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Vaccination without Autoantigen Protects against Collagen II-Induced Arthritis via Immune Deviation and Regulatory T Cells

Irina Kochetkova, Theresa Trunkle, Gayle Callis, and David W. Pascual

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 Ag 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. The incidence of arthritis and cartilage loss in vaccinated DBA/1 mice was remarkably lower when compared with 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-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 postchallenge. 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 collagen-induced arthritis by stimulating TGF-β- and IL-4-producing regulatory CD4⁺ T cells. The Journal of Immunology, 2008, 181: 2741–2752.

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.

Rheumatoid arthritis (RA) 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 *Escherichia 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 induce 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 provides 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 (PLP139–151) 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 regulatory (Treg) 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/I 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 autoantigen 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 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 independently of the autoantigen.

Materials and Methods

Mice

DBA/I male 6- to 8-wk-old mice were obtained from The Jackson Laboratory. 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 Δasd Salmonella enterica 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 × 10⁹ CFU Salmonella-CFA/I or its isogenic strain H647 (without CFA/I oporom) 7 days before CIA challenge. Control mice received sterile PBS. For induction of arthritis, 100 μg of bovine CII (Chondrex) emulsified in CFA containing LP50 M. tuberculosis (Chondrex) were injected s.c. in the tail 2 cm from mouse body (20). Generally, by the end of wk 5 postchallenge, 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; and 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 CIA 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. H&E 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 degradation; and 3 - heavy inflammatory infiltration and debris in the joint, severe chondrocytes and cartilage matrix loss with new bone tissue substitution, bone destruction. Paws and joint knee 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; and 3 - severe cartilage degeneration through tidemark.

Anti-CFA/I and anti-CHAb ELISA

Serum and fecal samples were collected on day 21 after immunization with Salmonella-CFA/I. Samples were tested against purified CFA/I fimbriae, as previously described (14, 17), and against CII (Chondrex). Serum samples from mice with induced arthritis were collected on day 21 postchallenge with CIA, and samples of dilute sera were added to microtiter wells (Maxisorp ImmunoPlate II microtiter plates; Nunc) coated with 2 μg/ml ELISA Grade bovine or mouse CII (Chondrex). For both Ab ELISAs goat anti-mouse HRP-labeled IgG1, IgG2a, IgG2b, or IgA (Southern Biotechnology Associates) were used as detecting Abs. Enzymatic reaction was developed with ABTS (Moss). OD was read at 415 nm using ELx808 microplate reader (Bio-Tek Instruments). 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 postinduction of arthritis challenge), axillary, inguinal, and popliteal lymph node (LN) cells were purified and total mononuclear cells (5 × 10⁶ 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), 2 mM l-glutamine, 50 μM 2-ME (Sigma-Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 0.1 μM nonessential amino acids. TNF-α, IL-6, IL-17, and IL-27 were measured from culture supernatants (16). In brief, wells were coated with purified anti-mouse mAbs: TNF-α (5 μg/ml; BD Pharmingen), anti-IL-1β (3 μg/ml; R&D Systems), 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 h at 37°C, supernatants were incubated overnight at 4°C. For detection, 0.5 μg/ml 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 h at 37°C. Reactions were developed using 1/1000 HRP-goat-anti-biotin Ab (Vector Laboratories) for 1 h at room temperature RT followed by ABTS substrate (Moss) Recombinant mouse TNF-α, IL-6, IL-17, and IL-27 (R&D Systems), and IL-1β (PeproTech) were used to generate standard curves.

T cell assays

Axillary, popliteal, and inguinal LNs were isolated on day 15 postchallenge. CD4+ T cells were purified using Dynal Mouse CD4 Negative Isolation Kit (Invitrogen) to >95% purity. Purified CD4+ T cells were resuspended in complete medium, and 2.5 × 10⁶ cells/well in 200 μl were restimulated with varying doses of T Cell Proliferation Grade CII (Chondrex) in the presence of syngenic mitomycin C-treated APCs. After 48 h, cells were pulsed with 0.5 μCi/well of [3H]HTdR for 18 h. Samples were harvested, and incorporated [3H]HTdR was measured by scintillation counting.

To measure cytokine-forming cells (CFCs), CD4+ T cells were restimulated at 10⁴/ml with 50 μg/ml CII for 3 days, then added to nitrocellulose-based wells (MultiScreen-HA; Millipore) 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) with precipitable substrate, AEC (Moss). Spots were enumerated and normalized per 10⁶ cells.

To characterize the cytokine profiles of CD4+ T cells induced by Salmonella-CFA/I or those induced by Salmonella vector, on day 15 postimmunization, mesenteric LN (MLN) CD4+ T cells were sorted for CD4+CD25+ and CD4+CD25− T lymphocytes using CELLection Biotin Binder Kit (Invitrogen). Each CD4+ T cell subset (10⁵ cells) was stimulated with 10 μg/ml plate-bound anti-CD3 mAb (BD Pharmingen) and 10 μg/ml 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).

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Adaptive 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 postimmunization, 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 2 × 10⁷ CD4⁺ T cells or an equivalent amount of CD4⁺ T cell subset: ~1.5 × 10⁶ CD4⁺CD25⁻ T cells and ~5 × 10⁴ 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) and anti-mouse TGF-β (1D11.16.8; ATCC) were expanded in serum-free supplemented HB101 media (Irvine Scientific). The mAbs were purified from culture supernatants using protein G (Sigma-Aldrich) affinity chromatography. CD4⁺ T cells from mice immunized with Salmonella-CFA/I were sorted for adoptive transfer into mice challenged 15 days earlier with CIA, 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 (HNHL) and MLN were purified. Immunofluorescent staining for surface CD4, CD25, CD39, glucocorticoid-induced TNF receptor (GITR), and intracellular Foxp3 was performed using fluorescence-labeled mAbs: CD4-FITC, CD4-PE-CY7, CD25-Percp-CY5.5, GITR-PE (BD Pharmingen), Foxp3-PE, Foxp3-AlexaFluor 647 (eBioscience), and polyclonal rabbit anti-CD39 (H-85) IgG (Santa Cruz Biosciences) paired with anti-rabbit IgG-FITC or IgG-PE (Jackson ImmunoResearch Laboratories). For proliferation assay on day 14 after immunization with Salmonella-CFA/I, CD4⁺ T cells from pooled HNHL and peripheral LN (effectors) were restimulated with 10 µg/ml CFA/I during 24 h. CII-specific CD4⁺ T cells (responders) were purified on day 15 after induction of arthritis, labeled by CFSE, as previously described (24), and cocultured 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 3 × 10⁶/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 FACSCalibur, LSRII, or Canto (BD Biosciences). CellQuest (BD Biosciences) and FlowJo (Tree Star) 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, and 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 whether Salmonella-CFA/I vaccine could result in protection against CIA, DBA/1 mice were orally vaccinated with 5 × 10⁹ CFU of Salmonella-CFA/I (strain H696), and then CIA was induced 1 wk later. Control mice were given the isogenic Salmonella vector (strain H647) or sterile PBS. Three weeks following CII challenge, ~90% of the PBS-dosed group developed CIA with a maximum average clinical score of ~4 (Fig. 1A). 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. 1B).

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 postchallenge, 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 with 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. 2A), similar to that obtained with other mouse strains (13, 14, 17, 18). Likewise, oral immunization with this vaccine does not induce production of Abs that cross-react to CII (Fig. 2B).

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 whether immunization with Salmonella-CFA/I protects from excessive inflammation by altering the Ab response to CII, serum samples were collected on day 21 postinduction of arthritis, and Ab titers to CII were measured. All groups developed equivalent IgG1, IgG2a, and IgG2b responses to CII (Fig. 2C). 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. 3A). 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. CFCs were incubated with CII for 72 h before cytokine production was measured. Purified LN CD4⁺ T cells restimulated with CII for 72 h resulted in a 3- and 4-fold increase of CII-specific CD4⁺ T cells producing TGF-β and IL-4, respectively, by comparison with CII-specific CD4⁺ T cells from mice given the Salmonella-CFA/I-vaccinated mice when compared with PBS- and vector-immunized mice (p ≤ 0.01; Fig. 3B). No significant differences in IL-13 or IL-10 CFCs were observed between Salmonella-CFA/I-immunized and PBS-dosed groups. Significant suppression of IFN-γ and IL-17-producing CD4⁺ T cells from Salmonella-CFA/I-vaccinated mice when compared with Salmonella-CFA/I-immunized mice or PBS group was also noted (Fig. 3B). Along with the decreased Th1 and Th17 cell activities, suppressed production of IL-27 was observed by whole
TNF-α results in suppression of proinflammatory cytokines (H696). D was obtained using a Mann-Whitney U mean protected and unprotected mice. Results depict the loss evaluated on sections stained with toluidine blue in termined on H&E-stained sections and level of cartilage score analysis; arthritis; Mann-Whitney ability test was applied to analyze the incidence of ar-thritis developed in mice immunized with vector; synovial hyperplasia and mild edema in the knee joint of mice vaccinated with vector. Student’s t test was used for statistical analysis. One of three experiments is depicted.

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 paw joints sections were prepared on day 50 after challenge with bovine CII and stained with H&E (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 postchallenge and stimulated for 3 days with CII. Cytokines concentrations were determined from collected supernatants by ELISA. *, p < 0.001 as compared with PBS group and mice immunized with Salmonella vector; and **, p < 0.05 as compared with PBS group and mice immunized with Salmonella vector. Student’s t test was used for statistical analysis. One of three experiments is depicted.

LN cultures derived from protected mice upon in vitro CII re-stimulation (Fig. 3C). 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 about 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. 4B). IL-17 was significantly reduced (p < 0.05) in both CD4+ T cell subsets from Salmonella-CFA/I-vaccinated mice (Fig. 4B). 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. 4B). 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. 4B), 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. 4C) 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 with untreated control mice (Fig. 4C). 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. 4C); 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. 4C). 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. 4C). 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 postchallenge 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 with diseased (PBS-dosed) mice (Fig. 5A). Cytokine production, except for IL-27, by recipients given Salmonella vector-primed cells was also lower compared with diseased mice, but TNF-α and especially IL-6 (which was still 5-fold greater) remained elevated when compared with control.
with recipients given Salmonella-CFA/I-primed CD4+ T cells (Fig. 5A).

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. 5B) with concomitant reduction in IL-17 (Fig. 5B). 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 with 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 CIA-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 CIA-specific proliferation by effector CD4+ T cells.

**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 six replicates. *, p < 0.001 as compared with PBS control and Salmonella vector (H647) primed CD4+ T cells recipients; **, p < 0.01; and ***, p < 0.05 as compared with 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; and ***, p < 0.05 as compared with PBS group and recipients of Salmonella vector-primed CD4+ T cells; ++, p < 0.001 as compared with 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 CIA-specific CD4+ T cell proliferation. A, Mice were immunized with Salmonella-CFA/I, as previously described, and 14 days later, MLN and HLN CD4+ T cells were cell-sorted and restimulated with CFA/I fimbriae for 24 h. Effector CD4+ T cells from mice with CIA were purified 15 days after CIA induction and labeled with CFSE. Flow cytometry analysis of CFSE+CD4+ T cells was performed after 5 days of incubation. B, Frequency of FoxP3 expression by CIA-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 with control and anti-IL-4, anti-TGF-β, or anti-IL-10 mAb-treated cells.
Salmonella-CFA/I CD4+ T cells were purified from combined LNs on day 14 postimmunization. These cells were restimulated with purified CFA/I fimbriae for 24 h in the presence of 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. 6A). 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 cocultures.

Table I. Treatment with anti-TGF-β and anti-IL-4 mAbs weakens therapeutic potential of Salmonella-CFA/I induced CD4+ T cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CIA/Total</th>
<th>Day Onset</th>
<th>Maximum Score</th>
<th>Average Score</th>
<th>Cumulative Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>10/10</td>
<td>27.8 ± 1.6</td>
<td>10</td>
<td>7.2 ± 0.78</td>
<td>50.4 ± 7.11</td>
</tr>
<tr>
<td>H696CD4⁺ T cells and IgG</td>
<td>4/10</td>
<td>33 ± 5.35</td>
<td>4</td>
<td>1.44 ± 0.65*</td>
<td>7.78 ± 4.65*</td>
</tr>
<tr>
<td>H696CD4⁺ T cells and anti-IL-4</td>
<td>8/8</td>
<td>26.71 ± 2.29</td>
<td>8</td>
<td>3.62 ± 0.96</td>
<td>34.87 ± 11.09</td>
</tr>
<tr>
<td>H696CD4⁺ T cells and anti-TGF-β</td>
<td>8/8</td>
<td>27.25 ± 2.43</td>
<td>12</td>
<td>5.75 ± 0.86</td>
<td>49.37 ± 10.13</td>
</tr>
</tbody>
</table>

a Arthritis was induced in DBA/1 mice with 100 μg bovine CII in CFA on day 0.

Salmonella-CFA/I induced CD4+ T cells were adoptively transferred to mice with induced arthritis on day 15. i.p. mAb treatments were initiated on day of transfer, then weekly for a total of four doses (1 mg total of mAb/mouse).

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

c Mean day ± SEM of first symptoms onset in diseased mice only.

d Maximum score in group in entire observation period.

e Average clinical score per group on day 43 postinduction (end of observation period) calculated as sum of individual scores divided by the number of mice in group ± SEM. *, p < 0.005 as compared with PBS group; †, p < 0.05 as compared with anti-IL-4 or anti-TGF-β mAb-treated group.

f Cumulative scores calculated as all scores during the period of observation divided by number of mice in each group. *, p < 0.005 as compared with PBS- and anti-TGF-β-treated groups; †, p < 0.05 as compared with anti-IL-4-treated group.
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 with CIA 2 wk 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. 7A and Table I). Treatment with anti-IL-4 mAb resulted in significantly higher clinical scores and incidences of CIA to 90% (Fig. 7A). Although the clinical score of anti-IL-4 mAb-treated mice was lower than in PBS control mice or in anti-TGF-β mAb-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 CIA 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. 7B). 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. 7B). Such an inflammatory cytokine profile in anti-IL-4 mAb-treated mice is also inconsistent 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 2 wk 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 with 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+ Treg cells (26, 27), CD39 expression was also evaluated on both Treg and CD4+CD25− T cells. Nearly half of FoxP3+ CD25−CD4+ T cells were also CD39 positive, whereas only ~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 ~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 CFA. 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 CIA-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 whether 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 with 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 course of the joint pathology. Treatment of DBA/1 mice with

<table>
<thead>
<tr>
<th>T Cell Subset</th>
<th>HNLN</th>
<th>MLN</th>
</tr>
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<tbody>
<tr>
<td>%CD25+ CD4+</td>
<td>9.60 ± 0.11</td>
<td>10.38 ± 0.29</td>
</tr>
<tr>
<td>%FoxP3+ CD25+ CD4+</td>
<td>9.96 ± 0.53</td>
<td>9.92 ± 1.19</td>
</tr>
<tr>
<td>%CD39+ FoxP3+ CD25+ CD4+</td>
<td>5.34 ± 0.76</td>
<td>6.38 ± 1.32</td>
</tr>
<tr>
<td>%CD39+ FoxP3+ CD25+ CD4+</td>
<td>14.21 ± 0.75</td>
<td>19.54 ± 2.79</td>
</tr>
<tr>
<td>%FoxP3+ CD25+ CD4+</td>
<td>3.83 ± 0.42</td>
<td>4.82 ± 1.43</td>
</tr>
<tr>
<td>%FoxP3+ CD25+ CD4+</td>
<td>1.30 ± 0.24</td>
<td>0.62 ± 0.08</td>
</tr>
<tr>
<td>%CD39+ CD4+</td>
<td>2.90 ± 0.75</td>
<td>3.42 ± 0.55</td>
</tr>
<tr>
<td>%FoxP3+ CD25+ CD4+</td>
<td>9.75 ± 1.44</td>
<td>9.43 ± 1.65</td>
</tr>
<tr>
<td>%FoxP3+ FoxP3+ CD25+ CD4+</td>
<td>4.1 ± 1.12</td>
<td>3.51 ± 0.89</td>
</tr>
<tr>
<td>%CD39+ CD4+</td>
<td>1.92 ± 3.16</td>
<td>3.81 ± 0.89</td>
</tr>
</tbody>
</table>

* p < 0.001; †, p < 0.005; and ‡, p < 0.05 as compared with PBS and H647 groups.
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 fimbrae 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). In contrast, exacerbated arthritis in IL-10-deficient mice and IFN-γRα-knockout 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 in 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 CIA challenge. Likewise, IL-4−/− mice could not be tolerized with CIA, 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. (41) 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. 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 before immunization with CII resulted in a more severe CIA in DBA/1 mice, and the adoptive transfer of CD4+CD25+ T cells from naive mice to CD25− 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 Treg 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 (reviewed in Ref. 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 as a possible alternative marker for Treg cells (26, 27). Oral immunization with Salmonella-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-expressing 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 cosegregation 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 to assess the impact of 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 with 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 reduce 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 with protection conferred by combined CD25− and CD25+ CII−/− T cells. These results suggest that Salmonella-CFA/I may induce regulatory cells in both subsets, CD4+CII−/− 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 (reviewed in Ref. 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. (49) succeeded in inhibiting the development of CIA, Ab-induced arthritis, as well as suppressing EAE by immunization against IL-17 before induction of diseases. 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 a 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 rIL-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 production. CD4+ T cells, and neutralization of IL-4 significantly weakened the pro-inflammatory and enhanced IL-4 (53). In contrast, mice with CIA treated with rIL-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.

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Disclosures
The authors have no financial conflict of interest.

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Borregaard, N., J. M. Seyer, C. O. Tacket, and M. B. Sztein. 2001. Host-directed vaccines that express colonization factor antigen 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.

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