – mfinas)/mfinas, where mfiexp is the mean fluorescence intensity per cell and mfinas is the mean fluorescence intensity of the background. Mean fluorescence intensity at each time point was calculated individually in 20 cells per assay using LSM 510 Meta software (Carl Zeiss, Munich, Germany).

RNA extraction and quantitative real-time RT-PCR

Total RNA extraction from cell cultures was performed as previously described (29). Murine Foxp3, IL-10, IFN-γ, and β-actin expression was measured by quantitative real-time PCR using specific primers for each gene as described previously (30). The level of expression of β-actin was used to normalize gene expression.

In vitro assays for murine or human Treg function

Inhibition of murine or human Treg was measured in two different in vitro assays of T cell stimulation. CD25-depleted spleen cells (105 cells/well) from BALB/c mice or 105 human PBMCs/well were stimulated in vitro with 1) 0.5 μg/ml anti-mouse CD3 Ab (Pharmingen, San Diego, CA) or with anti-human CD3/CD28 beads (Dynal, Oslo, Norway), respectively; or with 2) 105 bone marrow dendritic cells from C57BL/6 mice (prepared as described previously [31]) or with 105 human PBMCs (from a different donor) to induce an MLR, in the presence or absence of purified Tregs (105 cells/well). T cell proliferation was measured 3 d later, as previously described (32). In some experiments, Karpas cell line was used as a source of human Tregs in MLR reactions. Thus, 105 PBMCs from two different
donors were mixed with 10^7 Karpas cells in the presence or absence of 100 μM of different peptides. IFN-γ secretion to the culture supernatant was measured by ELISA (PharMingen) according to manufacturer’s instructions.

**Antitumor peptide vaccination**

Animals immunized with 50 nmol/mouse of peptide AH1 emulsified in IFA, as previously described (14), were treated i.p. with 50 nmol/mouse of peptide P60, control peptide, or saline daily during 10 d. At day 10, splenocytes from immunized animals were cultured in the presence or absence of peptide AH1 for 48 h. IFN-γ released to the supernatant was measured by a commercial ELISA (PharMingen), according to manufacturer’s instructions. Alternatively, mice were injected s.c. with 5 × 10^7 CT26 tumor cells. Tumor size was monitored twice a week with a caliper, and it was expressed according to the formula: V = (length × width)^2/2. Mice were killed when tumor size reached a volume >4 cm^3. Alternatively, splenocytes from immunized animals were cultured in the presence or absence of peptide AH1 for 48 h. IFN-γ released to the supernatant was measured by a commercial ELISA (PharMingen) according to manufacturer’s instructions.

**In vivo protection against infection with a recombinant vaccinia virus expressing HCV proteins**

Mice immunized i.p. with 5 × 10^6 PFU of the recombinant adenovirus RAdNS3 were treated i.p. with 50 nmol/mouse of peptide P60 or with saline from days 0–10. As control animals, nonimmunized mice were treated with saline on the same schedule. At day 10, mice were challenged i.p. with 5 × 10^6 PFU of the recombinant vaccinia HCV1-3011 expressing HCV polyprotein. Three days after vaccinia challenge, mice were sacrificed and spleens were removed. Spleen cell preparations were performed and assayed for HCV1-3011 titer by plating serial 10-fold dilutions of samples on a plate of BSC-1 indicator cells. Antiviral activity was calculated by comparing the virus titers in a dilution series in control and vaccinated mice and were expressed as log10 reduction in virus titer. The results were confirmed by ELISA using plates coated with GST-FOXP3 (not shown).

**Results**

**Identification of potential peptide inhibitors of FOXP3**

To identify peptide inhibitors of FOXP3 of potential use in therapy, we used a phage-display randomized 15-mer peptide library. This library was allowed to interact with GST-FOXP3 immobilized on plastic plates. After three cycles of elution-amplification at decreasing concentrations of GST-FOXP3, a total of 100 bacterial clones (infected with a unique phage) were obtained, and their phage DNA insert coding for their corresponding 15-mer peptides was sequenced. The binding capacity of 14 of the selected phages was confirmed by ELISA using plates coated with GST-FOXP3 (not shown). Fifteen-mer peptides codified by these 14 phages were synthesized and tested in an in vitro assay of inhibition of the immune response of T reg cells. Furthermore, the selected peptides were able to translocate across cell membranes and were characterized as short (<30 aa), water-soluble, and cationic or amphipathic in nature. Membrane translocation of the potential FOXP3 inhibitory peptide P60 is necessary to reach inhibition of Treg activity. To address whether P60 was able to enter into the cell, we synthesized the CF-P60 peptide containing a molecule of CF linked to the N terminus of the peptide, to visualize directly peptide internalization in live Jurkat cells by confocal fluorescence microscopy. As control, we used the irrelevant CF-labeled peptide CF-OVA(235–264). Time-course imaging analysis of the cells revealed a significant increase in their intracellular mean fluorescence intensity after the addition of CF-P60 peptide, but not of CF-OVA(235–264), reaching a maximum after 10 min of incubation (Fig. 1C, 1D), suggesting that peptide P60 is able to enter into the cell.

The FOX3 protein encodes at least three discernible functional domains, a carboxyl-termination forhhead domain (FKH), a single C2H2 zinc finger, and a leucine zipper-like motif. The ability of FOX3 to act as a transcriptional repressor requires the presence of the FKH domain, which is critical for the nuclear translocation of FOX3 and for its DNA-binding capacity (34). To study whether binding of P60 to FOXP3 could prevent its nuclear localization, we generated a plasmid expressing FOXP3-EGFP fusion protein. The construct was transfected into 293 cells to evaluate by fluorescence microscopy the subcellular localization of FOXP3-EGFP. It was found that 24 h after transfection, GFP was mainly localized into the nucleus of the cells. However, the addition of P60, but not the irrelevant peptide, to the transfected cells reduced significantly FOXP3-EGFP nuclear translocation (Fig. 1E). The addition of P60 to the cultures did not affect the level of expression of FOXP3-GFP, which was equivalent for P60 and for the control peptide (mean fluorescence intensity of 321 ± 19.1 versus 317 ± 15.6, respectively). Similar results were obtained when we studied by immunohistochemistry the cellular localization of Foxp3 in primary splenocytes incubated for 24 h in the presence or absence of P60 or the control peptide. Indeed, whereas Foxp3 staining was localized into the nucleus when cells were incubated with culture medium alone or with control peptide, it was found that incubation of cells with P60 favored cytoplasm localization of Foxp3 staining (Supplemental Fig. 1), suggesting that P60 can inhibit nuclear translocation of Foxp3 in primary Treg.

**P60 overcomes the inhibitory effect of FOXP3 on the activity of the transcription factors NF-κB and NFAT**

It has been described that FOXP3 interacts with NFAT and with NF-κB to repress cytokine gene expression and effector functions of T helper cells (35). When FOXP3 is overexpressed in 293 cells, it inhibits the basal level of NF-κB activation (35). When we measured activation of NF-κB on 293 cells transfected with the reporter plasmid NF-κB–luciferase in the presence or absence of a plasmid coding for FOXP3, we confirmed its inhibitory activity. However, the addition of P60 to the cultures was able to restore, at least in part, NF-κB activity (Fig. 2A). Similarly, when Jurkat cells
were transfected with the reporter plasmid NFAT-luciferase and stimulated with PMA and ionomycin, we observed the induction of NFAT activity that was repressed in the presence of FOXP3. Interestingly, the addition of P60 to the cultures was able to restore NFAT activity (Fig. 2B), a result in line with the inhibition of FOXP3.

**Peptide P60 inhibits human or murine Treg in vitro**

We then studied the capacity of P60 to inhibit the suppressor activity of natural Tregs in vitro. We conducted this experiment using both human and murine Treg to evaluate the possibility to study the potential effect of P60 in vivo in murine models. Thus, CD4⁺CD25⁺ T cells from murine splenocytes or from human peripheral blood were purified to analyze their immunosuppressive activity over effector T cells activated with different stimuli. We found that P60 (100 μM) was able to inhibit Treg suppressive function, restoring the proliferation of murine effector T cells or human PBMCs in response to soluble anti-mouse CD3 or anti-human CD3/CD28 Ab, respectively (Fig. 3A,3B). To study the longevity of peptide activity, we carried out some in vitro assays...
using Tregs pulsed during 2 h with P60 and washed before their coculture with effector T cells and anti-CD3 stimulus. After 24 h of culture, we found that, although some reversal of suppression was observed on Treg treated with P60, this effect did not reach statistical significance, suggesting that P60 must be present throughout all the experiment (Supplemental Fig. 2). P60 was also able to restore proliferation of effector T cells in MLR, when bone marrow-derived dendritic cells from C57BL6 were cocultured with spleen cells from BALB/c mice in the presence of Treg (Fig. 3C), or when human PBMCs from two different donors were mixed in the presence of human purified Treg (Fig. 3D). The percentage of inhibition of the Treg effect of P60 was found to vary between 35 and 100% (with an average of reversal of suppression of 66.8 ± 33.5%) depending on the different in vitro models.

To study the potential direct effect of P60 on the viability or the proliferative capacity of effector T cells or Tregs, we studied their proliferation in response to IL-2 and anti-CD3 stimulation. It was found that P60 did not alter conventional T cell or Treg proliferation in response to stimulation (measured by flow cytometry in a CFSE dilution assay or by [3H]thymidine incorporation; Supplemental Fig. 3). The capacity of P60 to restore the proliferation of effector T cells in the presence of Tregs was not related to its potential effects on APCs, because the addition of P60 to bone marrow-derived dendritic cells did not alter their maturation status (expression of costimulatory molecules or IL-12 production; data not shown).

**P60 inhibits upregulation of mRNA for Foxp3 and IL-10 on effector T cells and enhances mRNA for IFN-γ after anti-CD3 stimulation**

It has been described that anti-CD3 stimulation of human effector CD4+ T cells induced a transient upregulation of FOXP3 that, although it did not confer immunosuppressive activity to the T cell, was strongly associated with hyporesponsiveness of activated T cells to subsequent stimulation (36). We studied the effect of P60 on effector CD4+CD25− T cell stimulation with anti-CD3 by measuring the expression of mRNA for Foxp3, IL-10, and IFN-γ at different time points. It was found that anti-CD3 stimulation induced a transient expression of Foxp3 (8 h after stimulation) that was followed by a later upregulation of IL-10 (48 h). However, if T cell stimulation was carried out in the presence of P60, Foxp3 upregulation and the subsequent peak in IL-10 mRNA expression were significantly reduced (p < 0.05; Fig. 4A, 4B, respectively). Moreover, the presence of P60 during stimulation resulted in greater levels of IFN-γ mRNA at 24 h (p < 0.01; Fig. 4C).

We also analyzed by real-time PCR the expression of mRNA for Foxp3, IL-2, or IFN-γ in Tregs stimulated with IL-2 and anti-CD3 in the presence or absence of P60. Although we could not detect significant changes on the expression of Foxp3 or IFN-γ in the presence of P60 during the first 48 h (Fig. 4D, 4F), we found a significant upregulation of mRNA expression for IL-2 after 24 h of stimulation (p < 0.05; Fig. 4E).

**In vivo administration of P60 to newborn mice induces a scurfy-like disease**

The scurfy mutant mouse strain, harboring a mutation in the Foxp3 gene coding for a product that lacks the FKH, suffers from a fatal lymphoproliferative disease (37) that is characterized, among other symptoms, by gross internal lesions such as splenomegaly, enlarged lymph nodes, anemia, and massive infiltration in lungs, the acini of the pancreas, and the liver (38, 39). Selective depletion of Foxp3+ Tregs by diphtheria toxin administration to newborn DEREG mice expressing the diphtheria toxin receptor under the control of the Foxp3 locus leads to the development of scurfy-like symptoms (39). To test the effect of P60 peptide as a Foxp3 inhibitor in vivo, we treated ICR newborn mice daily with saline as control littermates or with 25 nmol/mouse of P60 from day 1 to 10 after birth. A group of mice were killed at day 14 for histologic analyses. It was found that P60-treated mice developed a lymphoproliferative autoimmune syndrome resembling the reported pathology in scurfy mice or in newborn DEREG mice treated with diphtheria toxin. Thus, the spleens of P60-treated mice were enlarged (not shown), and we observed a massive inflammatory infiltrate in various organs (Fig. 5A). The lungs of P60-treated mice showed peribronchial and perivascular infiltrations as compared with saline-treated mice. The liver exhibited periportal infiltrations, and the acini of the pancreas presented infiltrates and hemorrhagic lesions.

Moreover, mice treated with P60 had lower numbers of CD4+CD25+Foxp3+ T cells in the spleen (Fig. 5B), as well as in lymph nodes (not shown). Moreover, when we compared in the CD4+ T cell subpopulation the numbers of CD25+ versus Foxp3+ cells,
we found that in mice treated with P60, only 59.3 ± 2.19% of CD25+ cells were Foxp3+, as compared with the 72.7 ± 4.29% and 78.7 ± 1.29 found in mice treated with saline or control peptide, respectively (p < 0.05). These results might suggest that treatment with P60 is also favoring the activation of effector T cells. Interestingly, male mice treated with P60 during the 10 first days after birth developed shoulder ulcers and skin hyperplasia after 3 mo of life (Supplemental Fig. 4), suggesting exacerbation of inflammatory and autoimmune processes.

In vivo administration of P60 improves immunogenicity of AH1 peptide vaccination leading to protection against CT26 tumor challenge

Immunization of BALB/c mice with peptide AH1 emulsified in IFA is unable to induce a protective CTL response against challenge with CT26 tumor cells (26). However, depletion of CD25+ Tregs with anti-CD25 Abs before immunization with peptide AH1 resulted in the induction of a long-lasting antitumoral immune response (14). These observations led us to speculate that in vivo inhibition of Treg by peptide P60 (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 and treated daily with saline or with 50 nmol/mouse peptide P60 i.p. from days 0–9 after immunization. At day 10, when we analyzed the anti-AH1 immune response, we found that splenocytes from animals treated with P60 produced greater amounts of IFN-γ in response to AH1 peptide (Fig. 6A).

FIGURE 4. Expression of different mRNAs on effector T cells or Tregs after incubation with P60. Purified CD4+CD25− effector T cells (A–C) or CD4+CD25+ Tregs (D–F) were stimulated with anti-CD3 or with IL-2 plus anti-CD3, respectively, in the absence or presence of P60 or control peptide (ctrl pept). mRNA expression for the indicated genes was measured at different time points of stimulation by real-time PCR. Figures are representative of two independent experiments.
groups of mice were injected with AH1, AH1 plus a control peptide, or treated with P60 peptide alone. Treatment of mice with P60 in the absence of immunization did not protect the animals against CT26 tumor challenge. Similarly, the therapeutic efficacy of immunization with AH1 alone or with AH1 plus a control peptide was very poor (0–20% of protection). However, when mice immunized with AH1 were treated with P60, 9 of 11 mice (82%) remained free of tumors ($p < 0.05$; Fig. 6B).

**In vivo administration of P60 improves vaccination efficacy of an RAdNS3 protein**

In a previous study, we showed that i.p. immunization with an RAdNS3 was able to induce both T helper and T cytotoxic anti-NS3 responses (28). The responses thus elicited were able to protect ~60% of mice against challenge with the replicative recombinant vaccinia vHCV1-3011–expressing HCV proteins. Because Tregs may hamper the induction of protective cellular immune responses in several viral infections (7, 8, 40), we tested the capacity of P60 administration to improve immunogenicity of RAdNS3 vaccine. This was done by comparing the protective effects of the vaccine, with and without P60 administration, against a challenge with the recombinant vaccinia virus vHCV1-3011. Thus, BALB/c mice were immunized i.p. with $5 \times 10^8$ PFU of RAdNS3 and treated daily from days 0–10 with 50 nmol/mouse P60 i.p. or with saline. Ten days after immunization, animals were challenged with $5 \times 10^5$ CT26 tumor cells, as a surrogate of HCV infection. Three days later, viral load was measured in the ovaries. As expected, immunization with RAdNS3 was able to protect ~60% of the animals from recombinant vaccinia infection. However, P60 treatment of RAdNS3-immunized mice was able to protect 100% of mice (Fig. 6C). Interestingly, P60 alone was also able to induce a significant protection against challenge with vHCV1-3011, as compared with the effect of the administration of an irrelevant peptide.

**Discussion**

The aim of this work was to develop peptide inhibitors of protein FOXP3 to temporally overcome the immunosuppressive effect of Tregs. Using a phage-displayed random peptide library, we id-
identified a number of potential binders to FOXP3. Among them, P60 was found to be able to bind to FOXP3 and inhibit Treg activity in vitro and in vivo.

Because the activity of FOXP3 takes place inside the cell, we speculated that P60 was capable of entering into the cell. Indeed, experiments conducted with CF-labeled peptide suggest that P60 is able to enter the cell as a CPP. Although the exact mechanism of cellular entry of these peptides is still elusive, P60 fulfills the sequence requirements described for CPPs (reviewed in Ref. 33). Indeed, P60 has cationic character and contains arginines, as well as tryptophan, and it is also predicted as an amphipathic helix (41). Most importantly, P60 is able to reduce FOXP3 nuclear translocation and consequently to inhibit Treg activity. This is shown by its capacity to restore proliferation of human or murine effector T cells, in response to anti-CD3 in the presence of inhibitory Tregs, and also by its ability to enhance human or murine T cell proliferation in an MLR inhibited by Treg.

FOXP3 exists as an homo-oligomeric component of a large supramolecular complex that may contain different proteins and transcription factors, such as BRG-1, HDAC7, TIP60, NFATc2, NF-κB, FOXP1, retinoic acid-related orphan receptor (ROR)-γt, MEF2D, or AML1 (35, 42–46). It is clear that interactions between these proteins and FOXP3 are crucial for the final function of FOXP3, and thus disruption of one or more of these interactions might impair the potential effects of this supramolecular complex. We found that P60 reduced the inhibitory effect of FOXP3 on NF-κB and NFAT activity, suggesting that the binding of P60 to FOXP3 might take place in a region affecting the interaction with these transcription factors. These data are consistent with the capacity of P60 to upregulate the expression of mRNA for IL-2 on Tregs, which has been shown to be repressed by the interaction between NFAT and FOXP3 (47). We could not detect any effect of P60 on the capacity of FOXP3 to inhibit the transcriptional activation of the ROR-α (44) (data not shown). It has been described that the region encoded by exon 2 from FOXP3 is involved in the interaction with ROR-α (44). Thus, it is likely that different regions of FOXP3 may be involved in the interaction with the different transcription factors, and consequently, P60 could only be able to inhibit some of them. The capacity of P60 to inhibit FOXP3 nuclear translocation might suggest that the peptide binds to FKHD domain or to a region affecting its ability to target FOXP3 to the nucleus. However, we do not have direct evidence to support this hypothesis. We are currently attempting to determine the region of interaction between P60 and FOXP3.

It has been described that activation of human naïve T cells through TCR cross-linking can activate FOXP3 expression (reviewed in Ref. 48). In most cases, this expression is transient, and although it does not confer immunosuppressive activity to the T cell, it is strongly associated with hyperresponsiveness of activated T cells to subsequent stimulation (36). We identified a transient expression of Foxp3 soon after stimulation with anti-CD3, which was accompanied by a peak on the expression of mRNA for IL-10. Thus, P60 could have a direct effect on conventional T cells after their activation. Indeed, the addition of P60 reduced this Foxp3 expression and the subsequent peak of IL-10. Moreover, P60 increased IFN-γ induced by anti-CD3, suggesting that P60 could favor T cell activation after TCR stimulation by inhibiting Foxp3 expression. The regulation of FOXP3 transcription is a complex process, and there is considerable uncertainty on the multiple signal pathways implicated (reviewed in Ref. 49). The characterization of the core promoter of human FOXP3 revealed the presence of six NFAT and AP-1 binding sites, which are positively regulating the transactivation of the FOXP3 promoter after triggering of the TCR (50). Because FOXP3 can cooperate in a DNA-binding complex with NFAT to regulate the transcription of target genes (47), it is tempting to postulate that FOXP3 might also be implicated in its own transcription, and thus explain the effect of P60 at this level.

The scurfy mutant mouse strain, harboring a mutation in the Foxp3 gene coding for a product that lacks the FKHD, suffers from a fatal lymphoproliferative disease (37). Lack of functional Tregs has been suggested to cause the disease in scurfy mice, which is characterized, among other symptoms, by gross internal lesions such as splenomegaly, enlarged lymph nodes, anemia, and massive infiltration in lungs, the acini of the pancreas and the liver (38, 39). It has recently been shown that selective depletion of Foxp3+ Tregs by diphtheria toxin administration to newborn DEREG mice (expressing the diphtheria toxin receptor under the control of the Foxp3 locus) leads to the development of scurfy-like symptoms (39). We have found that newborn ICR mice treated daily with P60 during 10 d after birth develop a lymphoproliferative autoimmune syndrome, resembling the reported pathology in scurfy mice or in newborn DEREG mice treated with diphtheria toxin. Treatment with P60 was also able to reduce the number of CD4+ CD25+Foxp3+ T cells in the spleen, suggesting that inhibition of FOXP3 might have an effect in the homeostasis of Tregs. P60 administration was limited to the first 10 d after birth. This treatment did not lead to an early death (15–25 d) as occurred in the scurfy mutant mice (38). However, survival of male mice was highly compromised, with ~50% of deaths 3 mo after birth. It is interesting to note that all male mice treated with P60 developed shoulder ulcers after 3 mo of life, with skin hyperplasia (Supplemental Fig. 4) and persisting peribronchial and perivascular infiltrations in the lungs, indicating the presence of an unremitting and uncontrolled inflammatory syndrome. But interestingly, in adult mice, neither Treg depletion with anti-CD25 Abs (15), depletion of total Foxp3+ Tregs by diphtheria toxin treatment in DEREG mice (39), nor treatment of mice with P60 peptide resulted in the development of inflammatory autoimmune disorders (Supplemental Fig. 5). As it has been suggested by others, this finding could be explained by the lack of lymphopenia-induced proliferation, a physiologic process that regularly occurs during early postnatal life (51), preventing the activation of autoreactive T cells (52).

Downregulation of Treg suppressor activity in vivo in adults may be beneficial to enhance the immunogenicity of a vaccine (14). In a previous work, we showed that in vivo CD25+ T cell depletion improved immunogenicity of AH1 peptide vaccination and protected mice against CT26 tumor challenge (14). In this work, we have found that in vivo administration of P60, instead of Treg depletion, was also able to protect mice from tumor challenge. Similarly, in vivo administration of P60 improved the efficacy of an RAAdNS3-based vaccine against infection with a recombinant replicative vaccinia vHCV1-3011 expressing the HCV polyprotein. These results are in agreement with previous reports showing an enhancement of immunogenicity of a vaccine by Treg depletion (14, 16). However, P60 administration in vivo might allow a finer control on the intensity and duration of Treg suppression, in particular after vaccination, avoiding the risk for autoimmunity associated with depletion of Treg.

In summary, we have identified peptide P60, which is able to enter into the cells, bind to FOXP3, reduce its nuclear translocation, and inhibit its ability to downregulate the activity of the transcription factors NF-κB and NFAT. P60 is able to inhibit the immunosuppressive activity of murine and human-derived Tregs and enhances the effector T cell stimulation in vitro. Also, and most importantly, we show that P60 can improve the immunogenicity of cancer and viral vaccines.
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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Immunohistochemical analysis of Foxp3 on BALB/c mice splenocytes incubated with culture medium alone (A), with P60 (B) or with control peptide (C) for 24 h.
**Supplementary Figure 2. Effect of 2 hours-pretreatment of Treg cells with P60 on their suppressive activity in vitro.** CD4+CD25- spleen cells from BALB/c mice were stimulated with anti-CD3 monoclonal antibodies in the presence or absence of purified murine CD4+CD25+ Treg cells previously pulsed for 2 h with 100 μM of P60 or control peptide and washed. Cell proliferation was analyzed after 24h of culture by measuring trititated thymidine incorporation in the harvested cells using a scintillation counter.
**Supplementary Figure 3.** Effect of P60 peptide on the proliferation of Treg or effector T cells in response to IL-2 and anti-CD3. CFSE labelled Treg (A) or effector T cells (B) were cultured for 72 h with IL-2 or IL-2+antiCD3 in the presence or absence of P60 or P60Scramble peptide and analyzed by flow cytometry for CFSE dilution. ³H Thymidine incorporation of Treg (C) or effector T cells (D) cultured for 72 h with IL-2+antiCD3 or IL-2 in the presence or absence of P60 or P60Scramble peptide.
Supplementary Figure 4A. ICR mice treated during 10 days after birth with 25 nanomol/mice of P60 developed shoulder ulcers. Photographs were taken when mice were three months old.

Supplementary Figure 4B. Histological analysis of the skin of ICR mice treated during 10 days after birth with 25 nanomol/mice of P60 (A) or with saline (B). Samples were obtained when mice were three months old.
Supplementary Figure 5. Hematoxilin and eosin staining for the histological analysis of different organs from adult mice treated daily with 50 nanomoles/mice of P60 i.p. during 10 days. Pictures are representative of 1 out of five analyzed mice per group.