Vaccination Without Auto-antigen Protects Against Collagen II-Induced Arthritis via Immune Deviation and Regulatory T Cells [1]

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Abstract

Anti-inflammation immunotherapy has been successfully applied for the treatment of autoimmune diseases. Mucosal vaccines against autoimmune disorders are beneficial by influencing the regulatory compartment of gut and systemic adaptive immune systems. A *Salmonella* vector expressing colonization factor antigen I (CFA/I), shown to behave as an anti-inflammatory vaccine, stimulates the production of $CD4^+ CD25^+$ T cells and regulatory cytokines. In this work, we queried whether *Salmonella*-CFA/I can protect DBA/1 mice from collagen-induced arthritis (CIA). The incidence of arthritis and cartilage loss in vaccinated DBA/1 mice was remarkably lower when compared to unprotected mice. Clinical findings were accompanied by the suppression of inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-27. Vaccination evoked a multi-tier response consisting of IL-4 producing Th2 cells, an increased production of TGF-β by $CD4^+$ T cells, and suppression of collagen II (CII)-specific $CD4^+$ T cell proliferation. To assess the contribution of *Salmonella*-CFA/I-primed $CD4^+$ T cells, adoptive transfer studies with total $CD4^+$, $CD4^+CD25^-$, or $CD4^+ CD25^+$ T cells were performed 15 days post-challenge. Mice receiving either subset showed reduced disease incidence and low clinical scores; however, mice receiving total $CD4^+$ T cells showed delayed disease onset by 10 days with reduced clinical scores, reduced IL-17 and IL-27, but enhanced IL-4, IL-10, IL-13, and TGF-β. Inhibition of TGF-β or IL-4 compromised protective immunity. These data show that *Salmonella*-CFA/I vaccination of DBA/1 mice protects against CIA by stimulating TGF-β- and IL-4-producing regulatory $CD4^+$ T cells.

Keywords

Th1/Th2 cells; tolerance; vaccination; mucosa

Introduction

Targeted immunotherapy is a highly developed approach for treatment of chronic infections, autoimmune diseases, allograft rejections, and malignancies (1–3). Immunotherapy for autoimmune disorders is also especially attractive for correcting inflammatory diseases without having to resort to immunosuppressive drug therapies. The two main goals of such an approach are 1) to "switch off" the immune response against the host's own tissues and 2) to maximally balance the relationships between effector cells of different lineages to prevent relapses of chronic inflammation.

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Rheumatoid arthritis (RA)[3] is a systemic inflammatory disease of the joints that disables almost half of the affected patients. The etiology of RA is still unknown, but hereditary factors and possible infectious agents (bacteria and viruses) are assumed to participate in the disease initiation (4). RA is mediated by T cells, predominantly CD4$^+$ T cells, and proinflammatory cytokines, such as TNF-α and IL-1, are considered responsible for orchestrating pathogenesis (5–7). Using anti-TNF-α antagonists has resulted in success when combined with cytostatic therapy (8). The design of vaccines capable of preventing or reversing chronic inflammation is of particular interest. Collagen-induced arthritis (CIA), a model of RA, can be induced upon immunization with heterologous collagen II (CII) in DBA/1 mice or by mAbs to CII combined with LPS (9–11). CIA shares with RA several critical characteristics of the disease pathogenesis, including CD4$^+$ T cells' mediated inflammation and extensive cartilage and bone damage, resulting in joint deformities. This similarity permits the use of the CIA model as an investigative tool to test novel approaches for prevention and treatment of RA.

Live attenuated, *Salmonella* vaccine vectors are widely used as delivery systems and demonstrate avirulence, safety, and the ability to induce effective immune responses not only to *Salmonella* but also to passenger Ags (12). The *Salmonella* vector delivers the Ag directly to innate immune cells, thus, enhancing the development of the adaptive immune responses. Along these lines, oral immunization with our diarrheal vaccine for enterotoxigenic *E. coli* (ETEC), *Salmonella* expressing colonization factor Ag I, (*Salmonella*-CFA/I), induces strong mucosal and serum Ab responses against CFA/I fimbriae (13,14). This immunity is supported by an induction of a sustained Th2 cell activation against CFA/I fimbriae (14), much like that against soluble Ags, followed by Th1 (IFN-γ) cytokine production. Such a biphasic response induced by *Salmonella*-CFA/I is atypical because intracellular infections with *Salmonella* generally induce Th1-type responses (15). To ascertain whether this delay in Th1 cell development was somehow related to detective infection of innate cells, subsequent studies evaluated the vaccine's ability to infect macrophages and showed an absence of proinflammatory cytokine production despite similar bacterial loads in macrophages (16). The presence of CFA/I fimbriae on the *Salmonella* vector's cell surface does not interfere in stimulating protective immunity to wild-type *Salmonella*, since the fimbriated *Salmonella* still provided similar survival efficacy as did *Salmonella* vector-immunized mice (17). Thus, *Salmonella*-CFA/I vaccine has the unique properties to protect against both wild-type *Salmonella* and ETEC.

Given these findings, we hypothesized that *Salmonella*-CFA/I is an anti-inflammatory vaccine in addition to having vaccine qualities for diarrheal diseases. When the anti-inflammatory properties of *Salmonella*-CFA/I were tested in an animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), *Salmonella*-CFA/I-vaccinated SJL mice were protected against proteolipid protein (PLP$_{139-151}$) challenge (18). This protection was mediated via Th2-type cytokines IL-4 and IL-13, as well as by TGF-β-producing regulatory CD4$^+$CD25$^+$ T (T$_{reg}$) cells and a reduction in IL-17 (18,19).

In this present work, we questioned whether oral immunization with *Salmonella*-CFA/I could inhibit the development of a different autoimmune disease, CIA, in DBA/1 mice. Such a study would address whether *Salmonella*-CFA/I could suppress the development of an autoimmune disease unrelated to myelin Ags and independent of auto-Ag in mice with a different genetic background. Oral immunization with *Salmonella*-CFA/I lowered CIA disease incidence and clinical scores. *Salmonella*-CFA/I-derived CD4$^+$ T cells from both CD25$^-$ and CD25$^+$ T cell

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[3]Abbreviations used in this paper: **RA**, rheumatoid arthritis; **CIA**, collagen-induced arthritis; **CII,** collagen II; **ETEC**, enterotoxigenic *E. coli*; **CFA/I**, colonization factor antigen/I; **EAE**, experimental autoimmune encephalomyelitis; **PLP**, proteolipid protein; **T$_{reg}$**, regulatory T cells; **LN**, lymph node; **CFC**, cytokine-forming cells; **MLN,** mesenteric LN; **HNLNs**, head and neck lymph nodes; **FoxP3**, forkhead box P3;

subsets produced the regulatory cytokines IL-4, IL-10, and TGF-β that contributed to the observed protection, and this protection emanated optimally from unseparated CD4^{+} T cells. The protective effect by *Salmonella*-CFA/I was lost upon anti-TGF-β mAb treatment and significantly weakened by anti-IL-4 mAb treatment, indicating the importance of their role to suppress autoimmune disease. Thus, these results show that *Salmonella*-CFA/I can modify host immunity to auto-Ag independently of the auto-Ag.

Materials and Methods

Mice

DBA/1 male 6–8 weeks old mice were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were maintained at Montana State University Animal Resources Center in individual ventilated cages under HEPA-filtered barrier conditions and were fed sterile food and water *ad libitum*. All animal care and procedures were in accordance with institutional policies for animal health and well-being.

Immunizations and clinical evaluations of CIA

The Δ*asd Salmonella enterica* serovar Typhimurium-CFA/I vaccine (strain H696) and its isogenic *Salmonella* vaccine vector (strain H647) were used for these studies (13,14). Mice were vaccinated *per os* with 5×10^9 CFU *Salmonella*-CFA/I or its isogenic strain H647 (without CFA/I operon) seven days prior to CII challenge. Control mice received sterile PBS. For induction of arthritis, 100 μg of bovine CII (Chondrex, Inc., Redmond, WA) emulsified in CFA containing 4 mg/ml of *M. tuberculosis* (Chondrex, Inc.) were injected subcutaneously in the tail 2 cm from mouse body (20). Generally, by the end of week 5 post-challenge, 80–100% of mice in control group showed fully developed disease.

Mice were scored for clinical disease three times a week using a graded scale 0–3 for each limb for a maximal total score of 12 possible, as previously described (21): 0 - normal; 1 - mild redness or swelling of single digits; 2 - significant swelling of ankle or wrist with erythema; 3 - severe swelling and erythema of multiple joints. Percent of animals with arthritic lesions in the group represent the incidence of arthritis. Average clinical score in the group reflects the severity of the disease.

Histopathological analysis

Fifty days after CII challenge, limbs were fixed in 10% neutral buffered formalin and decalcified in 5% formic acid for 3–6 days. The joints were embedded in paraffin and cut at 8 μm sections. Hematoxylin - eosin and toluidine blue staining were performed for each sample. Histopathological scores for each joint were determined on a graded scale 0 –3 similar to that previously described (22): 0 - no changes; 1 - synovial hyperplasia and mild inflammatory infiltration; 2 - pannus formation with cartilage degeneration; 3 - heavy inflammatory infiltration and debris in the joint, severe chondrocytes and cartilage matrix loss with new bone tissue substitution, bone destruction. Paws and knee joint scores were estimated, with a total maximum score of 18 possible per mouse. Cartilage degeneration was scored in toluidine blue-stained sections on a graded scale 0 – 3 similar to that previously described (23): 0 - no cartilage loss; 1 - minimal chondrocytes and proteoglycan loss in superficial zone; 2 - moderate chondrocytes and proteoglycan loss into middle zone but above tidemark; 3 - severe cartilage degeneration through tidemark.

Anti-CFA/1 and anti-CII Ab ELISA

Serum and fecal samples were collected on day 21 after immunization with *Salmonella*-CFA/1. Samples were tested against purified CFA/I fimbriae, as previously described (14,17), and

against CII (Chondrex, Inc.). Serum samples from mice with induced arthritis were collected on day 21 post-challenge with CII, and samples of dilute sera were added to microtiter wells (Maxisorp Immunoplate II microtiter plates; Nunc, Roskilde, Denmark) coated with 2 μg/ml of ELISA Grade bovine or mouse CII (Chondrex, Inc.). For both Ab ELISA assays, goat anti-mouse HRP-labeled IgG1, IgG2a, IgG2b, or IgA (Southern Biotechnology Associates, Inc., Birmingham, AL) were used as detecting Abs. Enzymatic reaction was developed with ABTS (Moss, Inc., Pasadena, CA). Optical density was read at 415 nm using ELx808 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Endpoint titer represents reciprocal logarithm of two for the serum dilution with OD equal or more 0.1 above negative control.

Cytokine ELISA

Upon termination of the study (day 50 post-induction of arthritis challenge), axillary, inguinal, popliteal lymph node (LN) cells were purified and total mononuclear cells (5×10^6 cells/ml) were cultured alone or with 50 μg/ml CII for 3 days in a complete medium: RPMI-1640 medium supplemented with 10% of FBS (Invitrogen, Corp., Carlsbad, CA), 2 mM L-glutamine, 50 μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. TNF-α, IL-1β, IL-6, IL-17, and IL-27 were measured from culture supernatants (16). Briefly, wells were coated with purified anti-mouse mAbs: TNF-α (5 μg/ml; BD Pharmingen), anti-IL-1β (3 μg/ml; R&D Systems, Minneapolis, MN), anti-IL-6 (2 μg/ml; BD Pharmingen), anti-IL-17 (2 μg/ml; BD Pharmingen), and anti-IL-27p28 (5 μg/ml; R&D Systems). Following blocking with PBS containing 1% BSA for 2 hrs at 37^0C, supernatants were incubated overnight at 4^0C. For detection, 0.5 μg/ml of biotinylated anti-mouse TNF-α, IL-6, IL-17 (BD Pharmingen), or IL-1β, IL-27p28 (R&D Systems) were added to wells for 1.5 hr at 37^0C. Reactions were developed using 1:1000 HRP-goat anti-biotin Ab (Vector Laboratories, Inc., Burlingame, CA) for 1 hr at RT followed by ABTS substrate (Moss, Inc.) Recombinant mouse TNF-α, IL-6, IL-17, IL-27 (R&D Systems), and IL-1β (PeproTech, Rocky Hill, NJ) were used to generate standard curves.

T cell assays

Axillary, popliteal, and inguinal LNs were isolated on day 15 post-challenge. CD4$^+$ T cells were purified using Dynal$^®$ Mouse CD4 Negative Isolation Kit (Invitrogen Corp, Carlsbad, CA) to > 95% purity. Purified CD4$^+$ T cells were resuspended in complete medium, and 2.5 x 10^5 cells/well in 200 μl were restimulated with varying doses of T Cell Proliferation Grade CII (Chondrex, Inc.) in the presence of syngenic mitomycin C-treated antigen-presenting cells. After 48 h, cells were pulsed with 0.5 μCi/well of ^{3}H-TdR for 18 h. Samples were harvested, and incorporated ^{3}H-TdR was measured by scintillation counting.

To measure cytokine-forming cells (CFCs), CD4$^+$ T cells were restimulated at 10^6/ml with 50 μg/ml CII for 3 days, then added to nitrocellulose-based wells (MultiScreen-HA; Millipore, Bedford, MA) coated with purified anti-mouse mAbs to IFN-γ, IL-4, IL-10, IL-17 (BD Pharmingen), IL-13, and TGF-β (R & D Systems), as previously described (18,19). After overnight incubation at 37° C, cells were removed, and to the washed wells, biotinylated anti-mouse IFN-γ, IL-4, IL-10, IL-17 mAbs (BD Pharmingen), and biotinylated TGF-β or IL-13 Abs (R&D Systems) were added. Reactions were developed after incubation with HRP-conjugated anti-biotin (Vector Laboratories, Inc.) with precipitable substrate, AEC (Moss, Inc.). Spots were enumerated and normalized per 10^6 cells.

To characterize the cytokine profiles of CD4$^+$ T cells induced by *Salmonella*-CFA/I or those induced by *Salmonella* vector, on day 15 post-immunization, mesenteric LN (MLN) CD4$^+$ T cells were sorted for CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T lymphocytes using CELLection™ Biotin Binder Kit (Invitrogen Corp.). Each CD4$^+$ T cell subset (10^6 cells) was stimulated with 10

μg/ml of plate-bound anti-CD3 mAb (BD Pharmingen) and 10 μg/ml of soluble anti-CD28 mAb (BD Pharmingen). After culturing for 4 days at 37° C, supernatants were collected and analyzed for cytokine production. Cytokine-specific capture ELISAs were performed, as previously described (18,19).

Adoptive transfer of CD4^{+}, CD4^{+}CD25^{−}, or CD4^{+}CD25^{+} T cells from Salmonella-vaccinated mice

Mice were immunized with *Salmonella*-CFA/I or *Salmonella* vector, as described above, on day 0. At the same time, CIA was induced in a separate group of mice. On day 15 post-immunization, LNs were harvested from *Salmonella*-CFA/I- or *Salmonella* vector-vaccinated mice, and CD4^{+} T cells, CD4^{+}CD25^{−}, and CD4^{+}CD25^{+} T cells were further sorted using magnetic beads as described. Purity > 92% was achieved for all procedures. Mice induced with CIA 15 days earlier were injected i.v. with $2x10^6$ CD4^{+} T cells or an equivalent amount of CD4^{+} T cell subset: ~$1.5x10^6$ CD4^{+}CD25^{−} T cells and ~$5x10^5$ CD4^{+}CD25^{+} T cells.

In vivo treatment with anti-IL-4 and anti-TGF-β mAbs

Hybridomas producing the anti-mouse IL-4 mAb (clone 11B11; ATCC Manassas, VA) and anti-mouse TGF-β (clone 1D11.16.8; ATCC) were expanded in serum-free supplemented HB101 media (Irvine Scientific, Santa Ana, CA). The mAbs were purified from culture supernatants using protein G (Sigma-Aldrich Co.) affinity chromatography. CD4^{+} T cells from mice immunized with *Salmonella*-CFA/I were sorted for adoptive transfer into mice challenged 15 days earlier with CII, as described above. Corresponding anti-IL-4, anti-TGF-β, or rat IgG *in vivo* Ab treatments was conducted weekly starting on the day of adoptive transfer for a total dose of 1 mg per mouse i.p.

Flow cytometry analysis

On day 14 after immunization with *Salmonella*-CFA/I or *Salmonella* vector, head and neck LN (HNLN) and MLN were purified. Immunofluorescent staining for surface CD4, CD25, CD39, GITR, and intracellular FoxP3 was performed using fluorochrome-labeled mAbs: CD4-FITC, CD4-PE-CY7, CD25-PerCp-CY5.5, GITR-PE (BD Pharmingen), FoxP3-PE, FoxP3-AlexaFluor® 647 (eBioscience, San Diego, CA) and polyclonal rabbit anti-CD39 (H-85) IgG (Santa Cruz Biotechnology, Santa Cruz, CA) paired with anti-rabbit IgG-FITC or IgG-PE (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). For proliferation assay on day 14 after immunization with *Salmonella*-CFA/I, CD4^{+} T cells from pooled HNLN and peripheral LN (effectors) were re-stimulated with 10 μg/ml of CFA/I during 24 hrs. CII specific CD4^{+} T cells (responders) were purified on day 15 after induction of arthritis, labeled by CFSE, as previously described (24), and co-cultured with CFA/I-specific CD4^{+} T cells in the presence of irradiated (3000 rad) APCs and 50 μg/ml CII. The ratio of responders: effectors: APCs was equal (1:1:1). Total cell density in cultures was $3x10^6$/ml, and mAbs against IL-4, TGF-β, and IL-10 were added to the corresponding cultures at 50 μg/ml. After 5 days, cells were harvested, washed, and then stained for CD4, CD25, FoxP3, and GITR. Fluorescence was acquired on FACS Caliber, LSRII, or Canto (BD Biosciences, Mountain View, CA). CellQuest (BD Biosciences) and FlowJo (Tree Star, Inc., Ashland, OR) software was used for analysis.

Statistics

Fisher's exact probability test was applied for incidence of arthritis. Mann-Whitney U-test was used for statistical evaluation of clinical scores, histology scores, and cartilage destruction. Student's *t*-test was performed to analyze T cells proliferation, flow cytometry data, CFC responses, CII- and cytokine-specific ELISA measurements. Results were considered statistically significant if *P*-value was less than 0.05.

Results

Oral immunization with Salmonella-CFA/I inhibits development of CIA

To test if *Salmonella*-CFA/I vaccine could result in protection against CIA, DBA/1 mice were orally vaccinated with 5×10^9 CFU of *Salmonella*-CFA/I (strain H696), and then CIA was induced one wk later. Control mice were given the isogenic *Salmonella* vector (strain H647) or sterile PBS. Three wks following CII challenge, ~ 90% of the PBS-dosed group developed CIA with a maximum average clinical score of ~ 4 (Fig. 1*A*). In the *Salmonella*-CFA/I-dosed group, a delay onset was observed of clinical symptoms with maximal incidence of arthritis being only ~ 20%, a remarkable suppression of CIA.

To determine whether oral immunization with *Salmonella*-CFA/I reduces joint degeneration, on day 50 after challenge, paw and knee joint sections were stained with H & E to evaluate the histopathology or stained with toluidine blue to assess the degree of cartilage degeneration, characterized by chondrocytes and proteoglycan matrix loss. In comparison with normal joint sections, *Salmonella*-CFA/I-immunized mice developed edema and synovial hyperplasia (Fig. 1*B* and *C*), in contrast to median samples from PBS-dosed mice that showed severe bone and cartilage erosions and deformities (Fig. 1*B* and *C*). Consistent with clinical observations, both the average histological score and the level of cartilage degeneration were substantially lower in the group immunized with *Salmonella*-CFA/I, as compared with both control groups (Fig. 1*B*).

To compare production of proinflammatory cytokines between *Salmonella*-CFA/I-immunized mice and control groups, draining axillary, popliteal, and inguinal LNs were collected 50 days post-challenge, and total mononuclear cells were restimulated with CII. Confirming the clinical and histological findings, the results showed significant suppression of TNF-α, IL-1β, and IL-6 production by mononuclear cells from mice vaccinated with *Salmonella*-CFA/I compared to mice vaccinated with *Salmonella* vector or dosed with PBS. Thus, oral *Salmonella*-CFA/I immunization can prevent the development of CIA.

Salmonella-CFA/I does not influence anti-CII Ab responses

Oral immunization with *Salmonella*-CFA/I produces the expected anti-CFA/I Ab titers in DBA/1 mice (Fig. 2*A*), similar to that obtained with other mouse strains (13,14,17,18). Likewise, oral immunization with this vaccine does not induce production of Abs which cross-react to CII (Fig. 2*B*).

The cartilage destruction in arthritis joints occurs as a result of an inflammatory response for which the production of anti-CII Abs is required (25). To determine if immunization with *Salmonella*-CFA/I protects from excessive inflammation by altering the Ab response to CII, serum samples were collected on day 21 post-induction of arthritis, and Ab titers to CII were measured. All groups developed equivalent IgG1, IgG2a, and IgG2b responses to CII (Fig. 2*C*). These data demonstrate that immunization with *Salmonella*-CFA/I does not inhibit anti-CII Ab production.

Oral immunization with Salmonella-CFA/I reduces CII-specific CD4$^+$ T cell proliferation and increases Th2-type/regulatory cytokine production

To assess the CD4$^+$ T cell responses to CII, CD4$^+$ T cells from diseased and protected mice were tested. Purified CD4$^+$ T cells restimulated with CII showed a dose-dependent proliferative response in all groups, but in mice that were immunized with *Salmonella*-CFA/I, this response was significantly reduced, unlike that obtained with CD4$^+$ T cells from mice given the *Salmonella* vector or PBS (Fig. 3*A*). To determine the types of cytokines produced upon CII restimulation, cytokine ELISPOT assays were performed to quantify IFN-γ, IL-4, IL-10, IL-13,

IL-17, and TGF-β cytokine-forming cells (CFCs) one week before first symptoms of arthritis appeared (day 15 post-challenge). Purified LN CD4$^+$ T cells restimulated *in vitro* with CII for 72 hr resulted in a 3- and 4-fold increase of CII-specific CD4$^+$ T cells producing TGF-β and IL-4, respectively, by *Salmonella*-CFA/I-vaccinated mice when compared to PBS- and vector-immunized mice ($P \le 0.01$; Fig. 3*B*). No significant differences in IL-13 or IL-10 CFC were observed between *Salmonella*-CFA/I, H647-immunized groups and the PBS-dosed group. Significant suppression of IFN-γ- and IL-17-producing CD4$^+$ T cells from *Salmonella*-CFA/I-vaccinated mice when compared to *Salmonella*-vector immunized mice or PBS group was also noted (Fig. 3*B*). Along with the decreased Th1 and Th17 cell activities, suppressed production of IL-27 was observed by whole LN cultures derived from protected mice upon *in vitro* CII restimulation (Fig. 3*C*). These results demonstrated that the *Salmonella*-CFA/I vaccine is capable of altering the function of CII- specific CD4$^+$ T cells, driving the development of IL-4- and TGF-β-producing CD4$^+$ T cells, accompanied by reduction of IFN-γ, IL-17, and IL-27 production.

Therapeutic potential of CD25$^+$ or CD25$^−$ CD4$^+$T cells from Salmonella-CFA/I-vaccinated mice against CIA

Since immunization with *Salmonella*-CFA/I results in Th2-type and regulatory cytokine responses to CII, we queried which CD4$^+$ T cell subset from *Salmonella*-CFA/I-immunized mice is responsible for protecting against the development of CIA. To determine the activity of MLN CD4$^+$CD25$^−$ and CD4$^+$CD25$^+$ T cells induced by *Salmonella*-CFA/I or *Salmonella* vector, these T cell subsets were purified on day 15 after immunization and stimulated *in vitro* with plate-bound anti-CD3 and soluble anti-CD28 mAbs to assess cytokine profiles. IFN-γ was equally produced by CD4$^+$CD25$^+$ T cells from *Salmonella*-CFA/I- and *Salmonella* vector-immunized mice, but its production by CD4$^+$CD25$^−$ T cells was significantly ($P<0.001$) lower in *Salmonella*-CFA/I-vaccinated mice (Fig. 4*B*). IL-17 was significantly reduced ($P < 0.05$) in both CD4$^+$ T cell subsets from *Salmonella*-CFA/I-vaccinated mice (Fig 4*B*). Unlike *Salmonella* vector-immunized mice, the CD4$^+$CD25$^+$ T cells from *Salmonella*-CFA/I-vaccinated mice showed elevated production of IL-4, IL-10, and TGF-β (Fig. 4*B*). While these anti-inflammatory cytokines were produced by CD4$^+$CD25$^−$ T cells from both *Salmonella*-immunized groups, IL-4 was elevated ($P < 0.001$) by *Salmonella*-CFA/I-vaccinated mice (Fig. 4*B*), but IL-10 and TGF-β production were not significantly different. Thus, both CD4$^+$ T cell subsets showed more of an anti-inflammatory response and increased FoxP3 expression in H696-vaccinated mice than those immunized with the *Salmonella* vector.

Given these findings, adoptive transfer studies were performed using purified CD4$^+$CD25$^−$, CD4$^+$CD25$^+$, or whole CD4$^+$ T cells from mice immunized with *Salmonella*-CFA/I or *Salmonella* vector (Fig. 4*C*) into CII-challenged mice on day 15 post-CII challenge. Disease progression was monitored, and it was found that in all recipients given *Salmonella*-CFA/I-primed cells, the average clinical scores were significantly less when compared to untreated control mice (Fig. 4*C*). In fact, the mice adoptively transferred with the total CD4$^+$ T cells showed the best protection as opposed to mice given the individual CD4$^+$CD25$^−$ or CD4$^+$CD25$^+$ T cell subsets. Interestingly, both CD4$^+$CD25$^−$ and CD4$^+$CD25$^+$ T cells demonstrated a similar potential to reverse the development of arthritis symptoms. Incidence of arthritis in these groups was reduced by half (Fig. 4*C*); however, only the whole CD4$^+$ T cells were able to delay the disease onset in 100% of mice for 10 days, and only low clinical scores were observed by day 40 (Fig. 4*C*). Notably, neither of the CD4$^+$ T cell subsets derived from immunization with *Salmonella* vector could prevent CIA development when adoptively transferred to CII-challenged mice (Fig. 4*C*). Thus, these experiments showed that CD4$^+$ T cells induced by *Salmonella*-CFA/I are able to protect against CIA during the development phase (15 days post-challenge with CII). This protection is equally conferred by either

CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ T cells, but the best protection required the participation of both CD4$^+$ T cell subsets.

Upon termination of the study, draining LN mononuclear cells were isolated from recipients adoptively transferred with total CD4$^+$ T cells from H696- or H647-primed mice, and were cultured *in vitro* without and with CII to assess proinflammatory cytokine production. TNF-α, IL-1β, IL-6, and IL-27 were significantly reduced in recipients given *Salmonella*-CFA/I-primed CD4$^+$ T cells when compared to diseased (PBS-dosed) mice (Fig. 5*A*). Cytokine production, except for IL-27, by recipients given *Salmonella* vector-primed cells was also lower compared to diseased (PBS-dosed) mice, but TNF-α and especially IL-6 (which was still 5-fold greater) remained elevated when compared to recipients given *Salmonella*-CFA/I-primed CD4$^+$ T cells (Fig. 5*A*).

In addition, CD4$^+$ T cell cytokine production in recipient mice was also measured. Adoptive transfer of CD4$^+$ T cells derived after *Salmonella*-CFA/I immunization caused an increase in production of Th2-type cytokines IL-4 and IL-13 and regulatory cytokines IL-10 and TGF-β by CD4$^+$ T cells in recipient mice (Fig. 5*B*) with concomitant reduction in IL-17 (Fig 5*B*). Consistent with the observed clinical findings, *Salmonella* vector-primed CD4$^+$ T cells could not alter the trend in enhanced Th2-type and regulatory cytokine production by recipients when compared to diseased (PBS-dosed) mice. Collectively, these experiments demonstrated the potential of *Salmonella*-CFA/I-derived CD4$^+$ T cells to suppress CIA suggestively through Th2 cytokines, IL-10, and/or TGF-β.

Salmonella-induced CFA/I CD4$^+$ T cells suppress CII-primed CD4$^+$ T cells

To evaluate the relative contribution of IL-4, IL-10, and TGF-β produced by *Salmonella*-CFA/I- induced CD4$^+$ T cells in the suppression of autoimmune inflammation, an *in vitro* analysis was performed on CII-specific proliferation by effector CD4$^+$ T cells. *Salmonella*-CFA/I CD4$^+$ T cells were purified from combined LNs on day 14 post-immunization. These cells were restimulated with purified CFA/I fimbriae for 24 hrs in the presence of irradiated APC. CII primed-CD4$^+$ T cells were purified on day 15 after arthritis challenge and labeled with CFSE. CFA/I-stimulated CD4$^+$ T cells and CII-primed CFSE$^+$ CD4$^+$ cells were co-cultured in the presence of CII and irradiated APCs with inhibitory concentrations of anti-IL-4, anti-IL-10, or anti-TGF-β mAbs, or rat IgG. Flow cytometry analysis revealed that neutralization of each cytokine could restore proliferation of CFSE$^+$CD4$^+$ T cells when in the presence of inhibitory CFA/I-primed CD4$^+$ T cells (Fig. 6*A*). This experiment demonstrates that IL-4, IL-10, and TGF-β can contribute to the observed suppression mediated by *Salmonella*-CFA/I-primed CD4$^+$ T cells. Interestingly, the percentage of CFSE$^+$ FoxP3$^+$CD25$^+$ CD4$^+$ T cells induced by CFA/I-primed CD4$^+$ T cells decreased nearly 2-fold if blocking mAbs were added to the co-cultures.

In vivo neutralization of IL-4 or TGF-β compromises the therapeutic capacity of Salmonella-CFA/I-primed CD4$^+$ T cells against CIA

To further assess the role of IL-4 and TGF-β-producing CFA/I-primed CD4$^+$ T cells in the protection against CIA, purified *Salmonella*-CFA/I-primed CD4$^+$ T cells were adoptively transferred into recipients induced with CIA two wks earlier. Anti-IL-4 or anti-TGF-β mAb treatment was initiated at the time of adoptive transfer and treated subsequently at weekly intervals for a total of four doses. The *in vivo* neutralization of TGF-β resulted in clinical scores and incidences of CIA in these recipients similar to unprotected mice (Fig. 7*A* and Table I). Treatment with anti-IL-4 mAb resulted in significantly higher clinical scores and incidences of CIA to 90% (Fig. 7*A*). Although the clinical score of anti-IL-4 mAb-treated mice was lower than in PBS control mice or in anti-TGF-β-treated mice, the disease incidence was significant. Upon termination of the study, draining LN cells were purified from each test group and

evaluated for proinflammatory cytokine production following CII restimulation. The amount of TNF-α, IL-6, IL-17, and IL-27 produced by anti-TGF-β mAb-treated mice was similar to the PBS control group (Fig. 7*B*). In the anti-IL-4 mAb-treated group, TNF-α and IL-17 were significantly greater than in recipient mice given rat IgG, and IL-6 and IL-27 production were similar to PBS control group (Fig.7*B*). Such an inflammatory cytokine profile in anti-IL-4 mAb-treated mice is also in consistent with clinical findings. Collectively, these studies show that *in vivo* anti-TGF-β mAb treatment at the time of adoptive transfer of protective *Salmonella*-CFA/I-derived CD4$^+$ T cells (day 15 after induction of arthritis) completely reverses their anti-inflammatory effect. This demonstrates that TGF-β is essential for protection with the *Salmonella*-CFA/I-induced CD4$^+$ T cells. Neutralization of IL-4 also compromises the protective properties of *Salmonella*-CFA/I, suggesting that IL-4 is also an important cytokine for protection against CIA.

FoxP3 correlate with both CD25$^+$ and CD25$^-$ CD4$^+$ T cell subsets

Considering that *Salmonella*-CFA/I stimulates development of FoxP3$^+$ CD4$^+$CD25$^+$ T cells responsible for protection from EAE (19), the CD4$^+$ T cell phenotype was analyzed two wks after oral immunization with *Salmonella*-CFA/I or *Salmonella* vector vaccines. The frequency of CD4$^+$CD25$^+$ T cells in HNLN and MLN increased by 30% in mice immunized with *Salmonella*-CFA/I compared to PBS- and empty vector-immunized mice (Table II). Within this subset, the percentage of FoxP3 expressing cells was also higher (1.5-fold for HNLN and almost 2-fold for MLN). Since it was recently shown that CD39 could associate with FoxP3$^+$ T$_{reg}$ cells (26,27), CD39 expression was also evaluated on both T$_{reg}$ and CD4$^+$ CD25$^-$T cells. Nearly half of FoxP3$^+$CD25$^+$CD4$^+$ T cells were also CD39 positive, whereas only about 10% of FoxP3$^-$CD25$^+$CD4$^+$ T cells expressed CD39 after immunization with *Salmonella*-CFA/I. Additionally, expression of FoxP3 by MLN CD4$^+$CD25$^-$ T cells induced by *Salmonella*-CFA/I was also higher (~2-fold) than in the corresponding subset from PBS and vector control mice, and about 1/3 of these FoxP3$^+$ CD4$^+$CD25$^-$ T cells was CD39 positive. Thus, we observed that the population of CD4$^+$ T cells expressing FoxP3 included both the CD25$^+$ and CD25$^-$T cell subsets after vaccination with *Salmonella*-CFA/I.

Discussion

Salmonella vaccine vectors have been successfully exploited for transporting vaccines into mucosal inductive sites, particularly for the GALT (12–15). Previously, we demonstrated that live *Salmonella*-based vaccines against ETEC can be protective not only from intestinal infection (28,29), but at the same time have distinctive properties of soluble Ag delivery (14, 28,29). These unique attributes include the absence of proinflammatory cytokine production following *in vitro* infection of macrophages (16), as well as induction of Th2-type cytokines, which provides an amplified *in vivo* secretory IgA response (14,28,29). Because of these anti-inflammatory properties, we questioned whether oral immunization with *Salmonella*-CFA/I vaccine could prevent CIA in susceptible DBA/1 mice.

CIA is an experimental inflammatory disease induced in susceptible mice challenged with heterologous CII in complete Freund's adjuvant. CIA shares certain manifestations with RA, as evidenced by the production of proinflammatory cytokines (TNF-α, IL-1, and IFN-γ), Abs to CII, Th1 and other mononuclear cells infiltration, and irreversible joint degeneration (7, 30–32). Arthritis in mice can be passively transferred by CII-reactive mAbs in the presence of LPS or by collagen-reactive T cells (33,34), proving the important role of both B and T cells in pathogenesis. This study was conducted to examine if the featured immune response to *Salmonella*-CFA/I could influence the development of specific immunity to CII and, consequently, inhibit systemic inflammation, such as CIA. Clearly from the results shown here, oral immunization of DBA/1 mice with *Salmonella*-CFA/I resulted in substantially reduced

incidence of clinical CIA when compared to mice treated with PBS or with the isogenic *Salmonella* vector. Clinical observations were further supported by decreased production of proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-27 in draining LN cells of protected mice.

Microbial products can positively or negatively affect the clinical coarse of the joint pathology. Treatment of DBA/1 mice with *E. coli* heat-labile enterotoxin B subunit before challenge with CII was shown to prevent arthritis (35). Administration of *E. coli* or *Salmonella* LPS to mice with CIA leads to enhancement of clinical symptoms (36). Despite presence of LPS, the H696 strain was still protective, unlike its parental strain H647. Perhaps, the expression of CFA/I fimbriae interferes or minimizes the *Salmonella*'s LPS effect or, alternatively, interrupts the normal host recognition mechanisms specifically for LPS, as suggested in earlier studies (16).

Experiments with the EAE model showed that immunization with *Salmonella* vector could reduce demyelination (18,19), although significantly less effective than the CFA/I-expressing (H696) strain. This partially protective effect rendered upon immunization with the *Salmonella* vector was not observed in the CIA model, and in fact, the average clinical score and disease frequency in this group was similar to PBS control.

One explanation that could account for reduced CIA in vaccinated mice is the suppressed production of anti-CII Abs, particularly IgG2a. However, published studies of others demonstrate that suppression of CIA does not always correlate with decreased anti-CII Ab responses (37). On the other hand, exacerbated arthritis in IL-10-deficient mice and IFN-γRμ-KO mice was observed despite lower anti-CII Abs (38,39). Investigating B cell responses to CII, we found that *Salmonella*-CFA/I-vaccinated DBA/1 failed to show reduced anti-CII Ab titers in any of the IgG subclasses.

In contrast, protection conferred by *Salmonella*-CFA/I was mediated by suppressed proliferation of CII-specific CD4+ T cells and immune deviation in their response toward Th2-type and regulatory cytokine production. The cytokine profile for CII-restimulated CD4+ T cells confirmed that *Salmonella*-CFA/I redirects these immune responses to be Th2 cell-biased, as evidenced by increased numbers of IL-4 CFC. Similar immune deviation accounted for protecting SJL mice against PLP-mediated EAE (18,19). However, in that model, IL-13 was substantially induced, more so than IL-4. In this current work, the amount of IL-13-producing CD4+ T cells by *Salmonella*-CFA/I-immunized mice was only slightly increased. This finding that IL-4 is protective against CIA is consistent with what others have reported to limit inflammation (40). In that previous study, DBA/1 mice that were adoptively transferred with tolerogenic splenocytes and treated with anti-IL-4 mAb lost their protection to CII challenge. Likewise, IL-4−/− mice could not be tolerized with CII, thus, emphasizing the relevance of IL-4 for protection against CIA.

The notion that regulatory cells could control CIA was alluded to in an early study by Kakimoto et al. in which they described a CII-specific T cell line that could suppress disease onset and disease severity when these cells were adoptively transferred into recipient DBA/1 mice (41). The adoptive transfer of those cells could, in a dose-dependent fashion, suppress CIA (41). More recently, in a similar vein, depletion of CD25+ T cells 2 wk prior to immunization with CII resulted in a more severe CIA in DBA/1 mice, and the adoptive transfer of CD4+CD25+ T cells from naïve mice to CD25+ T cell-depleted mice reversed the enhanced disease severity (42). In this context, CD4+ T cells' response to *Salmonella*-CFA/I provoked enhanced Th2-type and regulatory cytokines IL-4, IL-10, and TGF-β production, as well as enhanced numbers of FoxP3+CD4+CD25+ T cells. FoxP3 is a functional marker of regulatory T cells and can also be expressed by CD4+CD25− T cells. It has been suggested that FoxP3 directs the conversion

of induced regulatory $CD4^+CD25^+$ T cells (rev. in 43). CD39, ectonucleoside triphosphate diphosphohydrolase-1, expressed predominantly on $FoxP3^+$ cells, has been shown to limit inflammation via generation of extracellular adenosine and was recently implicated a possible alternative marker for T_{reg} cells (26,27). Oral immunization with S*almonella*-CFA/I was shown to enhance the expression of CD39 by $FoxP3^+$ T cells. In fact, very limited percentages of the $FoxP3^-$ T cells in both $CD25^+$ and $CD25^-$T cell subsets were $CD39^+$. These findings led us to question the role of *Salmonella*-CFA/I-primed $CD4^+$ T cells, including the significance of the both $CD25^-$ and $CD25^+$ T cell subsets in suppression of CIA. Flow cytometry analysis for $CD4^+CD25^+$ and $CD4^+CD25^-$T cells showed co-segregation with FoxP3 and CD39. An adoptive transfer of $CD4^+$, $CD4^+CD25^-$, or $CD4^+CD25^+$ T cells from mice immunized with *Salmonella*-CFA/I or *Salmonella* vector was performed to mice previously challenged with CII approximately 7 – 9 days before disease onset. As a result, recipients of total *Salmonella*-CFA/I-primed $CD4^+$ T cells did not show symptoms of CIA for 10 days when compared to control diseased group. Although within the subsequent week, the incidence of CIA in this group achieved 100% as did the control group, and the average clinical scores remained remarkably lower. Recipients of isolated $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells demonstrated ~50% less disease frequency and lower average clinical scores per group. Corresponding T cell subsets from *Salmonella* vector-primed could not reverse CIA; although some reduction in average clinical scores and incidence of the disease was observed. *Salmonella*-CFA/I-induced $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were not equally protective in EAE (19). Only partial protection was achieved by treatment with $CD4^+CD25^-$ T cells, whereas $CD4^+CD25^+$ T cells were completely protective. In contrast, $CD25^-$ and $CD25^+$ T cell subsets from *Salmonella*-CFA/I-vaccinated mice showed equivalent protection against CIA, but were suboptimal when compared to protection conferred by combined $CD25^-$ and $CD25^+CD4^+$ T cells. These results suggest that *Salmonella*-CFA/I may induce regulatory cells in both subsets, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells.

We investigated the role of regulatory cytokines IL-4, IL-10, and TGF-β in protection by *Salmonella*-CFA/I. *In vitro* experiments demonstrated that all three cytokines mediate suppression of CII-specific $CD4^+$ T cell proliferation by CFA/I-specific $CD4^+$ T cells. Previous studies have shown IL-4 to promote the development of regulatory $CD4^+CD25^+$ T cells (44 and our observations). In this current study, FoxP3 expression was reduced by 50% when IL-4, IL-10, or TGF-β was neutralized.

The relevance of TGF-β was further implicated when anti-TGF-β mAb was administered in vivo to recipients of *Salmonella*-CFA/I-primed $CD4^+$ T cells and nearly completely neutralized the protective effect by the adoptively transferred $CD4^+$ T cells. While anti-IL-4 mAb treatment did not completely neutralize the protective properties of the adoptively transferred $CD4^+$ T cells, it did essentially compromise protection, resulting in disease incidence to be similar to the PBS control group, and it significantly increased clinical scores for those treated mice. Clearly these experiments provide evidence of the importance of TGF-β and IL-4 in suppression of inflammation by *Salmonella*-CFA/I.

Weakened CIA in recipients could also have been attributed to depressing IL-17 production by CII-reactive $CD4^+$ T cells. IL-17 produced by Th17 cells (45,46), distinct from Th1 and Th2 cell populations, has been shown to play an important role in CIA (46,47). Significant acceleration of CIA onset was achieved after i.v. IL-17 gene transfer, and if IL-17 was over expressed in the joint cavity, aggravated cartilage and bone destruction were observed (48). Recently Röhn T.A. et al. succeeded in inhibiting the development of CIA, Ab-induced arthritis, as well as suppressing EAE by immunization against IL-17 prior to induction of diseases (49). In this study, IL-17 suppression was mediated by *Salmonella*-CFA/I-primed $CD4^+$ T cells, producing Th2-type and regulatory cytokines, as also shown in treating EAE (18).

Moreover, IL-27 was modestly depressed in *Salmonella*-CFA/I-vaccinated mice. IL-27 has been shown to enhance IFN-γ production by naive CD4$^+$ T cells and NK cells (50), and mice lacking the IL-27 receptor showed reduced Th1 cells (51). Although such findings suggest that IL-27 maybe Th1 cell-polarizing, there are opposing reports describing such role in different rodent arthritis models. In a rat adjuvant-induced arthritis model, treatment with a DNA construct expressing murine IL-27 induced a rat anti-IL-27 Ab response neutralizing rat IL-27 resulting in significantly diminished disease (52). Likewise, treatment with an anti-IL-27p28 Ab mimicked this protective effect (52). Using a proteoglycan-induced arthritis model, IL-27 receptor$^{-/-}$ mice showed reduced disease incidence and clinical scores that correlated with reduced IFN-γ and enhanced IL-4 (53). In contrast, mice with CIA treated with recombinant IL-27 at the onset of disease showed reduced disease incidence and clinical scores that correlated with reduced IL-6 and IL-17 (54). In our study, both IL-6 and IL-17 were diminished as IL-27 in *Salmonella*-CFA/I-vaccinated mice that correlated to the increased production of regulatory cytokines. Perhaps the increased production of IFN-γ relative to PBS-treated mice was in part sustained by the residual IL-27. Additional studies are planned to address such a possibility and determine whether IL-27 has a Th1-type-promoting effect in our system.

In conclusion, we have shown that oral immunization with *Salmonella*-CFA/I, a vaccine originally designed to protect against a diarrheal disease, is capable of inhibiting systemic inflammation in the CIA model causing development of Th2-type and TGF-β-producing CD4$^+$ T cells. The contribution of vaccine-induced CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ regulatory T cells and possibly CD39$^+$ regulatory cells in such protection is clearly demonstrated in experiments with adoptive transfer of these subsets to mice in which CIA was induced. Neutralizations of TGF-β compromised protection against CIA in recipients of *Salmonella*-CFA/I-primed CD4$^+$ T cells, and neutralization of IL-4 significantly weakened the protective properties of transferred cells. *Salmonella*-CFA/I-primed CD4$^+$ T cells producing regulatory cytokines IL-4, IL-10, and TGF-β modulated the Th cell response to CII in recipient mice, further promoting the production of regulatory cytokines and suppressing IFN-γ and IL-17 production.

Acknowledgements

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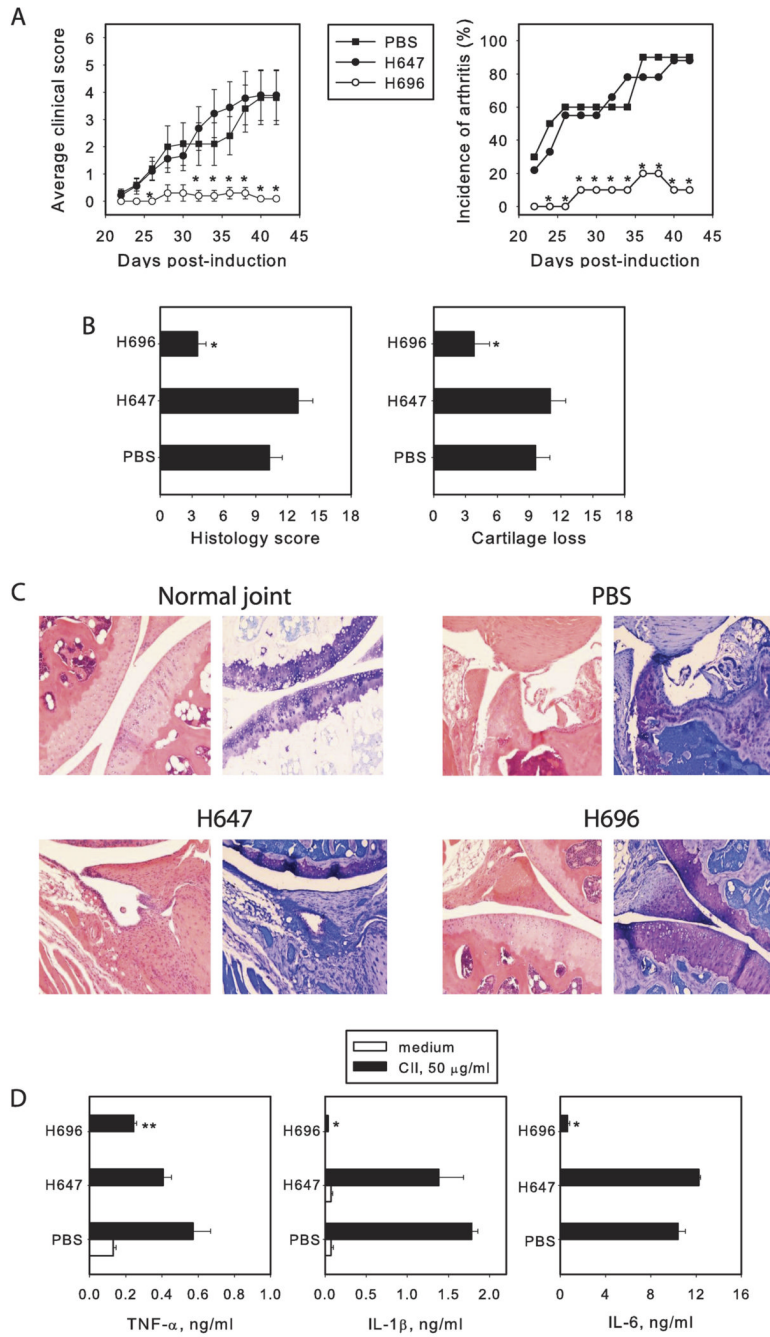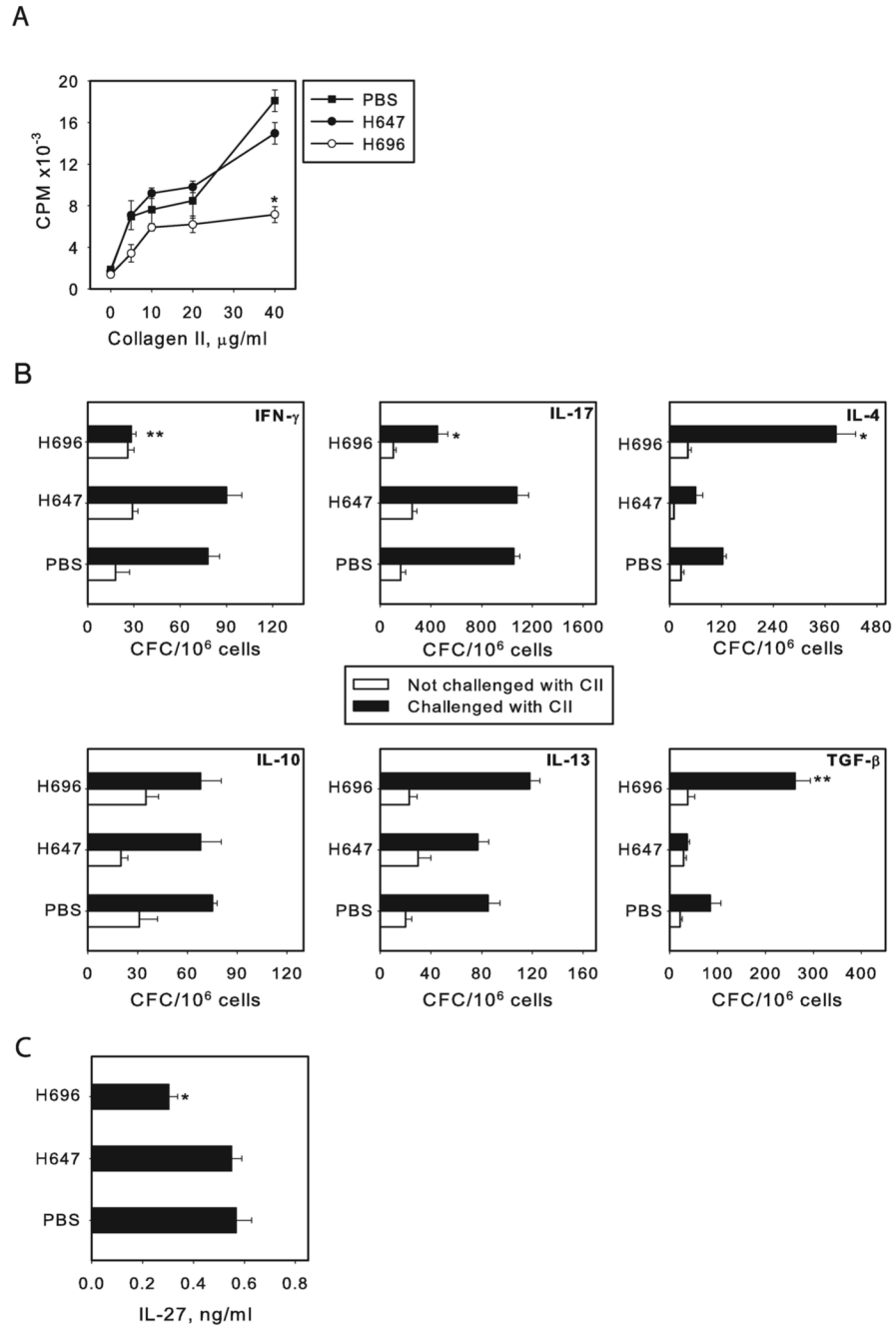
FIGURE 1.
Oral immunization with *Salmonella*-CFA/I (H696) suppresses the development of CIA. Eight-week-old DBA/1 male mice were orally vaccinated with *Salmonella*-CFA/I (H696), *Salmonella* vaccine vector (H647), or sterile PBS. (*A*) average clinical score and incidence of arthritis in mice challenged with bovine CII 7 days after oral vaccination. Data depict the mean of 10 mice per group ± SEM. Fisher's exact probability test was applied to analyze the incidence of arthritis; Mann-Whitney U-test was performed for clinical score analysis; *$P <0.05$. (*B*) Histological scores determined on H-E stained sections and level of cartilage loss evaluated on sections stained with toluidine blue in protected and unprotected mice. Results depict the mean ± SEM, and statistical significance of $P < 0.05$ was obtained using a Mann-Whitney U-

test. (*C*) Knees and paws joints sections were prepared on day 50 after challenge with bovine CII and stained with hematoxylin-eosin (left) and toluidine blue (right). The images are representative for each group from three experiments. Normal knee sections with undamaged cartilage; erosive bones and severe cartilage degeneration in PBS control group; arthritis developed in mice immunized with *Salmonella* vector; synovial hyperplasia and mild edema in the knee joint of mice vaccinated with *Salmonella*-CFA/I (H696). (*D*) Immunization with *Salmonella*-CFA/I results in suppression of proinflammatory cytokines TNF-α, IL-1β, and IL-6. Mice were immunized and challenged with CII as described. Total LN mononuclear cells were purified on day 50 post-challenge and stimulated for 3 days with CII. Cytokines concentrations were determined from collected supernatants by ELISA. * $p< 0.001$ as compared to PBS group and mice immunized with *Salmonella* vector; ** $p< 0.05$ as compared to PBS group and mice immunized with *Salmonella* vector. Student's *t*-test was used for statistical analysis. One of 3 experiments is depicted.
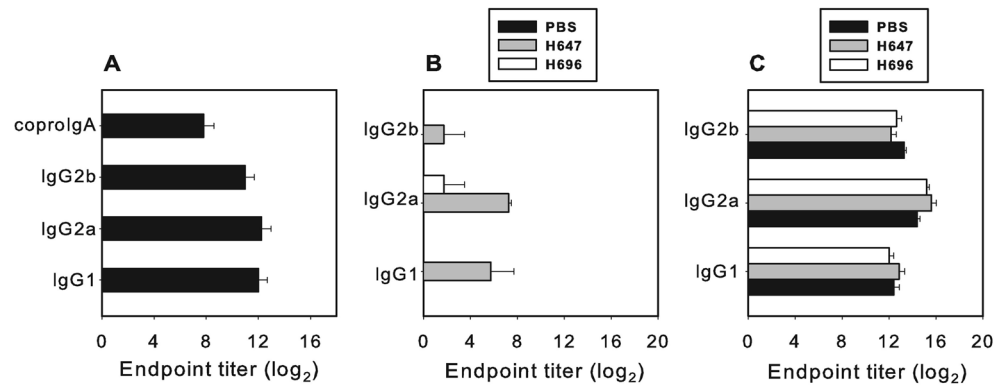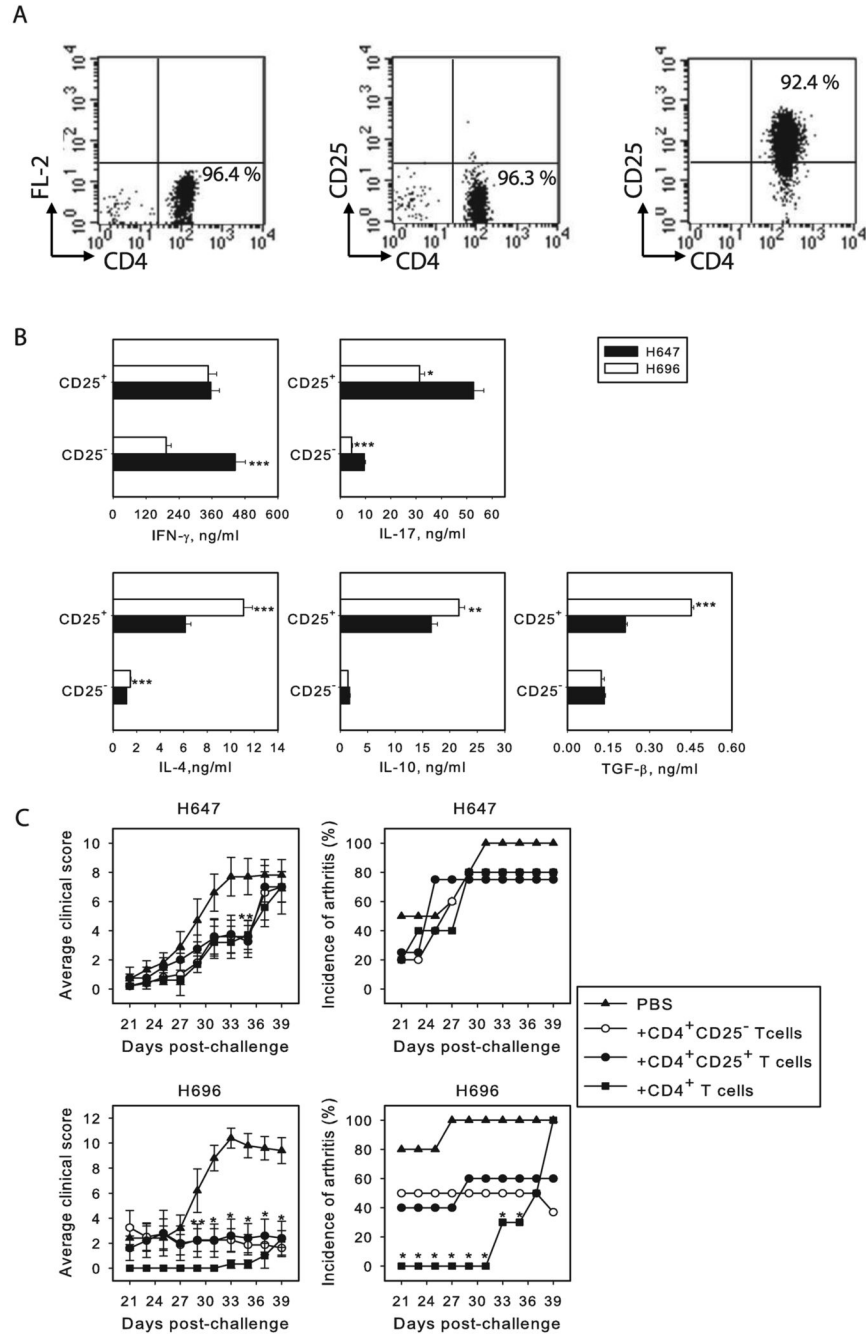
FIGURE 2.
Salmonella-CFA/1 induces Ab response to (*A*) CFA/I without cross-reaction with (*B*) CII. Sera and fecal samples were collected on day 28 after oral immunization with 5×10^9 CFU of *Salmonella*-CFA/I and tested in ELISA against CFA/I or CII. Results represent the mean of 10 mice per group ± SEM. (*C*) Oral immunization with *Salmonella*-CFA/I or *Salmonella* vector does not change Ab response to CII. Sera samples were collected on day 21 after induction of arthritis. Results depict the mean of 10 mice per group ±SEM.

FIGURE 3.
Immunization with *Salmonella*-CFA/I alters CII-specific CD4^{+} T cell responses. (*A*) Mice were immunized with *Salmonella*-CFA/I (H696), *Salmonella* vector (H647), or given sterile PBS. Challenge with CII was performed on day 7 post-immunization. Axillary, inguinal, and popliteal LN were collected 15 days after challenge, and purified CD4^{+} T cells were restimulated in culture with an equivalent amount of mitomycin C-treated syngenic APCs in the presence of varying amounts of CII for 72 h. Depressed CII-specific proliferative responses by draining LN CD4^{+} T cells were obtained upon restimulation with CII. Data represent the mean in CPM of five replicates ± SEM, and data are representative of three experiments. (*B*) Cytokine ELISPOT for draining LN CD4^{+} T cell responses upon CII restimulation. Open bars

show cytokine producing cells from mice in which arthritis was not induced. Data depict the mean CFC ± SEM and are representative of three experiments. *$P < 0.001$ as compared to PBS control and *Salmonella* vector-immunized group; **$P < 0.01$ as compared to PBS control and *Salmonella* vector-immunized group. (*C*) Immunization with *Salmonella*-CFA/I results in suppressed IL-27 response to CII upon restimulation of draining LN cells *in vitro*. * $P < 0.01$ as compared to PBS and *Salmonella* vector groups.
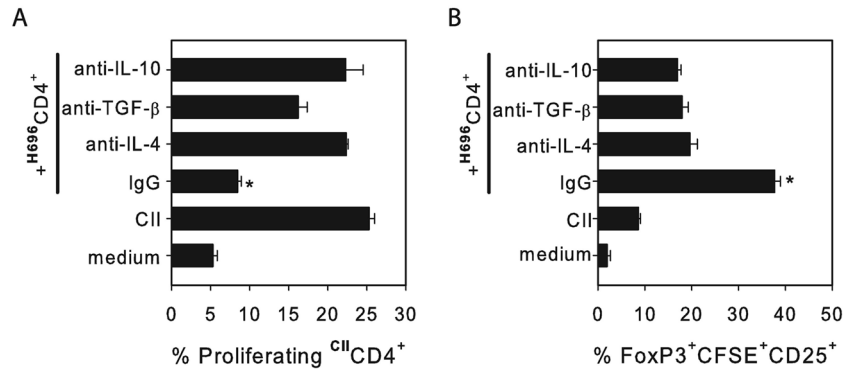
FIGURE 4.
CD4^{+} T cells activated by *Salmonella*-CFA/I are potent against CIA. (*A*) FACS profiles for sorted CD4^{+}, CD4^{+}CD25^{−} and CD4^{+}CD25^{+} T cells. (*B*) Cytokine production by MLN CD4^{+}CD25^{−} and CD4^{+}CD25^{+} T cells after immunization with *Salmonella*-CFA/I or *Salmonella* vector. Purified 10^6 CD4^{+}CD25^{−} and CD4^{+}CD25^{+} T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 mAbs for 3 days. Data show the mean concentration of cytokine ± SEM in series of 6 measurements. *$P < 0.001$, **$P < 0.005$, ***$P < 0.01$ depict significant differences between corresponding *Salmonella*-CFA/I-and *Salmonella* vector-primed cells. (*C*) CD4^{+} T cells induced by *Salmonella*-CFA/I, but not by *Salmonella* vector, confer protection from CIA. Recipient CII challenged DBA/1 mice were

adoptively transferred with purified CD4$^+$, CD4$^+$CD25$^-$, or CD4$^+$CD25$^+$ T cell subsets previously primed with *Salmonella*-CFA/I or *Salmonella* vector. Adoptive transfer was performed 15 days after induction of CIA (8 mice per group). Severity of arthritis symptoms is represented as average clinical score per group. Incidence of arthritis corresponds to the percentage of mice with arthritis in each group. *$P < 0.01$ as compared to control; **$P < 0.05$ as compared to control.
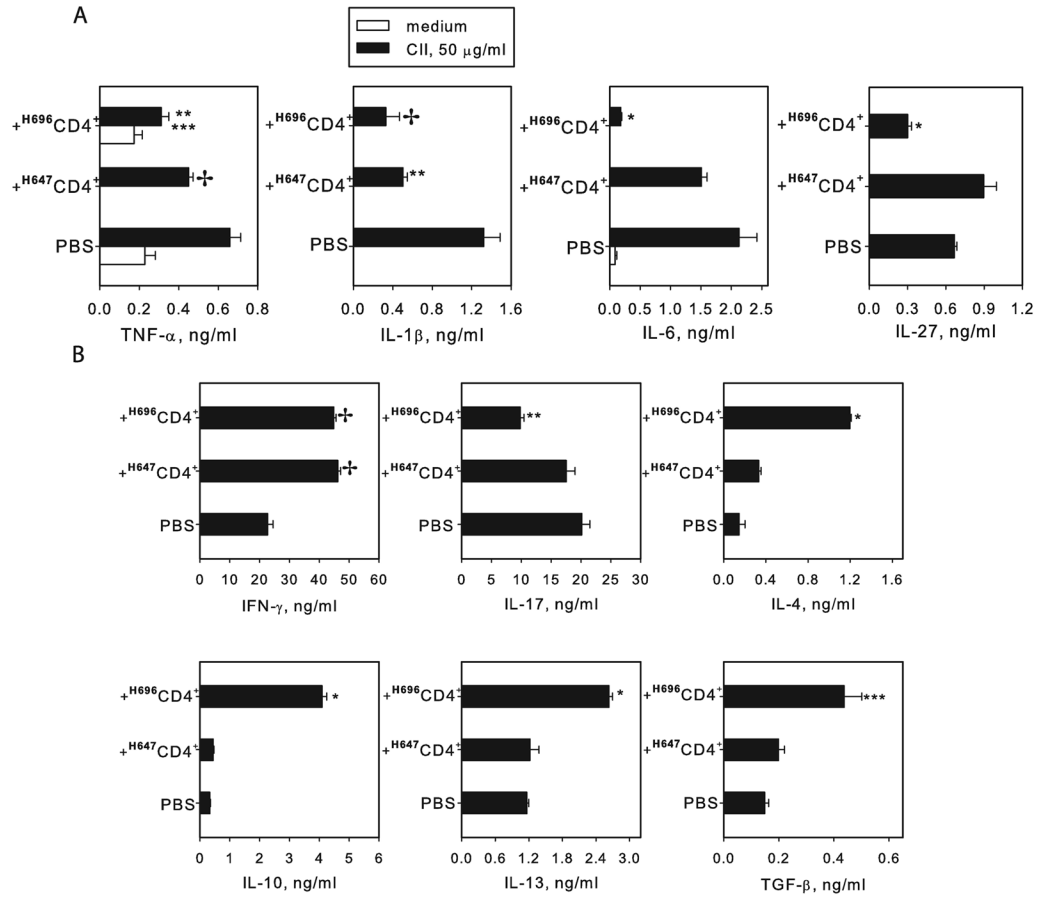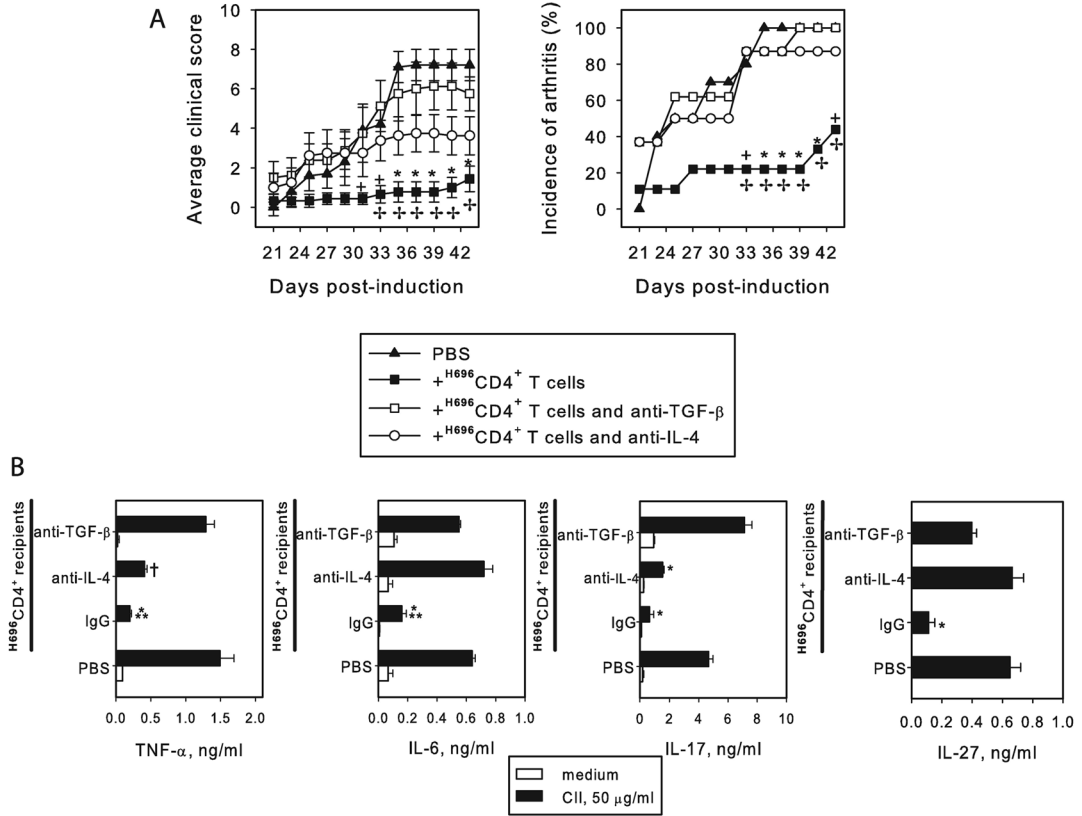
FIGURE 5.
Suppression of proinflammatory cytokine production by draining LN cells after adoptive transfer of *Salmonella*-CFA/I-primed CD4^{+} T cells. (*A*) Total mononuclear cells from the three treatment groups at the termination of the study in Fig. 4 were purified and cultured for 3 days with CII. TNF-α, IL-1β, IL-6, and IL-27 levels were measured in supernatants by ELISA. Data represent the mean ±SEM of 6 replicates. * $P < 0.001$ as compared to PBS control and *Salmonella* vector (H647) primed CD4^{+} T cells recipients; ** $P < 0.01$, *** $P < 0.05$ as compared to PBS control; ✥ $P < 0.05$ as compared to *Salmonella* vector (H647) primed CD4^{+} T cells recipients. (*B*) Recipients of total CD4^{+} T cells from *Salmonella*-CFA/I-vaccinated mice show enhanced production of Th2-type and regulatory cytokines. CD4^{+} T cells purified from draining LN of recipients given either *Salmonella*-CFA/I-, *Salmonella* vector-primed CD4^{+} T cells, or PBS control mice (at the termination of the study in Fig. 4) were restimulated with CII in presence of syngenic APC for 5 days. Data represent mean cytokine concentration ± SEM from two experiments. * $P < 0.001$, ** $P < 0.005$, *** $P < 0.05$ as compared to PBS group and recipients of *Salmonella* vector primed CD4^{+} T cells; ✥ $P < 0.001$ as compared to PBS control.

FIGURE 6.
The protective impact conferred by *Salmonella*-CFA/I-induced CD4^{+} T cells is abated upon anti-IL-4, anti-IL-10, or anti-TGF-β treatment resulting in the restoration of CII-specific CD4^{+} T cell proliferation. (*A*) Mice were immunized with *Salmonella*-CFA/I, as previously described, and 14 days later, MLN and HNLN CD4^{+} T cells were cell-sorted and restimulated with CFA/I fimbriae for 24 hrs. Effector CD4^{+} T cells from mice with CIA were purified 15 days after CII challenge and labeled with CFSE. These were added to CFA/I-restimulated CD4^{+} T cells in the presence of irradiated APCs, CII, and neutralizing quantities of anti-IL-4, anti-TGF-β, or anti-IL-10 mAbs or normal rat IgG. Flow cytometry analysis of CFSE^{+} CD4^{+} T cells was performed after 5 days of incubation. (*B*) Frequency of FoxP3 expression by CII-specific CFSE^{+} CD4^{+}CD25^{+} T cells. Data represent the mean of three replicates from each culture ± SEM. Significant differences are depicted: *P<0.001 as compared to control and anti-IL-4, anti-TGF-β, or anti-IL-10 mAb-treated cells.

FIGURE 7.
Treatment with anti-IL-4 or anti-TGF-β mAbs *in vivo* compromises protection to CIA by *Salmonella*-CFA/I-induced CD4$^+$ T cells. (*A*) CD4$^+$ T cells were purified from pooled HNLN and MLN of mice immunized with *Salmonella*-CFA/I 15 days post-immunization and adoptively transferred to mice in which CIA was induced 2 wks earlier. Corresponding Ab treatments were conducted at weekly intervals starting the day of adoptive transfer for a total of four doses. Each group contained 8 – 10 mice. * $P < 0.005$, $^+$ $P < 0.05$ as compared to PBS group; ✤ $P < 0.05$ as compared to anti-IL-4 or anti-TGF-β mAb-treated mice. (*B*) Restored production of proinflammatory cytokines in anti-IL-4 and anti-TGF-β mAb-treated mice. Draining LN were collected after following the disease course, and 5×10^6 cells/ml were re-stimulated with CII for 3 days. TNF-α, IL-6, and IL-17 were measured in supernatants by capture ELISA. Data depict mean value of 6 replicates ± SEM. * $P < 0.005$ as compared to PBS or anti-TGF-β mAb-treated group; ** $P < 0.05$ as compared to anti-IL-4 mAb-treated group; † $P < 0.005$ as compared to anti-TGF-β mAb-treated or PBS groups.

Table I

Treatment with anti-TGF-β and anti-IL-4 mAbs weakens therapeutic potential of *Salmonella*-CFA/1 induced CD4^{+} T cells. [a]

Treatment [b]	CIA/Total [c]	Day Onset [d]	Max. Score [e]	Average score [f]	CS [g]
PBS	10/10	27.8 ± 1.6	10	7.2 ± 0.78	50.4 ± 7.11
H696CD4^{+} T cells and IgG	4/10	33 ± 5.35	4	1.44 ± 0.65 [*] [†]	7.78 ± 4.65 [*] [†]
H696CD4^{+} T cells and anti-IL-4	8/8	26.71 ± 2.29	8	3.62 ± 0.96	34.87 ± 11.09
H696CD4^{+} T cells and anti-TGF-β	8/8	27.25 ± 2.43	12	5.75 ± 0.86	49.37 ± 10.13

[a] Arthritis was induced in DBA/1 mice with 100 μg bovine CII in complete Freund's adjuvant on day 0.

[b] *Salmonella*-CFA/1 primed CD4^{+} T cells were adoptively transferred to mice with induced arthritis on day 15. Intra-peritoneal mAb treatments were initiated on day of transfer, then weekly for a total of four doses (1 mg total of mAb/mouse).

[c] Number of mice with CIA /total in a group for 43 days after CII challenge.

[d] Mean day ± SEM of first symptoms onset in diseased mice only.

[e] Maximum score in group in entire observation period.

[f] Average clinical score per group on day 43 post-induction (end of observation period) calculated as sum of individual scores divided by the number of mice in group ± SEM.

[*] $P < 0.005$ as compared to PBS group;

[†] $P < 0.05$ as compared to anti-IL-4 or anti-TGF-β mAb treated group.

[g] Cumulative scores (CS) calculated as all scores during the period of observation divided by number of mice in each group.

[*] $P < 0.005$ as compared to PBS and anti-TGF-β treated groups;

[†] $P < 0.05$ as compared to anti-IL-4 treated group.

Table II

CD4^{+} T cells characterization on day 14 post-immunization with *Salmonella*-CFA/I

CD4^{+} T Cell Subset	Head and Neck LN			MLN		
	PBS	H647	H696	PBS	H647	H696
%CD25$^+$CD4$^+$	9.60 ± 0.11	10.38 ± 0.29	$\mathbf{12.87 \pm 0.58}^{\dagger}$	10.32 ± 0.23	9.37 ± 0.25	$\mathbf{13.73 \pm 0.34*}$
%FoxP3$^+$CD25$^+$CD4$^+$	9.96 ± 0.53	9.92 ± 1.19	$\mathbf{14.96 \pm 1.16}^{\dagger}$	24.08 ± 4.82	21.93 ± 1.1	$\mathbf{41.19 \pm 2.97}^{\dagger}$
%CD39$^+$CD25$^+$CD4$^+$	5.34 ± 0.76	6.38 ± 1.32	2.89 ± 1.38	4.96 ± 0.26	4.74 ± 1.40	$\mathbf{16.53 \pm 1.89}^{\ddagger}$
%CD39$^+$FoxP3$^+$CD25$^+$CD4$^+$	14.21 ± 0.75	19.54 ± 2.79	17.54 ± 2.56	18.14 ± 2.06	13.70 ± 2.20	$\mathbf{41.34 \pm 2.44}^{\ddagger}$
%CD39$^+$FoxP3$^-$CD25$^+$CD4$^+$	3.83 ± 0.42	4.82 ± 1.43	2.81 ± 0.14	6.64 ± 0.38	5.67 ± 1.44	$\mathbf{12.5 \pm 1.88}^{\dagger}$
%FoxP3$^+$CD25$^-$CD4$^+$	1.30 ± 0.24	0.62 ± 0.08	1.88 ± 0.45	6.48 ± 0.63	4.89 ± 1.52	$\mathbf{11.06 \pm 2.5}^{\dagger}$
%CD39$^+$CD25$^-$CD4$^+$	2.90 ± 0.75	3.42 ± 0.55	1.40 ± 0.07	3.05 ± 0.52	2.33 ± 0.77	$\mathbf{15.03 \pm 1.43*}$
%CD39$^+$FoxP3$^+$CD25$^-$CD4$^+$	9.75 ± 1.44	9.43 ± 1.65	8.41 ± 1.87	11.21 ± 0.95	8.77 ± 1.72	$\mathbf{31.97 \pm 2.67}^{\ddagger}$
%CD39$^+$FoxP3$^-$CD25$^-$CD4$^+$	4.1 ± 1.12	3.51 ± 0.89	1.70 ± 0.23	4.42 ± 0.84	2.67 ± 0.74	$\mathbf{17.44 \pm 1.27*}$
%CD39$^+$CD4$^+$	1.92 ± 3.16	3.81 ± 0.89	1.66 ± 0.22	3.02 ± 0.25	2.56 ± 0.61	$\mathbf{16.59 \pm 1.43*}$

* $P < 0.001$;

‡ $P < 0.005$;

† $P < 0.05$ as compared to PBS and H647 groups.