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In Vivo Delivery of Lentiviral Vectors Expressing Vasoactive Intestinal Peptide Complementary DNA as Gene Therapy for Collagen-Induced Arthritis

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Objective. **Vasoactive intestinal peptide (VIP) has been shown to exert potent immunomodulatory activity, and the use of lentiviral vectors has been found to be an effective means of gene delivery. The present study was therefore undertaken to investigate the feasibility and efficiency of gene therapy using lentiviral vectors expressing VIP (LentiVIP) for the treatment of rheumatoid arthritis (RA).**

Methods. **We evaluated the therapeutic potential of the gene therapy strategy in the collagen-induced arthritis (CIA) mouse model, administrating the vectors at different phases of the disease. The inflammatory response was determined by measuring the levels of various inflammatory cytokines and chemokines in the joints and serum. The Th1-mediated response was evaluated by determining the proliferative response and cytokine profile of T cells stimulated with autoantigen.**

Results. **A single intraperitoneal injection of LentiVIP was highly effective in treating CIA. Mice with established, severe arthritis showed complete regression of the disease. The therapeutic effect of LentiVIP was associated with widespread biodistribution of the vector and increased VIP levels, especially in joints and lymphoid organs, and was mediated through a striking reduction of the 2 deleterious components of the disease, i.e., the autoimmune response (self-reactive Th1 cell activity and autoantibody production) and the inflammatory response. LentiVIP treatment also induced the generation and/or activation of CD4+,CD25+,FoxP3+ Treg cells in arthritic mice.**

Conclusion. **Our findings show that in vivo administration of lentiviral vector expressing VIP produces one of the most potent therapeutic effects described so far in any animal model of RA. We propose that VIP gene transfer should be further investigated as a potential novel, effective treatment of RA and other chronic autoimmune disorders.**

The main advantage of gene-based therapy strategies is that they enable continuous and/or regulated synthesis of therapeutic molecules inside the target tissue for long periods, increasing potency and reducing systemic toxicity. In addition, the therapeutic molecules are more active than their recombinant counterparts (1,2). A wide range of gene therapy strategies directed against the inflammatory response or damaged synovium has been assayed in animal models of rheumatoid arthritis (RA) (refs. 3–7; for review, see ref. 3) and in clinical trials (8,9). These studies have demonstrated feasibility and safety of the gene therapy strategies, but new protocols are still needed to demonstrate their potency as a viable alternative to current RA treatments. In theory, a single injection of therapeutic vectors, administered locally or systemically, could be enough to have potent benefits. However, clinical trials of gene therapy for RA, involving direct injection of naked DNA and/or retrovirus, have shown poor results (http://

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www.wiley.co.uk/genmed/clinical/), due in part to poor transduction efficiency.

Plasmids as well as adenoviral and adeno-associated viral vectors have been used successfully for gene therapy of arthritis. They all prevented disease development (10–13) and also reduced severity when treatment was started shortly after disease onset (14–17). However, to date only partial therapeutic effect has been achieved when treatment is started in animals with established signs of severe arthritis (arthritis score >6) (for review, see ref. 3). Lentiviral vectors have been described as the most powerful of all integrative vector systems (18) and are one of the most efficient vectors for in vivo application (19,20). The latest generation of lentiviral vectors is one of the safest and most efficient tools for stable gene transfer (18,21), even eliminating the risk of vector mobilization, due to infection with a wild-type human immunodeficiency virus type 1 (HIV-1).

Vasoactive intestinal peptide (VIP) is a neuropeptide that elicits a broad spectrum of biologic functions (22), acting as a potent antiinflammatory factor, inhibiting Th1 responses, and promoting immune tolerance by inducing the generation of Treg cells (23). As a consequence, VIP has emerged as a promising tool for the treatment of autoimmune/inflammatory diseases (22,23), including RA, ulcerative colitis, multiple sclerosis, type 1 diabetes mellitus, and uveoretinitis (24–28). The therapeutic effect of VIP is associated with reduction of the 2 main phases of these immune disorders: autoimmunity to self tissue components and destructive inflammatory responses. Therefore, VIP shows therapeutic advantages versus agents directed against only 1 component of these diseases.

Despite these advantages, there are several obstacles to translating VIP-based treatment into viable clinical therapies. VIP is very unstable and extremely sensitive to the peptidases present in most tissues, requiring multiple injections of high doses of the peptide to achieve a sustained therapeutic effect. Therefore, in recent years, several groups have developed viral (29,30) and nonviral (27,31) vectors to deliver VIP for the treatment of autoimmune disorders, including experimental arthritis (31).

In this study we used lentiviral vectors to achieve steady expression of VIP for the treatment of experimental arthritis in a collagen-induced arthritis (CIA) model, in which Freund's complete adjuvant (CFA) was used instead of phosphate buffered saline (PBS) in the second immunization in order to simulate more aggressive disease. We showed that a single injection of lentiviral vectors expressing *VIP* complementary DNA (cDNA) provides highly effective treatment of CIA, with complete regression of established, severe arthritis correlating with a striking reduction of the autoimmune and inflammatory responses.

MATERIALS AND METHODS

Plasmids and lentiviral constructs. The HIV packaging (pCMVΔR8.91) and vesicular stomatitis virus G (pMD.G) plasmids (18) were kindly provided by Dr. D. Trono (University of Geneva, Geneva, Switzerland). The CEWP lentiviral vector expressing enhanced green fluorescent protein (EGFP) through the strong CMVTetO promoter was constructed by replacing the SFFV promoter in the pHRSIN-CSEW vector (SEWP) (32) as follows. A polymerase chain reaction (PCR) fragment containing the CMVTetO promoter and the *Eco* RI/*Bam* HI sites was obtained by PCR using the *Eco* RI forward (CCGGAATTCGTTGACATTGATTATTGACTA) and *Bam* HI reverse (CGCGGATCCCGGAAGATGGATCGGTCC) primers with pcDNA4/TO as template. The CE vector was obtained by inserting this fragment into the SEWP vector backbone by *Eco* RI/*Bam* HI direct ligation. The lentiviral vector expressing VIP (LentiVIP) was constructed by replacing *EGFP* in the CEWP plasmid with a 560-bp *VIP* cDNA fragment (nucleotides 173–724 from the human locus NM-003381). This fragment was obtained by PCR using the pCMV6-XL4-VIP plasmid containing the full-length *VIP* cDNA (Trueclone [catalog no. TC111704]; OriGene Technologies, Rockville, MD) using the *Bam* HI–VIP forward (5′-CGGGATCCATGGACACCAGAAATAAGG) and *Pst* I–VIP reverse (5′-CACTGCAGGGGAAGTTGTCATCAGC) primers. Direct cloning was performed after *Bam* HI/*Pst* I restriction of the PCR fragment and CEWP.

Vector production and titration. Lentiviral vectors were produced by cotransfection of 293T kidney cells (CRL11268; American Type Culture Collection; Rockville, MD) with 3 plasmids: 1) vector plasmid (CEWP or LentiVIP), 2) packaging plasmid (pCMVΔR8.91), and 3) envelope plasmid (pMD.G), as previously described (33), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Vector titration was performed in 293T cells. CEWP vector titration was determined based on the percentage of EGFP-positive cells calculated by fluorescence-activated cell sorter analysis 7 days after transduction. For titration of LentiVIP vectors, transduced cells were lysed and DNA extracted after 7–10 days. Vector copy number per cell was determined using quantitative PCR as described below.

DNA preparation and quantitative real-time PCR. Genomic DNA from culture cells and tissue samples was isolated by adding 1 ml of SNET extraction buffer (34). Quantitative real-time PCR were performed with an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Samples were mixed with 2× iQ SYBR Green Supermix (Bio-Rad, Richmond, CA). To quantitate LentiVIP integration, we used primers comprising vector (cytomegalovirus [CMV]) and *VIP* sequences (CMVVIP forward 5′-GAGCTCGTTTAGTGAACCGTCAGA-3′, reverse 5′-AAGGAGCTGGGCCTTATTTCTGGT-3′). For CEWP

quantification, EGFP forward primer (5′-GCCCGACAACC-ACTACCT-3′) and reverse primer (5′-CGTCCATGC-CGAGAGTGA-3′) were used.

Western blotting. Cells were lysed with 1% Nonidet P40 lysis buffer containing protease inhibitor cocktail (Sigma, St Louis, MO), resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% polyacrylamide gels under reducing conditions), and electrotransferred to Hybond P polyvinylidene difluoride membranes (Amersham Biosciences, Little Chalfont, UK). Membranes were blocked with 5% nonfat milk and probed for 1 hour at room temperature with 2 μg/ml of anti-VIP monoclonal antibody (mAb) (clone H16; Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with horseradish peroxidase–labeled goat anti-mouse antibody (1:10,000 dilution, Caltag, Burlingame, CA). Analysis was performed using the ECL Advanced Western Blotting Detection Kit (Amersham Biosciences).

Determination of VIP levels and bioactivity. VIP levels in culture supernatants of LentiVIP-transduced cells and in sera and tissue from mice treated with LentiVIP were determined with an enzyme immunoassay kit (Phoenix Pharmaceuticals, Karlsruhe, Germany). To measure secretion of bioactive VIP by LentiVIP-transduced cells, 1 ml of culture supernatants was lyophilized, resuspended (100 μl) in PBS, and added to the murine macrophage cell line RAW 264.7 (10^6 cells/ml). Cells were cultured for 20 minutes in the presence of 3-isobutyl-1-methyl xanthine, lysed, and intracellular cAMP levels determined with an enzyme immunoassay kit (Amersham Biosciences). In addition, RAW 264.7 cells were stimulated with lipopolysaccharide (500 ng/ml) for 8 hours, and levels of tumor necrosis factor α (TNFα) in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA).

Arthritis induction and treatment. Animal experimental protocols were reviewed and approved by the Ethics Committee of the Spanish Council of Scientific Research. To induce severe CIA, DBA/1J mice (7–10 weeks old; The Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously with 200 μg of type II collagen (CII; Sigma) emulsified in CFA containing 200 μg *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). On day 21 after primary immunization, mice were boosted by subcutaneous injection of 100 μg of CII in CFA. LentiVIP treatment consisted of intraperitoneal administration of lentiviral vectors as indicated below. Mice with CIA were injected intraperitoneally with PBS or with CEWP vectors (expressing EGFP) as controls. Mice were assessed under blinded conditions every other day by 2 independent examiners, and scored for signs of arthritis as follows: grade 0 = no swelling; grade 1 = slight swelling and erythema; grade 2 = moderate swelling and edema; grade 3 = extreme swelling and pronounced edema; grade 4 = joint rigidity. Each limb was graded, for a maximum possible score of 16 per animal.

Histologic analysis. For histologic analysis, paws were collected at random by 2 independent investigators on day 70 after primary immunization, fixed in 4% buffered formaldehyde, decalcified, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or Masson's-Goldner trichrome stain. Histopathologic changes were scored in a blinded manner based on cell infiltration, cartilage destruction, and bone erosion parameters as previously described (24). Neutrophil infiltration into the joints was monitored by measuring myeloperoxidase activity in joint extracts isolated on day 35 postimmunization as previously described (35).

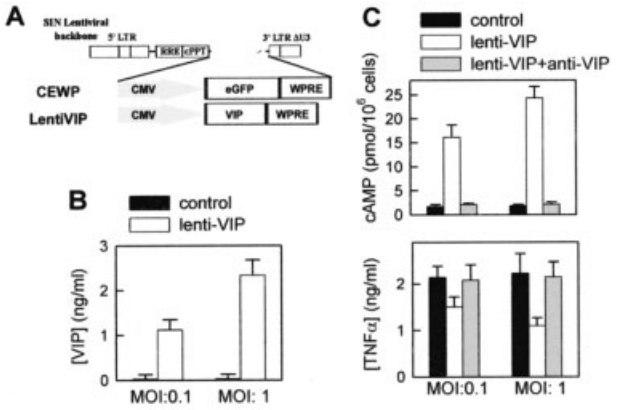

Figure 1. Evidence that lentiviral vectors driving the expression of vasoactive intestinal peptide (*VIP*) cDNA are efficient tools for expression of functional VIP. **A**, Maps of lentiviral vector used in the study. CEWP and LentiVIP are self-inactivated human immunodeficiency virus type 1–derived lentiviral vectors expressing enhanced green fluorescent protein (EGFP) and *VIP* cDNA, respectively, through the cytomegalovirus (CMV) minimal promoter. LTR = long terminal repeat; RRE = Rev-responsive element; cPPT = central polypurine tract; WPRE = woodchuck hepatitis posttranscriptional regulatory element. **B,** VIP levels in culture supernatants of cells transduced with LentiVIP at multiplicities of infection (MOI) of 0.1 and 1, determined by enzyme immunoassay. Controls were cells transduced with the same amount of CEWP vector. **C**, Production of bioactive VIP by LentiVIP-transduced cells. Culture supernatants of cells transduced with LentiVIP (MOI 0.1 and 1) were added to RAW 264.7 cells (10^6 cells/ml). In some samples, neutralizing concentrations of anti-VIP antibody (100 μg/ml) were added to cultures. Upper panel, Intracellular cAMP levels were determined by enzyme immunoassay after 20 minutes of culture. Lower panel, RAW 264.7 cells were stimulated with lipopolysaccharide (500 ng/ml) for 8 hours, and levels of tumor necrosis factor α (TNFα) in the culture supernatant were determined by enzyme-linked immunorsorbent assay. Controls were cells transduced with the same amount of CEWP vector. Values in **B** and **C** are the mean and SD from 3 experiments performed in duplicate.

Cytokine and autoantibody measurement. For determination of cytokine levels in the joints, protein extracts were isolated by homogenization of joints (50 mg tissue/ml) in 50 m*M* Tris HCl (pH 7.4) with 0.5 m*M* dithiothreitol and proteinase inhibitor cocktail (10 μg/ml; Sigma). Serum samples were collected at the peak of disease (day 35), and the levels of IgG, IgG1, and IgG2a anti-CII antibody were measured by ELISA as previously described (24). Cytokine and chemokine levels in the serum and joint protein extracts prepared on day 35 were determined with specific sandwich ELISAs using capture/biotinylated detection antibodies according to the recommendations of the manufacturer (BD PharMingen, San Diego, CA).

Assessment of T cell autoreactive response. Single-cell suspensions (10^6 cells/ml) pooled from spleen and draining lymph nodes or from knee joint synovial membrane were

obtained 35 days postimmunization. Cells were stimulated in complete medium (RPMI 1640 containing 10% fetal calf serum, 2 m*M* L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) with various concentrations of heat-inactivated CII for 48 hours (for cytokine determination) or for 72 hours (for proliferative response assays). Cell proliferation was evaluated using a bromodeoxyuridine-based cell proliferation assay (Roche Diagnostics, Mannheim, Germany). Cytokine content in culture supernatants was determined by specific sandwich ELISA as described above. For intracellular analysis of cytokines, draining lymph node and synovial cells were stimulated with inactivated CII (10 μg/ml) for 8 hours in the presence of monensin, and then stained with peridin chlorophyll protein (PerCP)–conjugated anti-CD4 mAb at 4°C, washed, fixed, saponin permeabilized, stained with fluorescein (FITC)– and phycoerythrin (PE)–conjugated anticytokine-specific mAb (BD PharMingen), and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). To distinguish between monocyte/macrophage and T cell sources, intracellular cytokine analysis was performed exclusively on the PerCP-labeled CD4+ T cell population.

Flow cytometric analysis of Treg cells. Synovial and draining lymph node cells isolated on different days (as indicated below) were incubated with various mAb (FITC-conjugated anti-CD25, PE-conjugated anti-CD45RB, PerCP-conjugated anti-CD4 [2.5 μg/ml final concentration]) for 1 hour at 4°C. After extensive washing, cells were fixed, saponin permeabilized, incubated for 45 minutes at 4°C with PE-conjugated anti–forkhead box P3 (anti-FoxP3) mAb (0.5 μg/sample), diluted in 0.5% saponin, and analyzed on a FACSCalibur flow cytometer. Isotype-matched antibodies were used as controls, and IgG block (Sigma) was used to avoid nonspecific binding to Fc receptors.

Statistical analysis. Values were expressed as the mean ± SD, and the differences between groups were analyzed by Mann-Whitney U test and, if appropriate, by Kruskal-Wallis analysis of variance.

RESULTS

Efficient expression of functional VIP by the LentiVIP vector in vitro. The HIV-1–derived lentiviral vector LentiVIP was constructed by insertion of a 560-bp human *VIP* cDNA fragment (nucleotides 173–724 from the human locus NM-003381) into the CEWP vector backbone by direct cloning using *Eco* RI/*Bam* HI restriction enzymes (Figure 1A). We first investigated whether cells transduced with LentiVIP produce bioactive VIP. Western blot analysis of 293T embryonic cells transduced with increasing numbers of LentiVIP vectors showed high content of preproVIP in cell lysates and culture supernatants compared with cells transduced with the CEWP vector (expressing EGFP) (details available from the author upon request). A VIP-specific ELISA demonstrated that 293T cells transduced with LentiVIP produced VIP, in a dose-dependent manner (Figure 1B).

Because VIP inhibits TNFα production by activated macrophages by increasing production of the second messenger cAMP (22), we next investigated whether the secreted VIP is bioactive. Thus, we added the conditioned medium of LentiVIP-transduced 293T cells to macrophage cultures and measured the levels of cAMP and TNFα. Supernatants of 293T cells transduced with LentiVIP, but not with CEWP, increased the production of intracellular cAMP and inhibited the secretion of TNFα by endotoxin-activated macrophages (Figure 1C). Both effects were specific for the secreted VIP, because the addition of a neutralizing anti-VIP antibody to the cultures reversed them (Figure 1C). These results therefore indicate that transduction of cells with LentiVIP results in the secretion of bioactive VIP and/or preproVIP with significant antiinflammatory actions.

Reduction of the severity of CIA by LentiVIP. We next investigated the potential therapeutic application of lentiviral vectors containing VIP, using the experimental CIA mouse model. A single administration of LentiVIP at the onset of the disease or when arthritis was already established completely abrogated the clinical signs and reduced the percentage of mice with arthritis, as compared with untreated or CEWP-treated mice (Figure 2A). Interestingly, we observed no loss of the therapeutic effects 4 weeks after the initial LentiVIP treatment. Importantly with regard to therapy, injection of LentiVIP into animals with severe clinical signs of arthritis (clinical score >10) progressively attenuated the severity of CIA (Figure 2A). Histopathologic analysis of joints showed that delayed treatment with LentiVIP significantly reduced CIA-characteristic chronic inflammation of synovial tissue (infiltration of inflammatory cells [lymphocytes, plasma cells, macrophages, and neutrophils] into the joint cavity and periarticular soft tissue), pannus formation, cartilage destruction, and bone erosion (Figure 2B). The LentiVIP-mediated inhibition of neutrophil infiltration was confirmed by the finding of decreased joint myeloperoxidase activity (Figure 2B).

In order to investigate the association of the therapeutic effects of LentiVIP in arthritic mice with increased secretion of VIP in these animals, we determined the biodistribution of the inoculated vector and the levels of VIP in various tissues and organs. Quantitative real-time PCR of CMV and VIP sequences revealed a significant rate of transduction of the LentiVIP in spleen (>50%; 0.5 vectors/cell) (Figure 3A). Liver, adipose tissue, and lymph nodes were also transduced

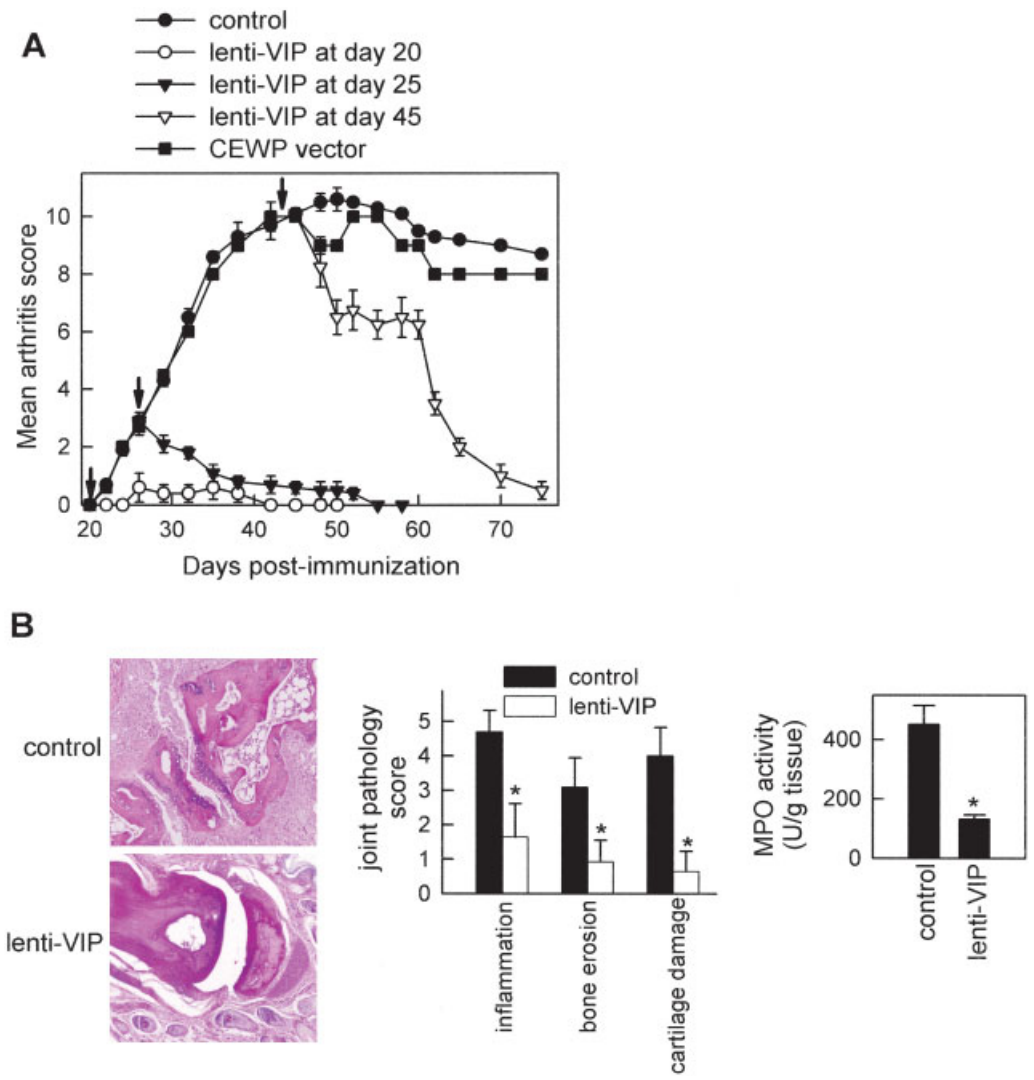

Figure 2. Evidence that lentiviral vector expressing vasoactive intestinal peptide (LentiVIP) reduces the severity of collagen-induced arthritis (CIA). **A**, DBA/1J mice with established CIA were injected intraperitoneally (arrows) either with phosphate buffered saline (PBS) (control) or with LentiVIP (10^8 copies of vector/mouse) on day 25 or day 45. In addition, prophylactic treatment with LentiVIP (10^7 copies of vector/mouse) was initiated on day 20. Clinical severity of arthritis was scored. Injection of the CEWP vector (10^7 copies of vector/mouse) into mice with CIA on day 20 did not affect disease progression. Values are the mean ± SD of 6–14 mice per group. **B**, Mice with severe CIA (mean arthritis score 10) were injected intraperitoneally either with PBS (control) or with LentiVIP (10^8 copies of vector/mouse) on day 45, and paw joints obtained on day 70 were assessed histologically (left panel) (original magnification × 200) and the results quantitated by scoring of inflammation, bone erosion, and cartilage damage (middle panel) and by measuring myeloperoxidase (MPO) activity in protein extracts isolated on day 70 to determine neutrophil infiltration into the joints (right panel). Values are the mean and SD of 6–14 mice per group. $* = P < 0.001$ versus controls.

with high efficiency (20%, 10%, and 3% respectively), whereas we did not observe detectable transduction of kidney cells (<0.1%) (Figure 3A). The wide spreading of the vector was paralleled by time-sustained increases in levels of VIP found in serum, spleen, and paws of animals treated with LentiVIP in comparison with arthritic mice injected with CEWP vector (Figure 3B).

Inhibition of the inflammatory response in CIA by LentiVIP. We next investigated the mechanisms underlying the decrease in severity of CIA following

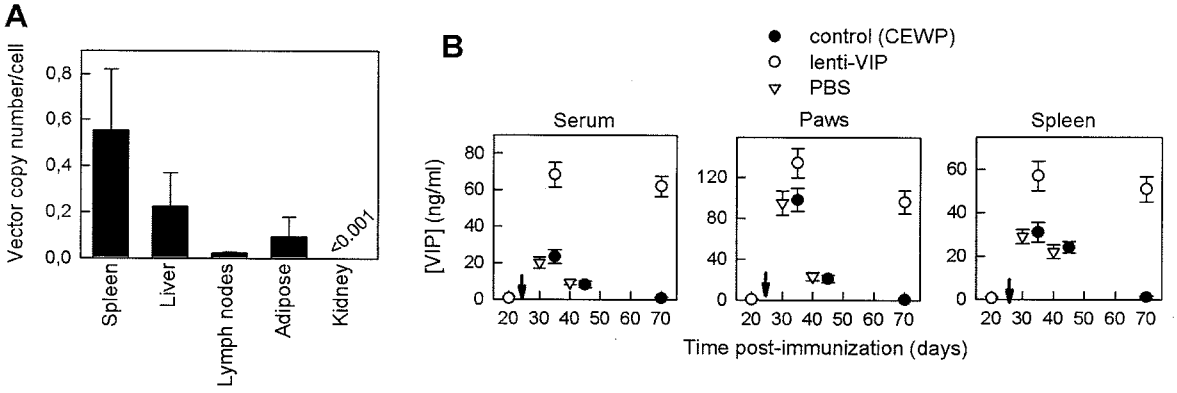

Figure 3. Vector distribution and VIP levels in LentiVIP-treated mice with CIA. DBA/1J mice with established CIA were injected intraperitoneally either with PBS, with CEWP vector (control), or with LentiVIP (10^8 copies of vector/mouse) on day 25. **A**, DNA from various tissues was obtained 15 days after LentiVIP inoculation, and the number of vector copies per cell was determined by quantitative polymerase chain reaction using cytomegalovirus and VIP cDNA sequences. Levels in the kidney were below the limit of detection (0.001 copy per cell = 0.1% of transduced cells). Values are the mean and SD of 3–5 mice per group. **B**, Serum and protein extracts of spleen and paws were obtained at different time points after LentiVIP injection (arrows), and VIP levels in serum and tissue were determined by enzyme immunoassay. Values are the mean ± SD of 3–5 mice per group. See Figure 2 for definitions.

LentiVIP treatment. We first evaluated the effect of LentiVIP on the production of inflammation mediators that are mechanistically linked to CIA severity. LentiVIP administration significantly reduced protein expression of inflammatory cytokines (TNFα, interferon-γ [IFNγ], interleukin-6 [IL-6], IL-1β, IL-12, and IL-17) and chemokines (RANTES and macrophage inhibitory protein 2) in the joints of arthritic mice (Figure 4A). In addition, joints of LentiVIP-treated mice showed increased levels of the regulatory cytokine IL-10 (Figure 4A). The broad antiinflammatory activity of LentiVIP in the inflamed joint was accompanied by down-regulation of the systemic inflammatory response, as demonstrated by the finding that LentiVIP decreased CIA-induced serum levels of the proinflammatory cytokines TNFα and IL-1β (Figure 4B).

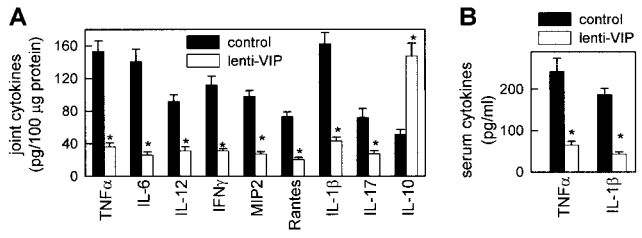

Figure 4. Evidence that vasoactive intestinal peptide (*VIP*) gene therapy inhibits the inflammatory response in collagen-induced arthritis (CIA). DBA/1J mice with established CIA (mean arthritis score >3) were injected intraperitoneally either with phosphate buffered saline (control) or with lentiviral vector expressing VIP (LentiVIP) (10^8 copies of vector/mouse) on day 25 postimmunization, and local and systemic expression of inflammation mediators in joint protein extracts (**A**) and sera (**B**) isolated on day 35 postimmunization was determined by enzyme-linked immunosorbent assay. Values are the mean and SD of 3–5 mice per group. $* = P < 0.001$ versus controls. TNFα = tumor necrosis factor α; IL-6 = interleukin-6; IFNγ = interferon-γ; MIP-2 = macrophage inhibitory protein 2.

Down-regulation of the Th1-mediated autoreactive response in CIA by LentiVIP. To determine whether LentiVIP could ameliorate CIA by reducing autoreactive T cell responses and/or their migration to the joints, we determined proliferation and cytokine profiles of pooled cells from spleen and draining lymph nodes isolated from control or LentiVIP-treated arthritic mice in response to antigen (CII) in vitro. Cells obtained from untreated mice with CIA showed marked CII-specific proliferation and effector T cell production of high levels of Th1-type cytokines (IFNγ, IL-2, and TNFα) and Th17-type cytokines (IL-17) and low levels of Th2-type cytokines (IL-4 and IL-10) (Figure 5A). In contrast, cells from LentiVIP-treated mice proliferated much less, and produced low levels of IL-17 and Th1-type cytokines and increased levels of suppressive cytokines (IL-10 and transforming growth factor β1 [TGFβ1]) and the Th2-type cytokine IL-4 (Figure 5A). This effect was antigen-specific, because LentiVIP treatment did not affect proliferation or cytokine production by anti-CD3–stimulated cells compared with findings in control mice with CIA (Figure 5A). This suggests that LentiVIP administration during CIA progression par-

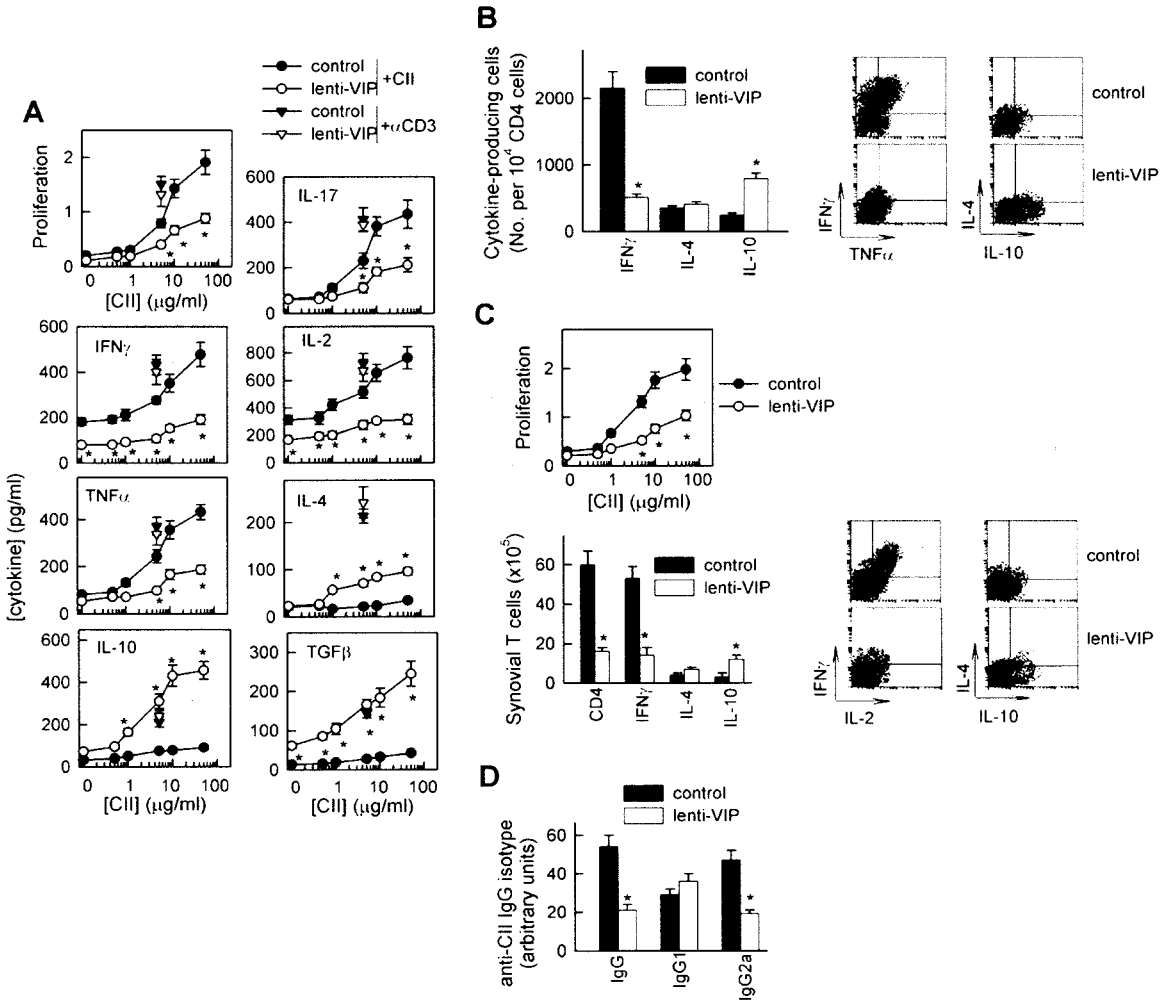

Figure 5. Evidence that LentiVIP down-regulates Th1-mediated responses in CIA. DBA/1J mice with established CIA (mean arthritis score >3) were injected intraperitoneally either with phosphate buffered saline (control) or with LentiVIP (10^8 copies of vector/mouse) on day 25 postimmunization. **A**, Proliferative response (absorbance at 405 nm) and cytokine production in pooled cells from spleen and draining lymph nodes isolated on day 30 from control or LentiVIP-treated mice with CIA after in vitro stimulation with type II collagen (CII) in various concentrations. Spleen cells were stimulated with anti-CD3 antibodies for assessment of nonspecific stimulation. A pool of 3 nonimmunized DBA/1J mouse cells was used for assessment of the basal response. No proliferation or cytokine production by T cells was detectable in the presence of an unrelated antigen (ovalbumin) (data not shown). Values are the mean ± SD of 3–5 mice per group. TGFβ = transforming growth factor β. **B,** Number of CII-specific cytokine-producing T cells. Cells from spleen and draining lymph nodes from control or LentiVIP-treated mice with CIA were restimulated in vitro with CII (10 μg/ml) and analyzed by flow cytometry for expression of CD4 and of intracellular cytokines in gated CD4+ T cells. Dot plots show representative double staining for IFNγ/TNFα or IL-4/IL-10 expression in gated CD4+ T cells. Mean and SD numbers of IFNγ-, IL-4–, and IL-10–expressing T cells are shown in the left panel. Data represent pooled values from 2 independent experiments. **C,** CII-specific proliferative response and number of cytokine-producing CD4+ T cells in synovial membrane cells isolated from control or LentiVIP-treated mice with CIA and stimulated in vitro with CII (10 μg/ml) for 48 hours. Dot plots show representative double staining for IFNγ/IL-2 or IL-4/IL-10 expression in gated CD4+ T cells. Mean ± SD levels of proliferation (absorbance at 405 nm) and numbers of CD4-, IFNγ-, IL-4–, and IL-10–expressing synovial membrane cells are shown in the left panels. Pooled synovial cells from 3 animals per group were used; data are from 2 independent experiments. **D,** Levels of CII-specific IgG, IgG1, and IgG2a antibodies in sera collected on day 35 from control or LentiVIP-treated mice with CIA, determined by enzyme-linked immunosorbent assay. Values are the mean and SD of 8–12 mice per group. $* = P < 0.001$ versus controls. See Figure 4 for other definitions.

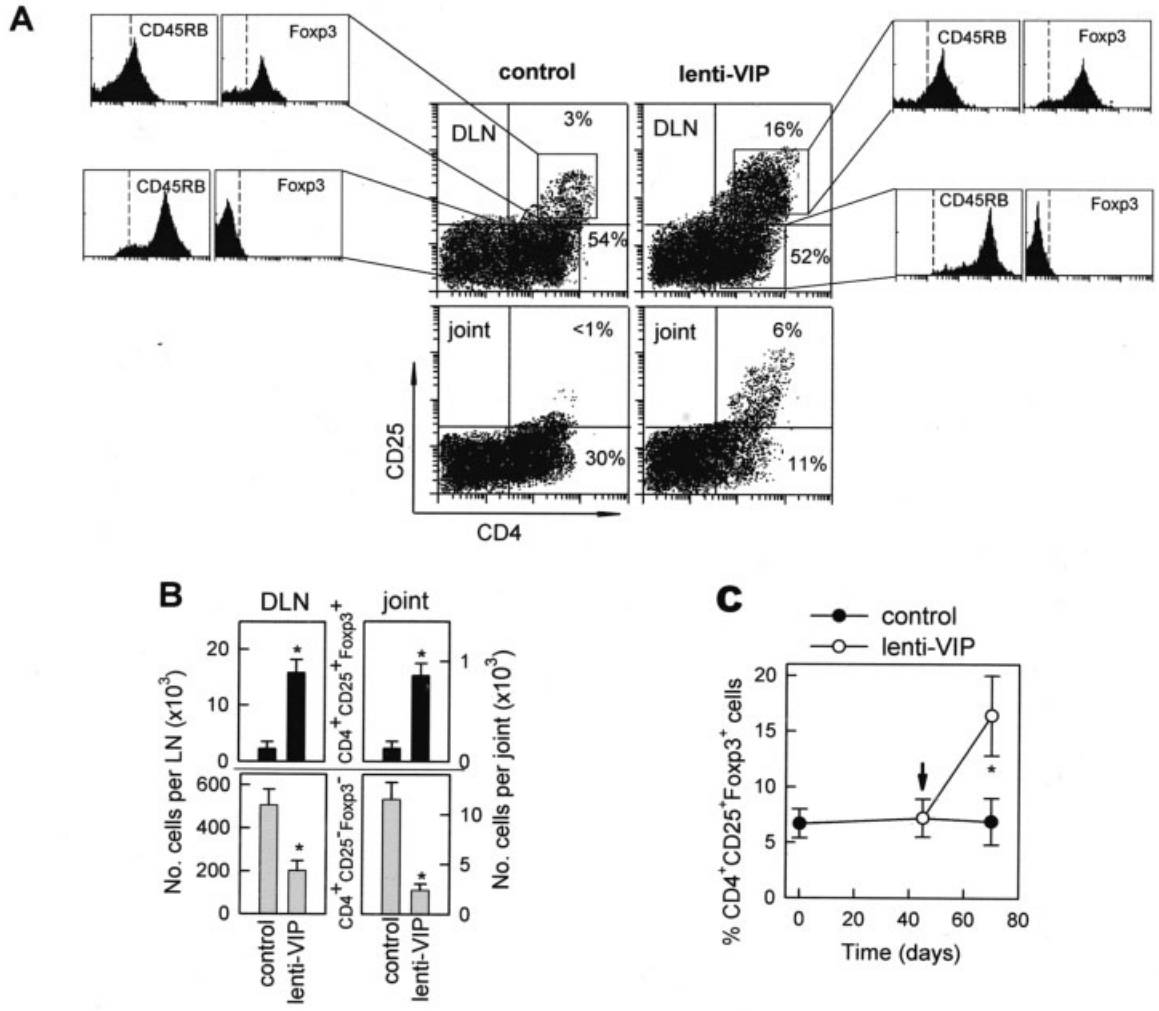

Figure 6. Evidence that LentiVIP induces the emergence of regulatory CD4+,CD25+,FoxP3+ T cells in CIA. **A,** DBA/1J mice with established CIA (mean arthritis score >3) were injected intraperitoneally either with PBS (control) or with LentiVIP (10^8 copies of vector/mouse) on day 25 postimmunization. Draining lymph node (DLN) and synovial (joint) cells isolated on day 35 were analyzed by flow cytometry for expression of CD4 and CD25. Numbers are the percentages of CD4+,CD25− and CD4+,CD25+ cells. Histograms show the expression of CD45RB and forkhead box P3 (FoxP3) in gated CD4+,CD25− and CD4+,CD25+ cells in draining lymph nodes. Similar histogram profiles were observed for synovial cells from LentiVIP-treated mice (data not shown). Dashed lines represent isotype antibody controls. **B**, Numbers of CD4+,CD25+,FoxP3+ cells and CD4+,CD25−,FoxP3− cells were determined in draining lymph nodes and synovial membrane (joint) isolated from control or LentiVIP-treated mice with CIA. Values are the mean and SD of 4 mice per group. $* = P < 0.001$ versus controls. **C,** DBA/1J mice with severe CIA (mean arthritis score >10) were injected intraperitoneally either with PBS (control) or with LentiVIP (10^8 copies of vector/mouse) on day 45 postimmunization (arrow). Draining lymph node cells were isolated at different time points, and the percentage of CD4+,CD25+,FoxP3+ cells was determined by flow cytometry. Values are the mean ± SD of 3 mice per group. $* = P < 0.001$ versus controls. See Figure 2 for other definitions.

tially inhibits CII-specific Th1 cell and, probably, Th17 cell clonal expansion.

In order to distinguish whether the decrease in Th1 cytokine production induced by LentiVIP treatment is a consequence of either down-regulation of cytokine release or inhibition of Th1 cell expansion, and to identify the source of IL-10 (macrophages or CD4+ T cells), we determined the intracellular expression of these cytokines by flow cytometry on sorted CD4+ T cells. LentiVIP treatment significantly reduced the number of IFNγ/TNFα-producing Th1 cells and increased the number of IL-10–producing CD4+ T cells from

draining lymph nodes and spleens (Figure 5B). We observed similar effects in synovial cells (Figure 5C). Thus, LentiVIP administration in mice with CIA regulates the expansion of autoreactive/inflammatory Th1 and Th17 cells and, presumably, IL-10–secreting T cells.

High levels of circulating antibodies directed against collagen-rich joint tissue invariably accompany the development of RA and CIA, and their production is a major factor in determining susceptibility to the disease. Administration of LentiVIP resulted in reduced serum levels of CII-specific IgG, particularly autoreactive IgG2a antibodies (Figure 5D), generally reflective of Th1 activity. These data provide further evidence that LentiVIP treatment during CIA reduces the autoreactive Th1 responses in both the joint and the periphery.

Emergence of regulatory CD4+,CD25+,FoxP3+ T cells induced by LentiVIP in CIA. Several studies have indicated that Treg cells confer significant protection against CIA by decreasing the activation and joint homing of autoreactive Th1 cells (36,37). Because delayed LentiVIP treatment also inhibits events during the inflammatory phase of CIA following activation of antigen-specific Th1 cells and induces the generation of IL-10/TGFβ1–producing T cells, we investigated whether LentiVIP induces Treg cells with suppressive activity during the progression of the disease. LentiVIP-treated mice with CIA were shown to have significantly higher percentages and numbers of CD4+,CD25+ cells in both draining lymph nodes and synovium compared with control mice with CIA (Figures 6A and B). Additionally, LentiVIP-induced CD4+,CD25+ cells from draining lymph nodes (Figure 6A) and synovium (data not shown) exhibited a Treg phenotype, i.e., CD45RB^{low},FoxP3+. Interestingly, late administration of LentiVIP was still able to induce the emergence of CD4+,CD25+,FoxP3+ cells in mice with severe established arthritis (Figure 6C).

DISCUSSION

Although the etiology of RA is unknown, there is evidence that the recruitment and activation of neutrophils, macrophages, and lymphocytes into joint tissue and the formation of pannus are hallmarks of RA pathogenesis. Several studies of animal models indicate that Th1-derived cytokines play a pathogenic role by promoting macrophage and neutrophil infiltration and activation (38). Inflammation mediators, such as cytokines and free radicals, produced by infiltrating inflammatory cells have a key role in joint damage (38). Recent evidence has demonstrated the involvement of IL-17 produced by Th17 cells as an additional critical player in the pathogenesis of RA (39). Available therapies based on immunosuppressive agents inhibit the inflammatory component of RA and have the potential to slow the progression of disability by delaying erosion and deformity (40). However, they do not reduce the relapse rate, and because continued treatment is required in most cases to maintain a beneficial effect, they have multiple side effects. This illustrates the need for novel therapeutic approaches to prevent the inflammatory and autoimmune components of the disease and to promote restoration of immune tolerance.

Gene therapy has been considered as a potential strategy for RA treatment or cure since the early 1990s. In most animal models of RA, gene therapy has resulted in prevention of disease development and/or moderate inhibition of disease progression (12,41,42). However very few studies (using gene therapy or any other treatment strategy) have demonstrated regression of established arthritis (16,17,43,44), and to date, total regression of established severe disease has not been achieved with any RA treatment.

Our rationale in the present study was to use one of the most potent tools for in vivo gene delivery (lentiviral vectors) to express one of the most potent immunomodulatory molecules (VIP) to treat severe arthritis. We chose to work with the HIV-1–derived lentiviral vectors since they have one of the best safety and efficiency profiles for in vivo gene delivery of all vectors described to date. In addition, they can be easily modified to hold drug- or inflammation-regulated promoters that will allow tight regulation of the transgene. Other vectors, such as plasmid, adenoviral, or adeno-associated viral vectors, could be used for this approach and it would be interesting to test them in similar settings. However, their low efficiency (plasmid), high immunogenicity (adenoviral), or low cargo capacity (adeno-associated) could be a disadvantage to developing an efficient, nonimmunogenic therapeutic vector able to achieve conditional or regulated expression of VIP.

The CIA model has numerous immunologic and pathologic similarities to human RA. In fact, treatment studies using this experimental model have provided the basis for development of new treatments for RA (43,45). In this study we found that LentiVIP treatment decreased the presence of autoreactive Th1 cells in the periphery and the joint and strongly reduced the inflammatory response during CIA progression. Of relevance is the fact that administration of LentiVIP to arthritic mice also resulted in a decreased CII-specific Th17-

mediated response, which is critical for RA progression. As previously reported for VIP (46), administration of LentiVIP to mice with CIA also induced the appearance of CD4+,CD25+,FoxP3+ cells with a Treg phenotype in draining lymph nodes and joints. This parallels the fact that CD4+ T cells from LentiVIP-treated animals showed increased production of IL-10 and TGFβ, 2 key mediators of Treg cell function. These data partially explain the finding that delayed LentiVIP administration inhibits events during the inflammatory phase of CIA following activation/differentiation of antigen-specific effector Th1/Th17 cells. Therefore, this treatment based on *VIP* gene delivery is able to restore immune tolerance in arthritic mice by improving the balance between Treg cells and autoreactive Th1/Th17 cells.

Of note was the improvement in bone erosion and cartilage damage after LentiVIP administration on day 45 to mice with severe CIA (arthritis score 10–11) (Figure 2B). To our knowledge, this is the first time such improvement has been observed after treatment of established disease. This improvement can be explained by the effect of VIP on factors involved in bone remodeling, i.e., reduction of RANKL, RANK, inducible nitric oxide synthase, IL-1β, TNFα, IL-6, IL-11, IL-17, and cyclooxygenase 2 levels and augmentation of IL-4, IL-10, and osteoprotegerin levels (47). In addition, the self-regulation of the autoimmune process probably allows natural mechanisms of regeneration to take place.

Taken together, our findings demonstrate that in vivo LentiVIP administration reduces the frequency of severe arthritis, ameliorates symptoms, and prevents joint damage. With regard to treatment, it is important to take into account the ability of delayed administration of LentiVIP to completely regress ongoing disease, which fulfills an essential prerequisite for antiarthritic therapy. The fact that we did not observe a loss of its beneficial effect with time suggests that a single injection of LentiVIP could induce remission of the disease. Compared with other treatments (including other gene therapy strategies or direct injection of synthetic VIP), intraperitoneal injection of LentiVIP offers the most potent therapeutic activity described so far in any animal model of RA. The higher efficiency of LentiVIP is probably due to the steady expression of VIP in immune organs (spleen and draining lymph nodes) and joints. The continuous presence of active VIP in these tissues allows very potent reduction of inflammation and autoimmunity, the 2 main processes involved in the pathology of RA and CIA. This certainly was crucial in the advantage of this strategy compared with other treatments directed against a single mediator, such as the new biologic agents (anti-TNFα, IL-1 receptor antagonist).

Systemic treatments with TNFα inhibitors are associated with severe side effects linked to systemic immunosuppression. However, the treated animals in the present study did not exhibit any apparent adverse effects, such as hypotension or diarrhea, resulting from steady expression of VIP in many tissues, probably due to the low concentration of circulating VIP. In addition, serious side effects due to sustained immunosuppression are minimized because LentiVIP treatment affects only CII-specific responses and induces CII-specific Treg cells. In any case, these potential side effects could be minimized by controlling VIP expression with drugs (doxycycline) or inflammation-related promoters in the final therapeutic vectors. Such transcriptional/transductional targeting is mandatory before this strategy can be used in clinical trials.

As previously described (48), our study shows that systemic and joint levels of VIP are increased in arthritic mice throughout the progression of the disease. Therefore, it is attractive to speculate that the body responds to an exacerbated inflammatory/autoimmune response by increasing peripheral production of endogenous antiarthritic factors, such as VIP, in an attempt to restore the inflammatory homeostasis. Interestingly, although LentiVIP was administered intraperitoneally, we detected higher amounts of VIP locally in the joints than systemically in the serum. A potential explanation for this finding is that LentiVIP could more efficiently transduce immune cells present at the site of injection (peritoneal cavity) or in draining lymph nodes, and after their migration to the inflamed joint, the local production of transgenic VIP would significantly increase. We also found that, in addition to the processed VIP, LentiVIP-transduced cells are able to secrete the preproVIP precursor. To our knowledge, this is the first study to demonstrate secretion of the VIP precursor, although we still do not know whether it exerts any VIP-like immunomodulatory activity and contributes to the therapeutic effect of LentiVIP in CIA.

In summary, the results of this study suggest a novel strategy for the treatment of RA and other chronic autoimmune disorders, based on *VIP* gene transfer with lentiviral vectors. This strategy has been shown to be safe, inexpensive, and more effective than any other therapeutic approach in the CIA model. Therefore, although further modifications of LentiVIP vector would be necessary prior to translation to the clinical setting, the basic findings described here provide

grounds for optimism about the applications of antiinflammatory neuropeptide gene therapy to human autoimmune disorders.

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AUTHOR CONTRIBUTIONS

Dr. Martín had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Delgado, Martín.
Acquisition of data. Delgado, Toscano, Benabdellah, Cobo, O'Valle, Gonzalez-Rey, Martín.
Analysis and interpretation of data. Delgado, Toscano, Benabdellah, Martín.
Manuscript preparation. Delgado, Martín.
Statistical analysis. Delgado, Gonzalez-Rey, Martín.

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