An epitope-specific DerG-PG70 LEAPS vaccine modulates T cell responses and suppresses arthritis progression in two related murine models of rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is an autoimmune joint disease maintained by aberrant immune responses involving CD4+ T helper (Th)1 and Th17 cells. In this study, we tested the therapeutic efficacy of Ligand Epitope Antigen Presentation System (LEAPS™) vaccines in two Th1 cell-driven mouse models of RA, cartilage proteoglycan (PG)-induced arthritis (PGIA) and PG G1-domain-induced arthritis (GIA). The immunodominant PG peptide PG70 was attached to a DerG or J immune cell binding peptide, and the DerG-PG70 and J-PG70 LEAPS vaccines were administered to the mice after the onset of PGIA or GIA symptoms. As indicated by significant decreases in visual and histopathological scores of arthritis, the DerG-PG70 vaccine inhibited disease progression in both PGIA and GIA, while the J-PG70 vaccine was ineffective. Splenic CD4+ cells from DerG-PG70-treated mice were diminished in Th1 and Th17 populations but enriched in Th2 and regulatory T (Treg) cells. *In vitro* spleen cell-secreted and serum cytokines from DerG-PG70-treated mice demonstrated a shift from a pro-inflammatory to an anti-inflammatory/regulatory profile. DerG-PG70 peptide tetramers preferentially bound to CD4+ T-cells of GIA spleen cells. We conclude that the DerG-PG70 vaccine (now designated CEL-4000)

exerts its therapeutic effect by interacting with CD4+ cells, which results in an antigen-specific down-modulation of pathogenic T-cell responses in both the PGIA and GIA models of RA. Future studies will need to determine the potential of LEAPS vaccination to provide disease suppression in patients with RA.

Keywords

Therapeutic vaccine; Peptide vaccine; Rheumatoid arthritis; Murine models; Autoimmunity; Immunotherapy; Cytokines

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by chronic inflammation and systemic destruction of the peripheral joints [1–6]. RA is a very heterogeneous disease that may have different initiators and be driven by different types of inflammatory responses for each individual. Although the initiating events of RA are unknown, the disease is maintained by pro-inflammatory mediators produced during T helper (primarily Th1 and Th17) cell-driven autoimmune responses [1].

Current treatment of RA largely focuses on alleviation of symptoms and delaying disease progression [7]. The two primary treatment modalities are synthetic, small molecule disease-modifying anti-rheumatic drugs (sDMARDs), and biologic large molecule DMARDs (bDMARDs) [8]. Both sDMARDs and bDMARDS suppress elements of the entire immune system in order to curtail the inflammatory process.

The pro-inflammatory cytokine interleukin (IL)17 has become a popular target for treating the inflammation associated with RA. IL17-targeting therapies still only treat the symptoms, do not act on the source of the inflammatory pathways, are not disease- or antigen-specific, and may ablate important anti-microbial immune responses [6,9].

Vaccination as an immunotherapy is an alternative to treatment with either sDMARDs or bDMARDs and has the potential to modulate the different cells and cytokines involved in the ongoing autoimmune and inflammatory responses. In contrast to other therapies for RA, therapeutic vaccines focus on the antigen specific disease-driving cells that are upstream of disease presentation to modulate the pro-inflammatory Th1 or Th17 responses or enhance regulatory T-cell (Treg) responses in a beneficial manner [10]. A key advantage of therapeutic vaccination is antigen specificity, which is focused on the disease initiators, but the vaccine must also modulate the aberrant ongoing immune reactions of the patient.

LEAPS[3] (Ligand Epitope Antigen Presentation System) vaccines offer the opportunity to immunize with a disease-related antigen and concurrently modulate the subsequent immune response. LEAPS vaccines are heteroconjugate peptides composed of a disease- specific antigenic epitope and an immune cell binding ligand (ICBL)[10]. The J peptide ICBL is from human β2 microglobulin and acts first on dendritic cells (DC) [11–13] and the DerG

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[3]For related information see Supplementary Information.

peptide from domain 2 of the β chain of the human MHC class II (HLA-DR) molecule acts on CD4+ T cells [14–16].

The LEAPS vaccine CEL-2000 (a conjugate composed of a J ICBL and an immunodominant type II collagen (CII) epitope) was shown to be therapeutic when administered after the onset of disease symptoms in collagen induced arthritis (CIA), a Th17-driven mouse model of RA [17]. The therapeutic effect was accompanied by reduced serum levels of pro-inflammatory cytokines. Other J-LEAPS heteroconjugate vaccines were effective in treating experimental autoimmune myocarditis [18] or viral infections in animal models [11,12,19,20].

Proteoglycan (PG)-induced arthritis (PGIA) and G1 domain (of PG) induced arthritis (GIA) are autoimmune animal models of RA,[3] induced by intraperitoneal (i.p.) immunization of BALB/c female mice with PG of human articular cartilage [21] or the recombinant G1 domain (rhG1) of PG [22]. Both the PGIA and GIA models are predominantly driven by Th1 interferon-gamma (IFNγ) responses [22–25], and generate Th17 as well as other pro- and anti-inflammatory cytokines responses. The PGIA [21,26] and GIA [22] models resemble human RA better than other animal models.

We report here the results of testing J-LEAPS (J-PG70) and DerG-LEAPS (DerG-PG70, also called CEL-4000) vaccines, heteroconjugates with the PG70 peptide,[3] in the PGIA and GIA models of RA. The effects of vaccines on the disease process, joint histopathology, and serum levels of anti-inflammatory and pro-inflammatory cytokines were evaluated. T-cell phenotypes and production of cytokines were also examined *in vitro* using spleen cells from vaccinated and control diseased animals. Binding of vaccine component peptides to different types of immune cells was examined to identify cells likely involved in the response to the vaccines.

2. Materials and methods[3]

2.1. Peptides

The sequences of the peptides used for the *in vivo* and *in vitro* studies were as follows: PG70 (ATEGRVRVNSAYQDK), DerG (DGQEE KAGVVSTGLIGGG), J (DLLKNGERIEKVEGGG), and conjugates DerG-PG70 (DGQEEKAGWSTGLIGGGATEGRVRVNSAYQDK) and J-PG70 (DLLKNGERIEKVEGGGATEGRVRVNSAYQDK). Peptides were purchased from 21st Century Biochemicals (Marlborough, MA) or Ambiopharm (North Augusta, SC) at 95% purity with amino acid sequences and mass confirmed by mass spectrometry. Biotinylated peptides were purchased from Biomatik, Inc. (Wilmington, DE).

2.2. Antigens used for inducing arthritis

Human cartilage PG and rhG1 protein were prepared as described.[3]

2.3. Mice, immunization, and visual assessment of arthritis

Adult (retired breeder) female BALB/c mice were obtained from the National Cancer Institute (NCI; Frederick, MD) or from the former NCI facility acquired by Charles River (Wilmington, MA).

To induce PGIA, mice were immunized i.p. with human PG (100 μg protein in 100 μl sterile phosphate-buffered saline (PBS, pH 7.2)) [21,26] emulsified in dimethyl-dioctadecyl ammonium bromide adjuvant (DDA; Sigma-Aldrich, St Louis, MO) three times, three weeks apart [25]. Mice were inspected for signs of arthritis (swelling and redness) twice a week after the second PG immunization. Upon disease onset, the degree of arthritis for each paw was visually scored every other day on a scale of 0 to 4 for each limb, summing the individual paw scores to a maximum visual arthritis (VA) score of 16 per animal[3] [25,27].

Similarly, groups of mice were immunized with rhG1 (40 μg per injection in DDA) i.p. three times to induce GIA [22]. The limbs of animals with GIA were visually scored for the degree of arthritis 3 times a week as described for PGIA.[3]

2.4. Vaccine treatment of mice with PGIA or GIA

Vaccine treatment was initiated after the mice developed arthritis (PGIA or GIA). Briefly, mice with a similar average VA score in each group (ranging from 2 to 4 in the different studies) were sorted into treatment groups. Doses (100 nmol) of LEAPS conjugates or individual peptides were prepared in 100 μl sterile PBS (pH 7.2) and emulsified at a 1:1 ratio with Montanide ISA-51$_{VG}$ adjuvant (Seppic, Paris, France). The vaccines (or control PBS in adjuvant) were administered s.c. at the nape of the neck into anesthetized mice. The mice were treated first on the day of initial grouping (day 0) and the same dose was administered s.c. on day 14. Monitoring of the VA scores continued three weeks after the second vaccination.[3]

2.5. Collection of blood and tissues from mice

At the end of the experiments, mice were anesthetized, and bled. Serum samples were stored at −70 °C until use. After blood draw, the anesthetized mice were euthanized via CO_2 inhalation. Spleens were harvested under aseptic conditions for *in vitro* studies, and hind limbs excised and fixed in formalin. All animal procedures[3] were approved by the Institutional Animal Care and Use Committee (IACUC) of Rush University Medical Center (IACUC permit number: 14-032).

2.6. Histology

Hind limbs tissue sections were prepared, processed, examined, photographed and scored as described[3] [21,25–27].

2.7. Spleen cell cultures

Single cell suspensions from the spleens of mice with PGIA or GIA were processed as described.[3] The spleen cells were seeded into 48-well culture plates (3×10^6 viable cells per well) in the absence or presence of PG (50 μg/ml) or rhG1 (7.5 μg/ml) for cells from PGIA and GIA mice, respectively, and cultured for 4 days.

2.8. Determination of T-cell phenotypes and cytokine profiles by flow cytometry

The percentages of Th (CD4$^+$) spleen cells containing intracellular cytokines or Foxp3 in cultures of antigen-stimulated spleen cells were determined by flow cytometry [28] on a BD FACS Canto II flow cytometer and analyzed using FACS Diva software (BD Flow Cytometry Systems, San Jose, CA).[3]

2.9. Assays of in vitro cytokine secretion

For cytokine secretion studies, spleen cells were either left untreated (native cultures) or treated with human PG or rhG1 (for PGIA and GIA cells, respectively) as specified above. On the fourth day of culture, the plates were centrifuged, supernatants were collected and frozen at −70 °C. For the PGIA study, the concentrations of cytokines were determined by ELISA (IFNγ, IL17, IL4, IL10 kits (Peprotech, Rocky Hill, NJ) and transforming growth factor (TGFβ1; R & D Systems, Minneapolis, MN), according to the manufacturers' instructions. For the GIA studies, multiple cytokines were measured using the multi-plex mouse Th17 kit (IL1β, IL2, IL4, IL6, IL10, IL12p70, IL17A, IL17F, IFNγ, TNFα) or a single-plex kit for TGFβ1 (both from EMD Millipore, Billerica, MA), according to the manufacturer's instructions and analyzed using a MagPix® reader with MilliPlex Analyst software (both from EMD Millipore).[3]

2.10. Serum cytokine assays

Cytokine levels in the serum samples of GIA mice were measured using the MagPix® technology as described above for cell culture supernatants.

2.11. Generation of bone marrow-derived dendritic cells (BM-DCs)

BM was obtained from naïve mice and was enriched in DCs as described previously [28,29]. DCs (MHC class II+ CD11c+ cells) constituted over 60% of the collected cell population, as determined by flow cytometry [29].[3]

2.12. Assay of peptide tetramer binding to cells

The avidity of peptide binding was increased by tetramerization of biotinylated (B)-peptides with streptavidin-phycoerythrin (SA-PE) according to a protein tetramerization protocol provided by the National Institutes of Health (NIH) Tetramer Core Facility (Emory University, Atlanta, GA). Peptide binding to cells was detected by flow cytometry as described.[3]

2.13. Statistical analysis

The GraphPad Prism 6 software (GraphPad, La Jolla, CA) was used for both statistical analysis and plotting of data. Results are presented as mean ± SEM. The statistical tests used as well as other details are provided in the Figure Captions and in the footnote to Table 1. *P* values less than 0.05 were accepted as statistically significant (Material handling and data collection details as described).[3]

3. Results

3.1. DerG-PG70 LEAPS vaccine limits arthritis progression in vivo

3.1.1. Visual arthritis (VA) scores—The therapeutic activities of LEAPS vaccines containing Der-G-PG70 or J-PG70 peptide conjugates (emulsified with Seppic ISA-51$_{VG}$ adjuvant) were compared to controls containing vehicle (PBS with or without adjuvant), in the PGIA (Fig. 1A: Study 1) and GIA (Fig. 1B: Study 2, and Fig. 1C: Study 3) models of RA. In Study 3 (Fig. 1C), additional controls of peptides PG70 and DerG in adjuvant as well as PBS without adjuvant were tested. As indicated by the VA scores, treatment with the DerG-PG70 vaccine significantly (*$p < 0.05$) limited disease progression within 3 weeks in all three studies whereas J-PG70 or individual peptides had no significant effects as compared with the PBS/adjuvant or PBS control (Fig. 1). Importantly, the DerG-PG70 vaccine effectively curtailed arthritis symptoms in GIA (Fig. 1B and 1C) despite its more aggressive disease course compared to PGIA (Fig. 1A).

3.1.2. Histopathological changes—Histopathological changes in the joints, induced by autoimmune inflammatory damage, or as modulated by vaccine treatment, were evaluated in comparison with normal joints of naïve mice (see Supplementary Figs. 1 and 2).[3]

Table 1 shows the individual and cumulative histopathology scores of the limbs of GIA mice (Study 2 and Study 3) after the animals underwent the *in vivo* treatments. In Study 2, mice treated with DerG-PG70 in adjuvant had significantly (*$p < 0.05$) reduced pannus formation and bone resorption as well as lower cumulative histopathology scores as compared to controls treated with PBS in adjuvant. In Study 3, DerG-PG70-treated animals demonstrated significantly reduced degree of inflammation, cartilage damage, and cumulative histopathology score, while J-PG70-treated mice generally had greater joint damage than PBS/adjuvant-treated controls. While the effect of DerG-PG70 on reduction of inflammation, pannus formation, cartilage damage, bone resorption, periosteal bone formation, or summed scores did not always reach the level of statistical significance in Studies 2 and 3, a protective effect was indicated by strong reductions in these parameters in both studies (Table 1). The *in vivo* VA scores (Fig. 1) and joint histopathology (Table 1 and Supplementary Figs. 1 and 2) indicate that DerG-PG70 treatment of PGIA and GIA mice had a consistent and reproducible therapeutic effect.

3.2. T-cell phenotypes of splenocytes from vaccine treated PGIA and GIA mice

The phenotypes of CD4$^+$ T-cells within the spleen cell cultures of the treatment groups of PGIA and GIA mice were determined by flow cytometric evaluation of intracellular cytokines IFNγ (Th1), IL17 (Th17), or IL10, or the Treg cell marker, Foxp3, following *in vitro* stimulation with antigen (PG or rhG1). Treatment of PGIA (Fig. 2A) or GIA (Fig. 2B) mice with the DerG-PG70 vaccine led to a reduction of pro-inflammatory Th1 and Th17 cells (even after antigen stimulation) and an increase in the frequency of anti-inflammatory IL10-producing as well as protective Treg cells in both models.

3.3. In vitro secretion of cytokines by splenocytes from vaccine-treated PGIA and GIA mice

Cytokine production from cell cultures prepared from the spleens of vaccinated mice were measured to evaluate ongoing or antigen-induced responses. Cytokine production by spleen cells receiving no additional *in vitro* antigen stimulation represent cellular responses resulting from the combination of the effects of disease and vaccine treatments on the mice, whereas cytokine production following *in vitro* antigen stimulation represents additional antigen-specific responses resulting from the combined effects of disease, vaccination, and *in vitro* antigen treatment. We specifically focused on the ratios of anti-inflammatory (IL4 or IL10) to pro-inflammatory (IFNγ or IL17) T-cell cytokines in the spleen cell cultures of PGIA and GIA mice (Fig. 3) as calculated from the concentrations of secreted cytokines (Supplementary Fig. 3).[3] In the PGIA study, these ratios were significantly increased in favor of anti-inflammatory cytokine release in both the non-stimulated (Fig. 3A) and PG-stimulated (Fig. 3B) spleen cell cultures of DerG-PG70-treated mice as compared to PBS/adjuvant-treated controls. In the GIA study, the IL4:IL17(A+F) ratio was significantly increased in the *in vitro* non-stimulated (Fig. 3C), but not in the rhG1-stimulated cultures (Fig. 3C). These ratios reflect the reductions in pro-inflammatory cytokines (IFNγ and IL17(A+F)) and increases in anti-inflammatory cytokines (IL4, TGFβ, and IL10) due to DerG-PG70 vaccine treatment, as compared to control treatment in all the PGIA cultures, although the differences did not always reach significance (Supplementary Figs. 3A and 3B).[3] In the cell cultures from GIA mice, DerG-PG70 vaccination resulted in significant reductions in the production of IL17(A + F) and IFNγ, but other cytokines were affected to a lesser degree (Supplementary Fig. 3C and 3D).[3]

Differences in the results between the PGIA and GIA spleen cell cytokine secretion studies may reflect differences in the detection methods (i.e., ELISA versus MagPix® with the latter being more sensitive than the former), but it is more likely that they reflect differences between the two animal models [22]. The relative inability of the DerG-PG70 vaccine to suppress IFNγ secretion by native or rhG1-stimulated spleen cells from GIA mice as opposed to a suppressive effect on IFNγ production by PGIA spleen cells might be due to the overproduction of IFNγ in the GIA model compared to the PGIA model, as demonstrated here and as reported previously [22]. Ultimately, the pro-inflammatory response was reduced by DerG-PG70 treatment in both models.

3.4. Serum levels of cytokines

Concentrations of 10 different cytokines (IL4, TNFa, IFNγ, IL2, IL1β, IL6, IL10, IL12p70, IL17A, and IL17F) in serum samples from LEAPS-vaccinated and control GIA mice (Studies 2 and 3) were evaluated as an additional indication of the action of DerG-PG70 on the immune and inflammatory processes. In both GIA studies, IL17F serum levels were significantly reduced in DerG-PG70-treated mice as compared to the PBS/adjuvant-treated controls, but the concentrations of other cytokines were comparable among the treatment groups (Supplementary Fig. 4). Ratios of anti-inflammatory T-cell cytokines (IL4 and IL10) to pro-inflammatory T-cell cytokines (IL17(A + F) and IFNγ) in serum from DerG-PG70 treated mice were significantly increased in favor of anti-inflammatory responses (Fig. 4A and B).

3.5. Binding of LEAPS conjugates and related individual peptides to immune cells in vitro

The binding of tetramers of DerG-PG70, J-PG70, and their component peptides to spleen cells from GIA mice and BM-derived DCs from normal mice was examined *in vitro* to identify the cell types that can be affected by treatment. *SA-PE-B-Peptide* tetramers were prepared to increase the avidity and add fluorescence to the peptide ligands to facilitate analysis of their binding to cells. Tetramers were formed with SA-PE using a method developed for detecting the binding of MHC-peptide complexes to T-cells by flow cytometry [30]. However, as the LEAPS heteroconjugate peptides contained a cell binding ligand (either DerG or J), addition of MHC was not necessary. Although the DerG-PG70 tetramer bound to CD4+ T-cells (Fig. 5A, top) better than the other ligands, binding specificity to the other cell types was relatively difficult to discern. Specifically, the binding patterns to DCs were obscured by the high background fluorescence (e.g., SA-PE alone), possibly due to phagocytic activity of DCs [31] (Fig. 5A, bottom). Therefore, the net mean fluorescence intensity levels (above the SA-PE background) were calculated as a measure of specific peptide binding to cells (Fig. 5B). By comparing the binding of DerG-PG70 and J-PG70, two similar-sized peptides differing in the ICBL, the binding preference of the DerG-PG70 tetramer for CD4+ T-cells was evident. Similarly, the binding preference of J-PG70 for MHC II-bearing cells (APCs) and CD11c+ BM-DCs became evident. Tetramers containing the individual peptides (PG70, DerG or J) bound very weakly to all of the cell types examined (Fig. 5B).

4. Discussion

Since Th1-dominated diseases might benefit from a shift away from the pro-inflammatory Th1 to a more balanced Th2 or Treg response, our hypothesis was that treatment with a Th2-promoting DerG LEAPS heteroconjugate incorporating PG70 would initiate an antigen specific response to ameliorate the arthritis symptoms, while a Th1-promoting J-LEAPS heteroconjugate with PG70 would either have no effect or exacerbate disease. Indeed, the DerG-PG70 vaccine reproducibly curtailed the progression of disease in PGIA and two independent GIA studies as indicated by the VA scores of *in vivo* monitored animals and joint histopathology. As expected, treatment with the J-PG70 vaccine was either ineffective or slightly exacerbated disease in these models. Although the outcomes in the two disease systems were similar, there were differences in the kinetics of both disease development and vaccine effects between PGIA and GIA.

As in RA, the inflammatory disease in both the PGIA and GIA models is driven and sustained primarily by Th1 but also by Th17 cells and cytokines produced by them. DerG-PG70 treatment promoted more balanced, less inflammatory cytokine responses by increasing Th2 (IL4+ and IL10+) and Treg (Foxp3+ and TGFβ1+) cells and reducing Th1 and Th17 cells in PGIA, and to a somewhat lesser extent in the GIA system, as represented by the T-cell phenotypes from the treated animals. The switch from a pro-inflammatory T-cell population to one favoring regulation and modulation by DerG-PG70 treatment in the spleen is likely to reflect the systemic changes seen in the curtailed progression of disease.

Although showing similar trends, the immune responses driving disease and subsequently, the responses to DerG-PG70 and J-PG70 for the PGIA and GIA models were subtly

different. There is more robust production of IFNγ in the GIA model compared to the PGIA model, as seen herein and an earlier study [22]. In both models, the therapeutic effect of DerG-PG70 treatment was reflected in the reduction in IL17 production and the proportion of Th17 cells, critical players in RA pathogenesis. The reduction in these pro-inflammatory responses was likely due to increases in Th2 and/or Treg activity.

The ICBL component of a LEAPS vaccine attached to the disease related antigenic peptide promotes interaction with immune cells and hence directs the subsequent immune response. Using the SA-PE-peptide tetramers it was possible to demonstrate that the DerG-PG70 conjugate preferentially bound to splenic CD4+ Th (or Treg) cells, whereas J-PG70 showed preference for binding to MHC II-bearing APCs and CD11c+ DCs, known to be involved in PGIA [32]. Preference for CD4+ T-cells is in keeping with previous studies for the DerG ICBL peptide [10,13–16,33,34], and preference for APCs for the J ICBL, is consistent with its ability to promote the maturation of DCs [11,12,20]. The binding studies suggest that the DerG-PG70 conjugate modulates T-cell responses via direct binding to CD4+ T-cells in the PGIA and GIA mice.

The ability of DerG-PG70 to steer T-cells away from pro-inflammatory responses and promote anti-inflammatory/regulatory activities explains the therapeutic success of DerG-PG70 in restricting the progression of disease in the treated PGIA and GIA mice. Unlike cytokine ablation [23,35], which also suppresses PGIA, DerG-PG70 appears to act directly on the T-cell drivers of the immune response rather than the subsequently elicited arthritis related cytokines to ameliorate disease progression. Focusing the therapy on the inflammatory disease-promoting immune responses in an antigen-specific manner with LEAPS vaccines appears to bring the system to an appropriate balance, presumably without loss of important immune protections.

RA is different for different patients and failure to respond to certain therapies may reflect differences in the initiators and responses driving their disease. In this study, the DerG-PG70 vaccine was effective in curtailing progression of arthritis driven by Th1 responses whereas a collagen peptide-containing J-LEAPS vaccine, CEL-2000, was effective in blocking the progression of arthritis in the Th17-driven CIA model [17]. As such, knowledge of signature T-cell cytokine phenotypes that drive the patient's disease can facilitate the choice of the appropriate LEAPS vaccine therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.05.009.

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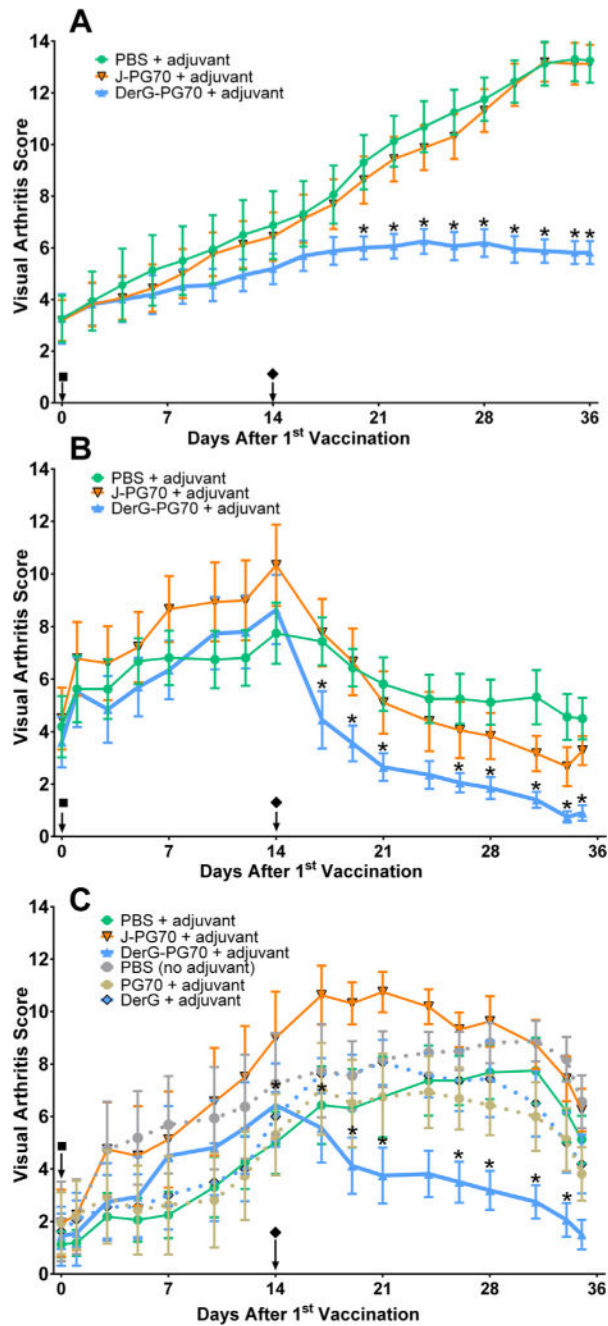
Fig. 1.
Visual arthritis (VA) scores of mice with PGIA or GIA treated with LEAPS conjugate vaccines or control agents. VA scores of mice in the (A) initial PGIA study (Study 1), (B) initial GIA study(Study 2) and (C) confirmatory GIA study (Study 3)with additional control groups (dotted lines). VA Scoring: sum of value for each paw: 0 = no evidence of inflammation, 1 = slight swelling of the paw or at least 3 finger joints, 2 = moderate swelling of the paw and fingers, 3 = moderate swelling of the paw, fingers, and ankle/wrist joints, 4 = severe swelling and redness of paw, fingers, and ankle/wrist joints, summing the individual paw scores to a maximum VA score of 16 per animal [34–38]. Groups of mice (n = 8–9

mice per group) with VA scores averaging ~3 in each group were injected s.c. on day 0 (black arrows with squares) and day 14 (black arrows with diamonds) with the following: (A) and (B) PBS emulsified with Seppic ISA-51VG adjuvant (solid green lines, vehicle control), J-PG70 in PBS + adjuvant (solid orange lines), or DerG-PG70 in PBS + adjuvant (solid blue lines). (C) Groups treated as shown in (A) and (B) and the three additional control groups were treated with PBS (no adjuvant) (dotted gray line), PG70in PBS + adjuvant (dotted brown line) or DerG in PBS + adjuvant (dotted blue line). Data are expressed as the mean ± SEM. Asterisks depict statistically significant (*$p < 0.05$) differences between the DerG-PG70-treated and PBS + adjuvant) control groups. Data were analyzed using (two-way repeated measures analysis of variance ANOVA with post hoc Fisher's least significant differences (LSD) test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
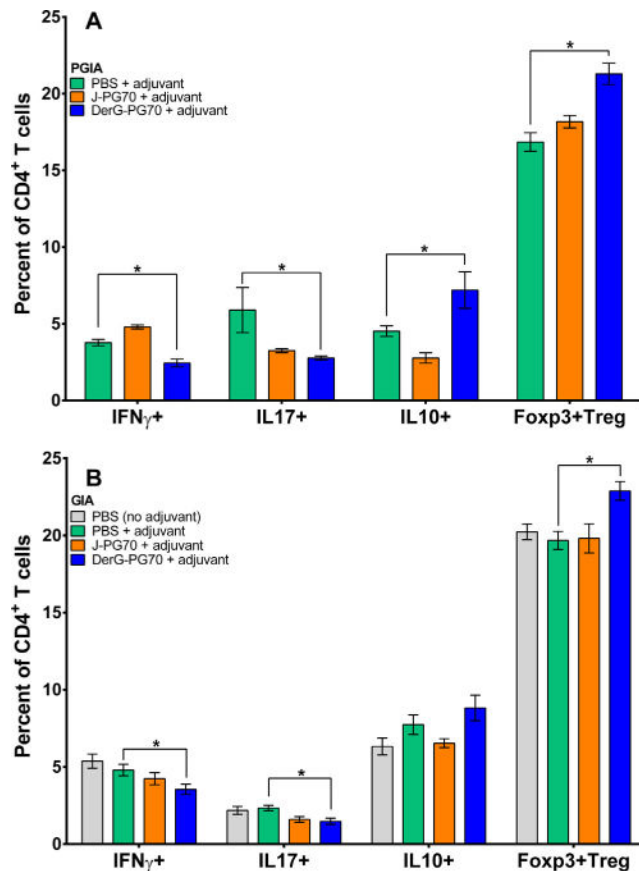
Fig. 2.
Effects of LEAPS vaccines on the phenotype of cultured CD4+ T-cell populations from the spleens of mice with (A) PGIA or (B) GIA. Percentages of Th1 (IFNγ+), Th17 (IL17+), Th2/Treg (IL10+), and CD25+ Tregs (Foxp3+) in PG-stimulated cultures of spleen cells from control or LEAPS-vaccine treated PGIA or GIA mice as determined by intracellular cytokine or Foxp3 staining and flow cytometry. For culture conditions see Materials and Methods. Data shown are the mean ± SEM of the percent of cytokine-expressing CD4+ T-cell populations in the cultures from the 3 groups of PGIA mice (spleen cells from n = 4 per group) and GIA mice (n = 8 per group). Data were analyzed in both (A) and (B) using one-way ANOVA with Fisher's LSD test. Asterisks depict statistically significant differences (*p < 0.05).
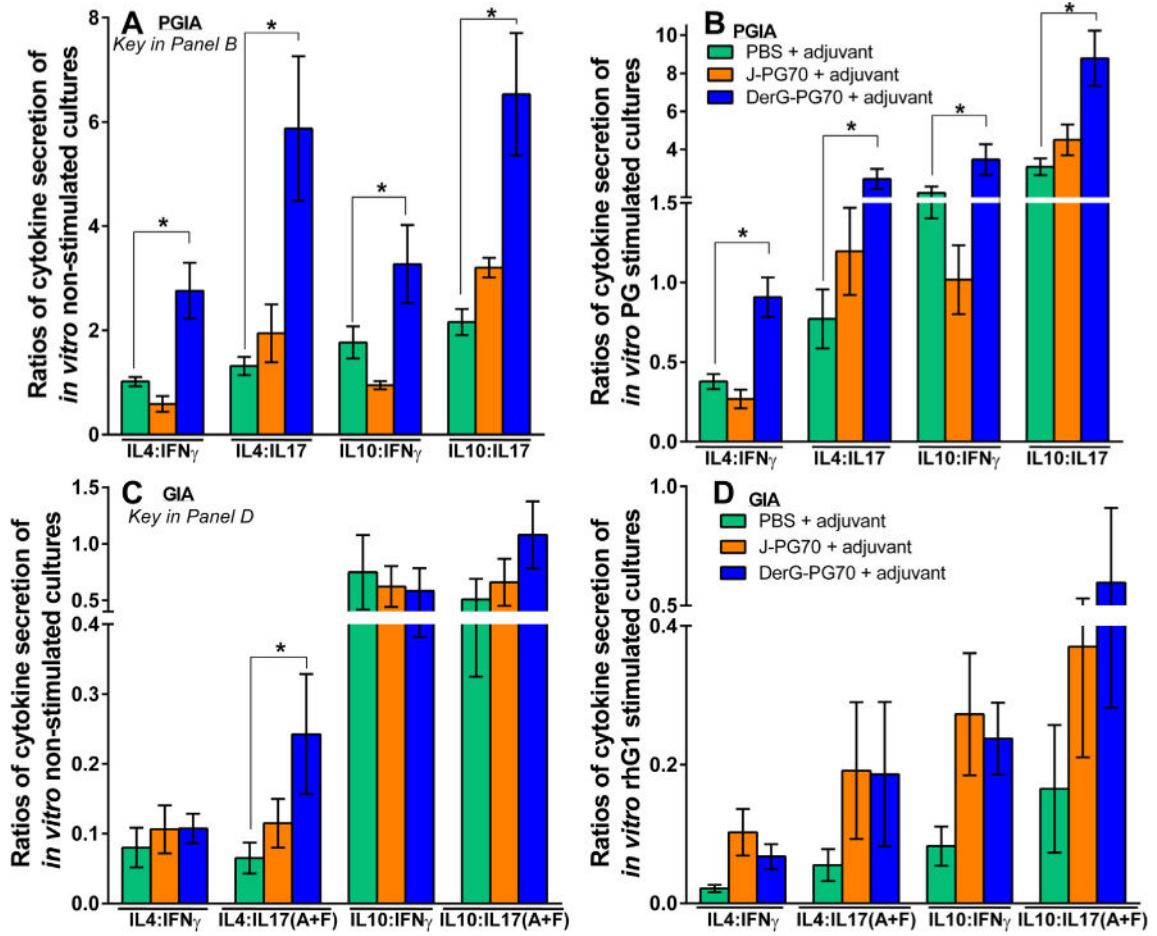
Fig. 3.

Ratios of key anti-inflammatory to pro-inflammatory T-cell cytokines in *in vitro* cultures of spleen cells from control and LEAPS vaccine-treated PGIA (A and B) and GIA (C and D) mice. Ratios of secreted anti-inflammatory (IL4 or IL10) to pro-inflammatory (IL17 or IFNγ) cytokines detected in the supernatants of spleen cells from mice with PGIA cultured in the absence of antigen (A) (*in vitro* non-stimulated cultures), or (B) in the presence of PG antigen (*in vitro* PG-stimulated cultures). The ratios of the same cytokines in the supernatants of spleen cells from GIA mice cultured in the absence of antigen (C) (*in vitro* non-stimulated cultures) or (D) in the presence of rhG1 antigen (in *vitro* rhG1-stimulated cultures) (see Supplementary Fig. 3[1] for cytokine concentrations). Data were analyzed with one-wayANOVA and pairwise comparisons between the control and LEAPS-treated groups were made using Fisher's LSD test. Asterisks depict statistically significant differences (*$p < 0.05$).
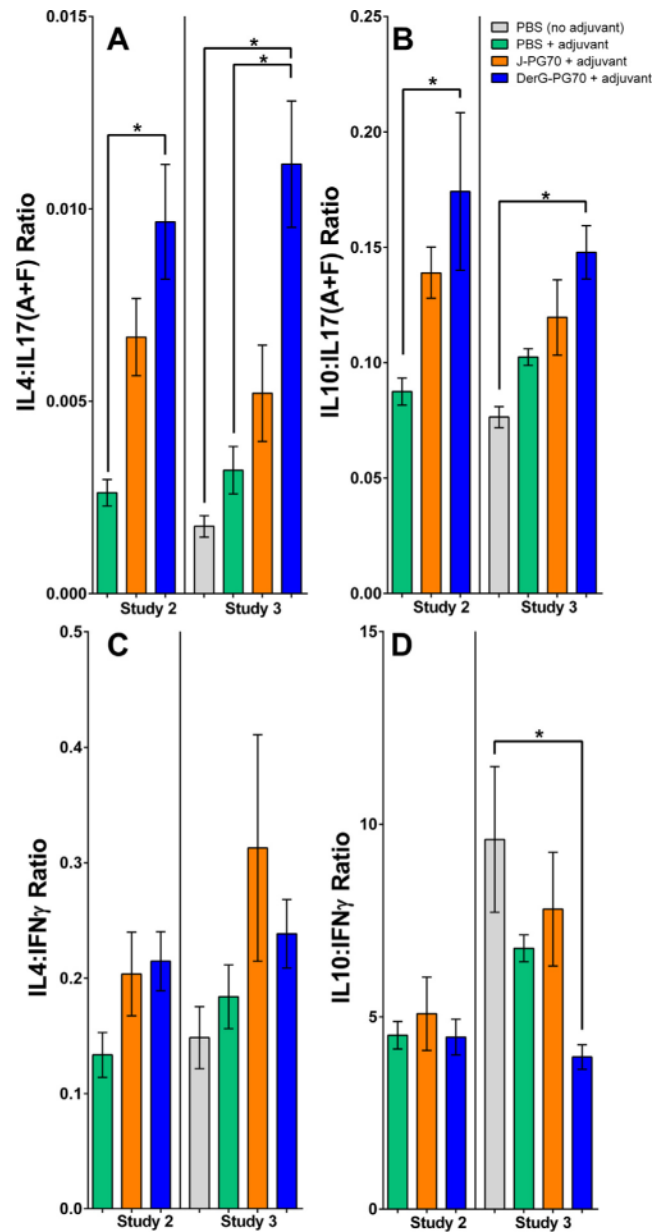
Fig. 4.
Ratios of key anti-inflammatory to pro-inflammatory cytokines in serum samples collected from control and LEAPS-treated GIA mice. Ratio of anti-inflammatory IL4 (A) or IL10 (B) to pro-inflammatory IL17(A + F) in the sera of GIA mice at the end of studies. Ratio of anti-inflammatory IL4 (C) or IL10 (D) to pro-inflammatory IFNγ in the sera of the same mice. Cytokine concentrations were determined using a multiplex Luminex MagPix® kit (serum concentrations of 10 different cytokines are shown in Supplementary Fig. 4).[3] Results are expressed as the mean $\pm$ SEM of cytokine ratios (n = 8 mice in Study 2 and n = 9 mice in Study 3 per group). Data analysis was performed using one-way ANOVA followed by Fisher's LSD test. Asterisks indicate statistically significant differences (*$p < 0.05$).
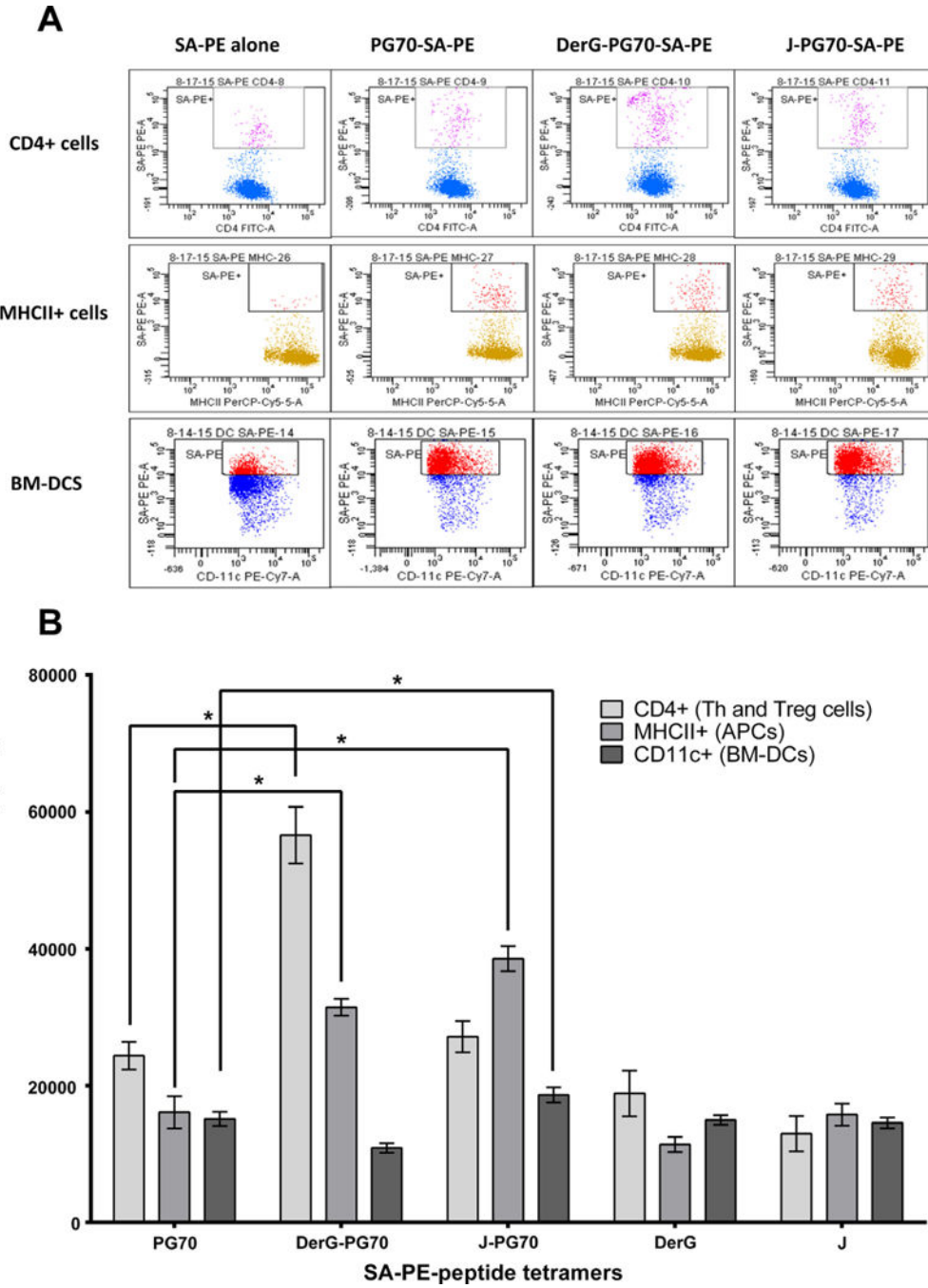
Fig. 5.
Binding of LEAPS conjugates or individual peptides to cells *in vitro* as determined by flow cytometry. (A) Representative flow cytometry panels of streptavidin-phycoerythrin (SA-PE)-biotinylated (B)-peptide tetramer binding to splenic CD4+ T-cells or APCs from GIA mice or to BM-DCs from naïve mice. Cells binding (SA-PE)-B-peptide tetramer are shown as dots in the box within each of the flow panel frames. (B) Summary of (SA-PE)-B-peptide tetramer binding flow cytometry data using GIA spleen cells (n = 8 mice) or BM-DCs from naïve mice (n = 6). Results are expressed as the means ± SEM of net mean fluorescence

intensity (net MFI: total MFI minus SA-PE alone background MFI). For statistical analysis, data were transformed ($Y = Y^2$) and then analyzed using one-way ANOVA followed by Dunnett's test for multiple comparisons. Asterisks indicate statistically significant differences (*$p < 0.05$).

Table 1

Histopathology scores of the hind limbs of mice with GIA following *in vivo* treatment with LEAPS peptide vaccines or control agents.[1]

	GIA (Study 2)[2]			GIA (Study 3)[2]					
	PBS + adj.	J-PG70 + adj.	DerG-PG70 + adj.	PBS + adj.	J-PG70 + adj.	DerG-PG70 + adj.	PG70 + adj.	DerG + adj.	PBS
Inflammation	4.70 ± 0.1	4.00 ± 0.9	3.45 ± 0.6	4.56 ± 0.2	5.00 ± 0.0	2.97 ± 0.6*,#	3.72 ± 0.6	4.88 ± 0.1	4.50 ± 0.2
Pannus formation	1.00 ± 0.0	0.80 ± 0.2	0.65 ± 0.1*	1.38 ± 0.2	2.13 ± 0.2	0.81 ± 0.2	1.13 ± 0.3	1.56 ± 0.1	1.38 ± 0.2
Cartilage damage	4.60 ± 0.2	3.95 ± 0.9	3.45 ± 0.6	3.84 ± 0.4	4.94 ± 0.1	2.34 ± 0.5#	3.41 ± 0.7	4.88 ± 0.1	4.13 ± 0.4
Bone resorption	1.00 ± 0.0	0.80 ± 0.2	0.65 ± 0.1*	1.38 ± 0.2	2.06 ± 0.2	0.78 ± 0.2	1.13 ± 0.3	1.56 ± 0.1	1.38 ± 0.2
Periosteal bone formation	2.10 ± 0.0	2.10 ± 0.4	1.70 ± 0.3	1.94 ± 0.2	2.06 ± 0.1	1.19 ± 0.3	1.88 ± 0.4	2.06 ± 0.1	2.19 ± 0.2
Cumulative scores	13.40 ± 0.2	11.65 ± 2.6	9.90 ± 1.6	13.09 ± 1.0	16.13 ± 0.5	8.09 ± 1.8#	11.25 ± 2.1	14.94 ± 0.2	13.09 ± 1.0

[1] Hind limbs of mice with rhG1-induced arthritis (GIA), treated with either LEAPS peptide vaccines in adjuvant (+adj.) or control agents, were subjected to joint histopathology evaluation as described in the Materials and Methods. Scores of individual histopathology parameters (inflammation, pannus formation, etc.) and cumulative scores identified as italized values are listed.

[2] Values are the means ± SEM; n = 5 mice/group (Study 2) and n = 8 mice/group (Study 3). Data were transformed ($Y = Y^2$) before statistical analysis using one-way ANOVA followed by Fisher's LSD test (Study 2) or by Dunnett's test (Study 3) for multiple comparisons. The lowest individual and cumulative scores were noted in mice treated with DerG-PG70 LEAPS vaccine in both studies. Statistically significant differences are indicated as:

*
$p < 0.05$ (DerG-PG70 + adj. vs. PBS + adj. control).

#
$p < 0.05$ (DerG-PG70 + adj. vs. PBS control).