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Tolerizing DNA vaccines for autoimmune arthritis

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Abstract

Current therapies for rheumatoid arthritis (RA) and other autoimmune diseases non-specifically suppress immune function, and there is great need for fundamental approaches such as antigen-specific tolerizing therapy. In this paper we describe development of antigen-specific tolerizing DNA vaccines to treat collagen-induced arthritis (CIA) in mice, and use of protein microarrays to monitor response to therapy and to identify potential additional autoimmune targets for next generation vaccines. We demonstrate that tolerizing DNA vaccines encoding type II collagen (CII) reduced the incidence and severity of CIA. Atorvastatin, a statin drug found to reduce the severity of autoimmunity, potentiated the effect of DNA vaccines encoding CII. Analysis of cytokines produced by collagen-reactive T cells derived from mice receiving tolerizing DNA encoding CII, as compared to control vaccines, revealed reduced production of the pro-inflammatory cytokines IFN-γ and TNF-α. Arthritis microarray analysis demonstrated reduced spreading of autoantibody responses in mice treated with DNA encoding CII. The development of tolerizing DNA vaccines, and the use of antibody profiling to guide design of and to monitor therapeutic responses to such vaccines, represents a promising approach for the treatment of RA and other autoimmune diseases.

Keywords: *Rheumatoid arthritis, DNA vaccine, proteomics, autoantibodies*

Introduction

Rheumatoid arthritis (RA) is an autoimmune synovitis affecting over 0.5% of the world population. Current therapies for RA include methotrexate, TNF-α-antagonists, CTLA4-Ig, anti-CD20, and corticosteroids, all of which non-specifically suppress or modulate immune function and thereby make patients more susceptible to infectious complications. The objective of antigen-specific therapy is to specifically attenuate autoreactive T and B cell responses, leaving intact global immune function. In this manuscript we describe development of antigen-specific tolerizing DNA vaccines to treat collagen-induced arthritis (CIA), a rodent model for RA, and use of synovial protein microarrays to monitor responses to tolerizing therapy and to identify additional autoimmune targets for development of next generation vaccines.

DNA immunization provides a non-replicating transcription unit that serves as a template for the synthesis of proteins or protein segments to induce antigen specific immune responses in the host. Injection of DNA encoding foreign antigens has been demonstrated to promote immunity against a variety of microbes and tumors [1–3]. In contrast, in autoimmune diseases DNA vaccines induce tolerance to the DNA-encoded self-antigens [4–9].

In experimental autoimmune encephalomyelitis (EAE), a Th1-mediated mouse model for multiple sclerosis, disease was prevented by DNA vaccination with a minigene encoding the dominant epitope of proteolipid autoantigen alone or in combination with

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interleukin-4 (IL-4) DNA [5,6]. Further, combination therapy with IL-4 DNA plus a DNA vaccine encoding whole myelin oligodendrocyte glycoprotein alone or a DNA vaccine cocktail encoding four myelin protein autoantigens successfully treated active EAE [6,10,11]. In EAE, tolerizing DNA vaccine therapy reduced autoantigen-specific T cell proliferative responses, and was associated with modulation of cytokine responses toward production of Th2 cytokines as well as with reduced epitope spreading of autoreactive B cell responses.

Atorvastatin, a 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor well-known for its lipid lowering properties, has also been shown to suppress the proinflammatory response [12]. Atorvastatin has provided efficacy in EAE [13,14] and CIA [15]. Further, based on its Th2-promoting properties, atorvastatin has been used as an adjuvant to enhance the efficacy of glatiramer acetate in treating EAE [16].

Our data demonstrating that tolerizing DNA vaccines provide efficacy in EAE and the non-obese diabetic (NOD) model for autoimmune diabetes [7–9,17–19], together with data demonstrating that DNA vaccines encoding heat shock proteins protect rats against adjuvant-induced arthritis [20,21], form the rationale for the development of antigen specific tolerizing DNA vaccines for CIA. CIA is induced by injecting genetically susceptible strains of mice with CII emulsified in complete Freund's adjuvant (CFA). The resulting severe polyarticular arthritis is characterized by synovitis and erosion of cartilage and bone that histologically resemble RA [22]. CII is the major constituent protein of cartilage in diarthrodial joints, the predominant site of inflammation in RA [23]. Although significant evidence suggests that CII is not a primary autoantigen in the majority of RA patients, both anti-CII IgG and anti-CII IgG-producing B cells have been detected in rheumatoid cartilage and synovium, and circulating autoantibodies to native and denatured CII have been detected in sera [24–26].

In this paper, we demonstrate that a tolerizing DNA vaccine encoding whole CII reduced the incidence and severity of CIA. Moreover, the protective effect of tolerizing DNA vaccine therapy was potentiated by co-administering CII DNA with a DNA vaccine encoding IL-4 or the small molecule drug atorvastatin. We further demonstrate that protein array profiling of autoantibody responses can be utilized to monitor responses to the tolerizing DNA vaccination therapies.

Materials and methods

Mice

Male DBA1/J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and were between 6 and 7 weeks of age when experiments were initiated. Each treatment group consisted of 20 mice. All animal protocols were approved by the Department of Comparative Medicine at Stanford University and the Committee of Animal Research at Stanford University, in accordance with the National Institutes of Health guidelines.

Construction of DNA vaccines

The mouse collagen type II gene was amplified by PCR from spleen cDNA (Clontech, Mountain View, CA, USA) by use of the following PCR primer pairs: 5′-TCGCGGTGAG CCATGATCCG CCTCGGG-GCTC-3′ and 5′-GGGTTTTACA AGAAGCAGAC AGGCCCTATG-3′ and cloned into the target CMV promoter-driven mammalian expression vector pTARGET (Promega, Madison, WI, USA). DNA plasmids were grown in the *E. coli* strain JM-109 (Promega), and plasmid DNA purification was performed using Qiagen Endo-Free Giga Prep kits (Qiagen, Valencia, CA, USA) and confirmed as previously described [6].

CIA induction

DBA1/J male mice were immunized intradermally at the tail base with 0.2 mg bovine Type II collagen (CII) in 0.05 M acetic acid (immunization grade, Chondrex, Redmond, WA, USA) emulsified with an equal volume of CFA, consisting of incomplete Freund's adjuvant (IFA, Sigma, St Louis, MO, USA) and 0.5 mg heat-inactivated *Mycobacterium tuberculosis* (strain H37 RA; Difco Laboratories, Detroit, MI, USA). Twenty-one days later, the mice were boosted subcutaneously at the base of the tail with a second injection of bovine CII in IFA. Animals were scored every 2–3 days for indications of paw inflammation using the following scale: grade 0, no erythema or swelling; grade 1, erythema and mild swelling extending from the ankle to the midfoot; grade 2, erythema and moderate swelling extending from the ankle to the metatarsal joint; grade 3, erythema and severe swelling encompassing the ankle, foot and digits. Each paw was graded and the four scores were totalled so that the maximal possible score per mouse was 12 [27].

DNA vaccination and atorvastatin treatment regimen

Naïve mice were injected in both quadriceps with a total of 0.1 ml of 0.25% bupivacaine–HCL (Sigma, St Louis, MO, USA) in PBS (Gibco #9236, Invitrogen, Carlsbad, CA, USA) on day zero. Two days later, naïve mice were injected intramuscularly in the quadriceps with a total of 0.1 mg of DNA, in a total volume of 0.1 ml PBS per quadricep. Control mice were treated with PBS alone. Mice were vaccinated again on day nine. Mice were induced with CIA on day 16 and vaccinated a third time on day 23. Atorvastatin

(Pfizer, New York, NY, USA) was administered orally as a 10 mg/kg dose suspension in 0.5 ml PBS once daily using 20 mm feeding needles (Popper and Sons, New Hyde Park, NY, USA). Control mice were administered PBS alone. Treatment with atorvastatin or PBS was initiated on the same day as the first DNA vaccination injection (day 2).

Proliferation assay

About 1×10^6 bulk splenocytes were cultured in a 96-well microtiter plate and stimulated with 0.1 mg/ml denatured whole bovine CII (Chondrex) in triplicate wells for 72 h. Enriched tissue culture media for the assay consisted of RPMI 1640 supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-mercaptoethanol (5×10^{-5} M), and 10% fetal bovine serum. Wells were pulsed with 1 μCi[^{3}H]TdR (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) for the final 16 h of culture, and incorporated radioactivity was measured using a betaplate scintillation counter.

Cytokine analysis

Bulk splenocytes were incubated in enriched RPMI-1640 and 0.1 mg/ml denatured whole bovine CII. After 72 h of culture, the supernatants were collected and assayed in triplicate for the production of IFN-γ and TNF-α by sandwich ELISA using commercial ELISA kits (BD PharMingen, San Diego, CA, USA).

Array data analysis

Synovial arrays and the associated methods used in this work were previously described in detail [28]. The 1536-feature synovial antigen arrays contain approximately 225 antigens including proteins and sets of overlapping peptides representing antigens relevant to RA. Antigen arrays were produced using a robotic microarrayer to attach candidate RA and control peptides and proteins to ArrayIt SuperEpoxy microscope slides (TeleChem International, Sunnyvale, CA, USA). Arrays were probed with 1:200 dilutions of serum from individual mice. Reactive antibodies were detected using Cy3-conjugated goat-anti-mouse IgG/IgM (1:4000 dilution, Jackson Immunoresearch) prior to scanning. GenePix Pro 5.0 software (Axon Instruments) was used to determine the net median pixel intensities for each antigen feature. Data analysis was performed using Significance Analysis of Microarrays (SAM) software (http://www-stat-class.stanford.edu/SAM/servlet/SAMServlet) to identify antigen features with statistically significant differences in reactivities between the treatment groups. The reported antigen lists are SAM-identified features with false discovery rates (q-values) $<4.8\%$ for Figure 3C, and $<3.4\%$ for Figure 3D. The displayed SAM-identified antigen features were further selected using a numerator threshold of 1.0. Cluster software was then used to hierarchically group the mice and antigen features based on a pairwise similarity function, and TreeView software was used to display the data as a heat map ([29], http://rana.lbl.gov/EisenSoftware.htm).

Pathology

Mice were euthanized and both hind limbs were dissected, formalin fixed and decalcified. The samples were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin according to standard procedures. Representative sections are shown at 4× and 20× magnifications.

Results

A DNA vaccine encoding type II collagen (CII) prevents CIA

To determine whether a DNA vaccine encoding CII could prevent CIA, the full-length cDNA encoding type II collagen (CII) was isolated using PCR, cloned into the mammalian expression vector pTARGET, and the clone confirmed by DNA sequencing. Groups of 20 DBA/1 mice were treated with 50 μg CII DNA 14 and 7 days prior to induction of CIA with CII emulsified in CFA, and a third CII DNA dose delivered 7 days following induction. Mice were boosted with CII emulsified in IFA 21 days following induction, and monitored for the development of inflammatory arthritis using the visual scoring system. DNA encoding CII reduced the disease severity (mean clinical score; $p < 0.05$ by Mann–Whitney) and disease incidence of CIA to 50% as compared to the PBS-treated control group in which the disease was more severe and the disease incidence was 89%. Treatment of CIA mice with CII DNA in combination with IL-4 DNA demonstrated a trend toward a further reduction in disease severity and incidence (45%) (Figure 1A,B). Treatment with control vector + IL-4 did not impact disease severity as compared to PBS treated control mice with CIA, although this group did exhibited a lower disease incidence of 63%.

Atorvastatin potentiates the efficacy of DNA encoding CII

Treatment with oral atorvastatin 10 mg/kg/day also protected against disease severity and reduced disease incidence to 40%, compared to 89% in control PBS treated mice. As compared to tolerizing DNA encoding CII alone or atorvastatin alone, co-treatment with CII DNA plus daily atorvastatin further reduced disease severity (CII versus CII + Atorva $p < 0.05$ on days 20 and 28) and disease incidence (17%) (Figure 1C,D).

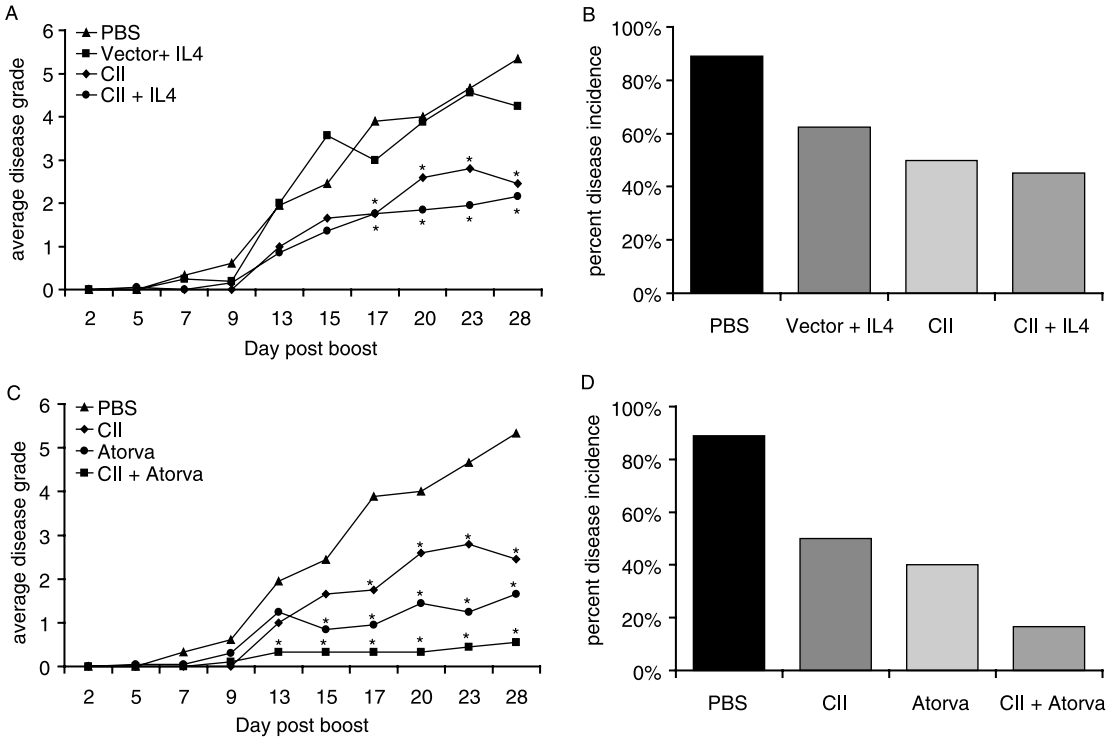

Figure 1. A DNA vaccine encoding CII prevents CIA and its efficacy is potentiated by combination therapy with atorvastatin but not DNA encoding IL-4. Male DBA1/J mice were treated with intramuscular injections of control vector, DNA encoding CII with or without co-delivery of DNA encoding IL-4 or daily oral gavages of atorvastatin. Mean CIA scores using the visual scoring system are plotted against the number of days post CII/IFA boost. Treatment with DNA encoding whole CII alone or in combination with IL-4 DNA resulted in the reduction of CIA disease scores (A) and overall percent disease incidence (B). Treatment with atorvastatin alone or in combination with DNA encoding whole CII also significantly reduced CIA disease scores (C) and overall percent disease incidence (D). The asterisks indicate time points at which there were statistically significant differences ($p < 0.05$ by the Mann–Whitney test) comparing the treatment group versus the PBS treated group. The presented data are representative of two independent experiments.

CII DNA vaccines reduce joint destruction in CIA

Four weeks post-boosting for CIA, mice were sacrificed and representative joints harvested for histology. Histopathology performed on representative hind limbs from each group demonstrated dense inflammatory infiltrates and destruction of the articular surface in mice treated with PBS (Figure 2A,B) and the vector + IL-4 DNA vaccine control (Figure 2C,D). Groups of mice treated with CII DNA alone (Figure 2E,F), CII DNA + IL-4 DNA (Figure 2G,H), atorvastatin alone (Figure 2I,J), and CII DNA + atorvastatin (Figure 2K,L) all exhibited significantly reduced inflammatory infiltrates and joint destruction.

DNA vaccines reduce the production of pro-inflammatory cytokines

To characterize the cytokines produced by CII-specific T cells, draining lymph nodes were harvested from all treated mice 4 weeks post-boosting for CIA. Lymphocytes were stimulated *in vitro* with 100 μg/ml denatured CII. Following 72 h of stimulation, culture supernatants were harvested for ELISA analyses to determine levels of IFN-γ (Figure 3A) and TNF-α (Figure 3B). CII-stimulated lymphocytes isolated from CIA mice treated with a DNA vaccine encoding CII produced significantly less IFN-γ and TNF-α compared to PBS and DNA vector + IL-4 treated mice.

DNA vaccines reduce autoantibody epitope spreading

Synovial antigen arrays were utilized to profile serum autoantibody levels derived from individual mice from each treatment group at the termination of the study. The SAM algorithm was applied to identify antigen features with significant differences in autoantibody reactivity between DNA vaccine treated and control groups. SAM demonstrated that autoantibody production to candidate RA and CIA autoantigens was significantly reduced after treatment with the tolerizing CII DNA vaccine alone as compared to PBS treated mice (Figure 3D,E). In CII DNA vaccinated mice, significant reductions in autoantibody titers to epitopes in CII, collagen type I, human cartilage glycoprotein 39 (HCgp39), cyclic citrullinated peptide (CCP), glucose-6-phosphate isomerase (GPI), native fibrinogen and heat-shock proteins (HSP) 65, 70, and 90.

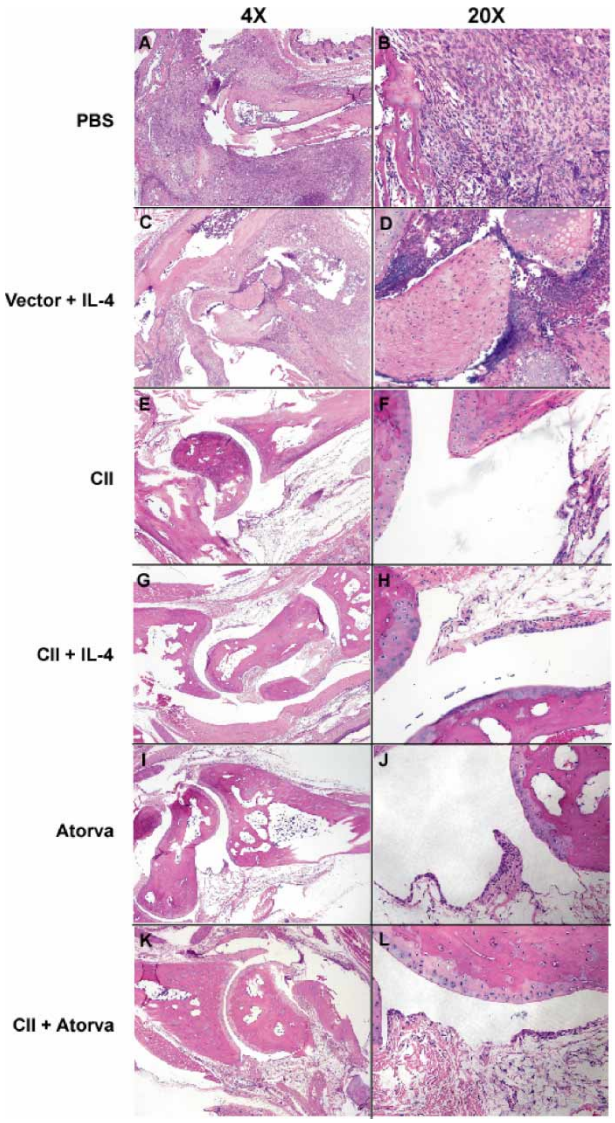

Figure 2. Combination therapy with CII tolerizing DNA vaccine plus IL-4 or atorvastatin reduces joint destruction. On day 28, hind limbs from representative mice described in Figure 1 were harvested, formalin fixed and decalcified. Samples were embedded in paraffin, sectioned, and H&E stained. Representative images are presented and exhibit the nature of infiltrating immune cells for each treatment group at 4 × (A, C, E, G, I, K) and 20 × (B, D, F, H, J, L) magnifications.

Discussion

In this "systems biology" issue of *Autoimmunity*, we present development of tolerizing DNA vaccines to treat the CIA model for RA. Such antigen-specific tolerizing therapies provide a fundamental approach to the treatment of RA and other autoimmune diseases. Major challenges for the development of antigen-specific tolerizing therapies include: (i) insufficient knowledge of the specificity of the autoimmune response, for which we have developed protein microarrays to profile the specificity of the autoantibody response; and (ii) the need for methods to induce specific tolerance, for which we are developing tolerizing DNA vaccines [10]. We previously demonstrated that tolerizing DNA vaccines encoding multiple protein array-identified autoantibody targets provided superior efficacy in treating autoimmunity in a murine model of multiple sclerosis [10]. Further, antibody profiling can be utilized to facilitate diagnosis, assess prognosis, and to monitor responses to therapy [10]. In this paper we extend this approach to develop tolerizing DNA vaccines for CIA, and utilize synovial protein microarrays to monitor responses to DNA vaccine therapy.

We herein describe successful prevention of CIA by vaccination of mice with DNA encoding CII. Disease incidence and severity were substantially reduced in the CII DNA treated groups, and co-administration of atorvastatin potentiated the protective effect. Mice protected against CIA by CII DNA vaccines exhibited reduced inflammation and destruction of joints on histopathologic analysis. *In vitro* analysis of anti-CII T cells demonstrated reduced production of the pro-inflammatory cytokines IFN-γ and TNF-α in CII vaccinated mice. Further, CII DNA vaccines reduced epitope spreading of auto-reactive B cell responses in CIA.

IL-4 is a potent Th2 cytokine. It is well established that delivery of IL-4 via gene therapy or administration of recombinant protein ameliorates arthritis in CIA [30,31]. These data combined with the potentiating effect of DNA encoding IL-4 in tolerizing DNA vaccines for EAE [6,10] and NOD [19] provide the rationale for testing its potential to augment CII-encoding DNA vaccines for CIA. In contrast to the results in EAE and NOD mice, co-treatment with DNA encoding CII plus IL-4 did not provide benefit over treatment with CII DNA alone.

Statins have been reported to block the induction of nitric oxide synthase and proinflammatory cytokines TNF-α, IFN-γ IL-1β, and IL-6, and to increase the production of Th2 type cytokines (IL-10) in rats [12]. Certain statins have been shown to reduce inflammatory responses and disease activity in the mouse model of RA and in patients with RA. When administered either prophylactically or one day following onset of clinical arthritis, simvastatin suppressed murine CIA by inhibiting collagen-specific Th1 humoral and cellular immune responses [15]. Clinical trials examining the potential efficacy of atorvastatin and simvastatin in human RA demonstrated that both could provide clinical benefit [32]. Further, atorvastatin has been used as a Th2-promoting adjuvant to enhance the efficacy of glatiramer acetate in EAE [16]. Based on these data, we tested the ability of atorvastatin to potentiate the efficacy of DNA encoding CII. We observed increased efficacy of atorvastatin-CII DNA as compared to CII DNA treatment on days 20 and 28 post antigenic boost ($p < 0.05$).

CIA is an autoimmune arthritis mediated by T and B lymphocytes induced to autoreact against CII. Recent clinical trials demonstrate that RA patients benefit from non-specific inhibition of T cell co-stimulation

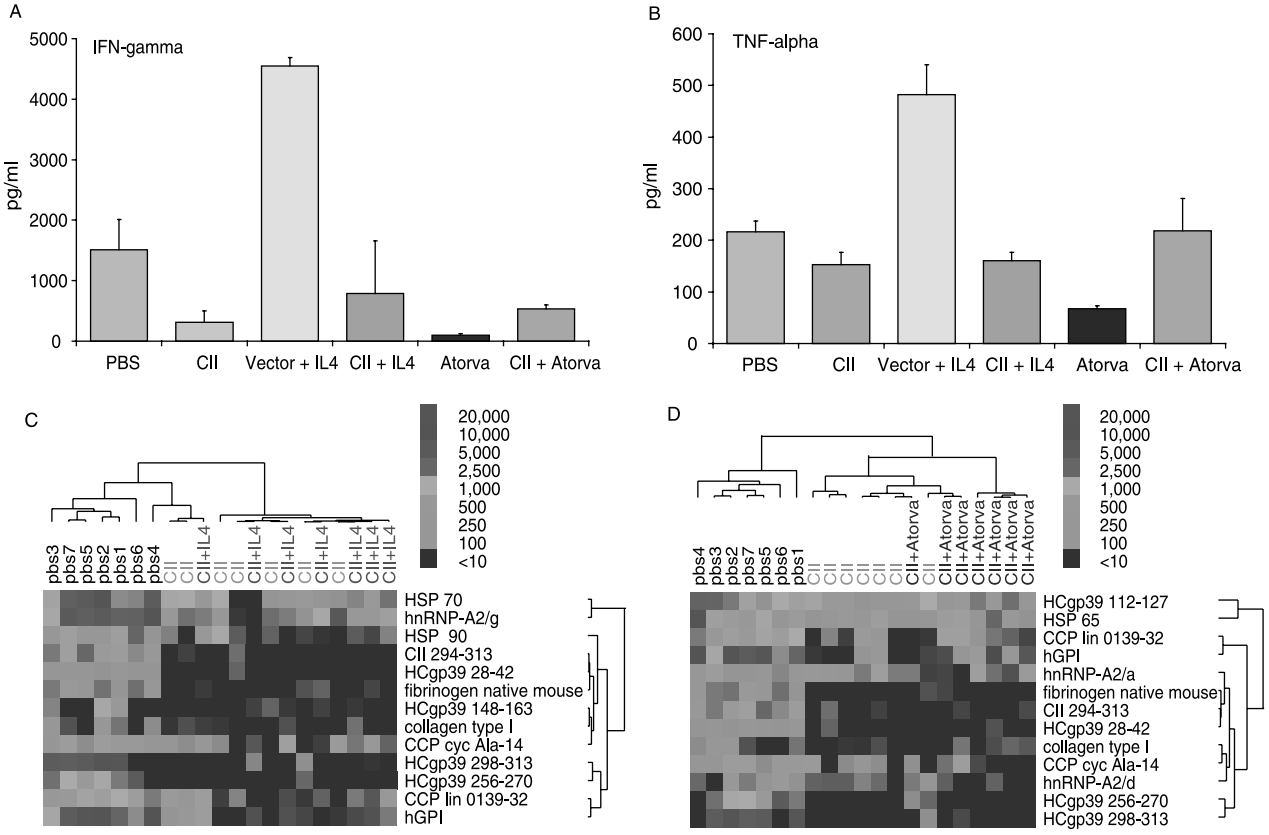

Figure 3. Combination therapy with CII tolerizing DNA vaccine plus DNA encoding IL-4 or atorvastatin suppresses CII specific Th1 cells and diversification of autoantibody responses. On day 28 post boost, lymphocytes harvested from mice described in Figure 1 were stimulated with denatured CII for 72 h and supernatants were collected and assayed for IFN-γ (A) and TNF-α (B). At the same time, sera were collected and analysed using synovial antigen arrays. (C) Heatmap display of hierarchically-clustered antigens identified by multiclass SAM analysis as exhibiting statistically significant differences in IgG and M autoantibody reactivities between groups of mice treated with PBS, DNA encoding CII, and DNA encoding CII plus IL-4, and (D) Heatmap display of hierarchically-clustered antigens identified by multiclass SAM analysis as exhibiting statistically significant differences in IgG and M autoantibody reactivities between groups of mice treated with PBS, DNA encoding CII, and DNA encoding CII in combination with atorvastatin. Antibody reactivity was consistently detected against the immunizing whole CII protein, and to several other RA relevant epitopes including peptides derived from HCgp 39, GPI, HSP, filaggrin and fibrinogen. Each column represents autoantibody reactivities from a single animal from each group, and each row reactivities against a peptide or protein contained on synovial arrays. Reactivity levels are based on the displayed color scale. Represented are only the SAM "hits", antigens identified by SAM as exhibiting significant differences between the groups compared. Prefixes denote the species from which each peptide was taken (h-human, m-mouse,); peptide abbreviations are as described in the text.

by a CTLA-4Ig fusion protein [33]. Such systemic immunosuppressive approaches place patients at increased risk for infectious and malignant complications. RA is likely mediated by autoreactive T cells coordinating autoimmune responses targeting specific synovial joint proteins, and tolerizing DNA vaccines represent a promising and highly-specific strategy to attenuate such autoimmune responses.

A critical aspect to developing antigen-specific tolerizing therapies for human disease is knowledge of the autoantigens targeted by the pathogenic immune responses. To characterize the specificity of autoimmune responses we have developed protein microarrays to determine the specificity of the autoantibody response. Recent studies demonstrate that approximately 60% of RA patients possess autoimmune responses against citrullinated epitopes and that autoantibody responses against such epitopes may contribute to joint tissue injury [34]. Since DNA vaccines encode native polypeptides, it is likely that DNA vaccines may only provide the potential to treat the subset of RA patients possessing autoimmune responses against native epitopes. Multiple laboratories, including our own, have ongoing efforts to delineate both the citrullinated and native targets of the autoimmune response in RA.

Based on our work in the EAE model demonstrating that tolerizing DNA vaccines encoding a greater number of autoantibody targets are more efficacious, we anticipate that development of tolerizing vaccines encoding additional synovial array-identified autoantibody targets (Figure 3C,D) could provide superior efficacy in CIA. Finally, autoantibody profiling could be applied to pre-select autoimmune patients for receipt of tolerizing DNA vaccines encoding the relevant autoimmune targets. Such a strategy would be analogous to pre-selection of breast cancer patients overexpressing Her-2 for treatment with Herceptin.

Conclusion

Major challenges exist in the diagnosis, classification and treatment of RA and other autoimmune diseases. In this paper we describe development of tolerizing DNA vaccines for autoimmune arthritis, and application of protein arrays to monitor responses to therapy and to identify potential targets for next generation vaccines. These studies reflect our greater objective of developing a comprehensive therapeutic approach for autoimmune diseases based on:

i) autoantibody profiling to:
   facilitate diagnosis, assess prognosis, and monitor responses to therapy
   pre-select patients for receipt of antigen-specific tolerizing therapies
   guide development of antigen-specific therapies.
ii) tolerizing DNA vaccines to attenuate autoimmune responses.

We anticipate that such a "systems biology-type" approach would revolutionize our ability to care for patients with RA and other autoimmune diseases.

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