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Location of CD4+ T Cell Priming Regulates the Differentiation of Th1 and Th17 Cells and Their Contribution to Arthritis

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Th cytokines IFN-γ and IL-17 are linked to the development of autoimmune disease. In models of rheumatoid arthritis, that is, proteoglycan (PG)-induced arthritis, IFN-γ is required, whereas in collagen-induced arthritis, IL-17 is necessary for development of arthritis. In this study we show that the route of immunization determines the requirement for either IFN-γ or IL-17 in arthritis. Intraperitoneal immunization with PG induces a CD4+ T cell IFN-γ response with little IL-17 in the spleen and peripheral lymph nodes. However, s.c. immunization induces both an IFN-γ and an IL-17 CD4+ T cell response in spleen and lymph nodes. The failure to induce a CD4+ T cell IL-17 response after i.p. immunization is associated with T cell priming, as naive T cells activated in vitro were fully capable of producing IL-17. Moreover, PG-induced arthritis is converted from an IFN-γ to an IL-17-mediated disease by altering the route of immunization from i.p. to s.c. The histological appearance of joint inflammation (cellular inflammation and bone erosion) is similar in the i.p. versus s.c. immunized mice despite the presence of CD4+ T cells producing IL-17 in joint tissues only after s.c. immunization. These data indicate a critical role for the site of initial T cell priming and the Th cytokines required for susceptibility to arthritis. Our findings suggest that T cell activation at different anatomical sites in rheumatoid arthritis patients may skew the T cells toward production of either IFN-γ or IL-17.

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease manifested primarily in the synovial joints (1). RA is characterized by infiltration of leukocytes into the synovial lining and hyperplasia of the resident synoviocytes. The overproduction of cytokines, chemokines, autoantibodies, and other inflammatory mediators results in cartilage destruction, bone erosion, and pathological remodeling of joint structures. The clinical presentation of RA reveals striking heterogeneity, with a spectrum ranging from mild to severe. There is marked variability in the features of synovial inflammation among RA patients. The wide variation in responsiveness to different modes of anti-rheumatic treatment is consistent with the concept of heterogeneity and indicates that distinct molecular mechanisms underlie the disease process in RA (2). It is unclear how RA is initiated, but particular MHC alleles are strongly linked to RA, implicating a CD4 T cell response to self-Ags in the autoimmune process (3).

CD4+ T cell play a central role in the immune response through the production of cytokines that orchestrate immune reactivity. Distinct populations of CD4+ T cells are identified based on their production of cytokines. The two CD4+ Th subsets that play a critical role in autoimmune disease are Th1 cells that produce IFN-γ as their signature cytokine and Th17 cells that produce IL-17A, IL-17F, and IL-22 (4). The major determinant of Th cell differentiation is the presence of cytokine at the time of naïve T cell activation. Th1 cell differentiation is selectively induced under the influence of IL-12 and IFN-γ. These cytokines are important in the upregulation of transcription factors and the STAT proteins that drive differentiation. Th-bet, STAT1, and STAT4 are the major transcription factors for transcription of the IFN-γ gene (5–7). Th17 cells differentiate effectively when stimulated with a combination of TGF-β and IL-6; however, IL-21 can substitute for IL-6 whereas IL-23 is important for the maintenance of IL-17 production (8–10). IL-1 is also an important signal for IL-17 differentiation in vivo (11). In Th17 differentiating cells, the major transcript factor is retinoic acid–related orphan receptor (ROR)γt and to a lesser extent RORα, both of which are upregulated with TCR stimulation in the presence of TGF-β and IL-6 (12, 13). STAT3 is also activated by IL-6, IL-21, and IL-23 and synergizes with RORγt for the differentiation and maintenance of IL-17 (14, 15).

Several autoimmune disease models manifest different requirements for Th subsets. Proteoglycan (PG)-induced arthritis (PGIA) is a model of arthritis mediated by Th1 effector cells. We previously demonstrated that induction of PGIA requires IL-12, the IL-27 receptor, STAT4, and IFN-γ and is independent of IL-17 (16–19). In other autoimmune disease models, including collagen-induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE),...
and experimental autoimmune uveitis (EAU), despite high levels of IFN-γ, the involvement of Th1 cells in disease was not substantiated. It was found that the absence of IFN-γ or signaling through the IFN-γ receptor did not inhibit disease but in fact exacerbated disease (20–24). The discovery that IFN-γ inhibits IL-17 production provided the explanation for these findings (25–27). The enhanced disease observed in CIA and EAE in the absence of IFN-γ was due to an increase in IL-17. Studies confirmed the importance of IL-17 in CIA and EAE using IL-17-deficient mice and neutralization of IL-17 (28–32).

The requirement for Th1 versus Th17 in similar models of autoimmune arthritis highlights an important question, the answer to which could address underlying mechanisms that account for the heterogeneity of human autoimmunity.

Ag-specific T cell priming is dependent on the activation of innate immune cells (33). Several reports suggest that the route of Ag exposure may affect the differentiation of Th1 and Th17 cell populations. Epicutaneous versus i.p. sensitization with an allergen induces Th17 response (34). Mucosal exposure to infectious agents preferentially induced a Th17 response (35–37). In contrast, splenic dendritic cells (DCs) produced IL-12, which is important for the differentiation of Th1 cells (38). These findings raise the question of whether EAE, EAU, and CIA are Th17-mediated autoimmune diseases because they are induced by s.c. and intradermal (i.d.) immunization, respectively.

We report in this study that tissue-specific microenvironments program the requirement for Th1 versus Th17 cells in the induction of arthritis. Exposure to Ag by the i.p. route induces a predominately IFN-γ response with very little IL-17, whereas exposure to Ag by the s.c. route induced both an IFN-γ and IL-17 response. We found that production of IL-17 correlates with the requirement for IL-17 in the development of arthritis. In PGIA, development of arthritis after immunization by the i.p. route is independent of IL-17; however, PGIA can be converted to an IL-17–dependent arthritis by immunization by the s.c. route.

Materials and Methods

Mice

BALB/c Charles River (Kingston colony) mice are the most PGIA-susceptible BALB/c subline. IL-17−/− mice were backcrossed to BALB/c for eight generations (39) and further backcrossed to BALB/c (Kingston colony) for two generations, and then intercrossed to obtain wild-type (WT) and IL-17−/− littermates. BALB/c IFN-γ−/− and BALB/c congenic CD90.1 (Thy1.1) mice were obtained from The Jackson Laboratory. BALB/c IFN-γ−/− mice were further backcrossed to BALB/c mice from the Kingston colony for three generations. IL-12p40−/− and IL-2p35−deficient mice were obtained from The Jackson Laboratory. IL-6−/− mice were provided by Dr. Ken Tung (University of Virginia). TCR-Tg 5/4E8, designated 5/4E8, which are specific for an immunodominant peptide in the human G1 domain of PG that cross-reacts with mouse G1, were generated as described (40). Female BALB/c mice age-matched for 12–14 wk were used in all experiments. Animal experiments were approved by the Institutional Animal Care and Use Committee.

Induction and assessment of arthritis

PG was obtained from human knee joint cartilage following joint replacement surgery and provided by the Orthopedic Transplant, Transplant, and Implant Repository at Rush University Medical Center with the approval of the Institution Review Board. PG was isolated as previously described (41). Recombinant human aggregan G1-domain protein cloned from human chondrocytes consists of 351 aa of the G1 domain and 59 aa of the interglobular domain of PG. The fusion protein composed of human G1 domain cDNA and the mouse IgG2a-Fc fragment construct was inserted into the mammalian expression vector Lonza PEE14.1 plasmid. G1/Fc was purified on a protein G-Sepharose column (Amersham/Fischer Scientific). The purity and quantity of the G1 domain was determined on Western blot using an anti-G1 Ab (G18mAb) (42).

For the induction of PGIA, mice were immunized either i.p. or s.c. with 100 μg PG emulsified in 2 mg dimethyldioctadecyl ammonium bromide (DDA) (Sigma-Aldrich) or 40 μg rG1 protein in 1 mg DDA in a volume of 100 μl. Mice were boosted at 3 and 6 wk. Development of arthritis was monitored twice weekly and scored in a blinded manner. An established scoring system was used to score paw swelling on a scale of 0–4 as follows: 0, normal; 1, mild erythema and swelling of several digits or part of the paw; 2, moderate erythema and swelling of digits and paw; 3, more diffuse erythema and swelling; and 4, severe erythema and swelling of the complete paw with ankylosis. Incidence of arthritis denotes the percentage of the total that develop PGIA. A cumulative score ranging from 0 to 16 is based on individual paw scores of 0–4. Groups of at least 8–10 mice were immunized and the experiments were repeated.

T cell proliferation in vivo and in vitro

To assess T cell proliferation in vivo, CD4+ 5/4E8 T cells were prepared from the spleen by MACS negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4+ 5/4E8 T cells were labeled with CFSE (Invitrogen, Carlsbad, CA). CFSE-labeled 5/4E8 Thy1.2 CD4+ T cells (5 × 10⁶) were injected i.v. into Thy1.1 congenic WT mice. Twenty-four hours after cell transfer, mice were immunized with saline, DDA, rG1, or rG1/DDA either i. p. or s.c. Proliferation of 5/4E8 Thy1.2 T cells in the spleen and peripheral lymph nodes (LN)s was measured at 24, 36, 48, and 72 h by the reduction of CFSE label over time by flow cytometry. Cytokine production by Thy1.2 5/4E8 T cells was measured on day 5 by intracellular staining by flow cytometry. In some experiments mice were treated orally with 30 μg FTY720/mouse or vehicle control on the day of immunization daily until day 5.

CD4+ T cells from spleen and peripheral LNs were purified by negative selection using MACS (Miltenyi Biotec), and in some cases naive and memory T cells were prepared by positive selection of CD62L+ by MACS (Miltenyi Biotec). CD4+ T cells (2.5 × 10⁶/well) were incubated with mitomycin-treated naive spleen cells (2.5 × 10⁶/well) as APCs in the absence or presence of PG (20 μg/ml) or rG1 (2 μg/ml). Spleen and periphe-

ical LNs (5.0 × 10⁶/well) were incubated in the absence or presence of PG (20 μg/ml) or rG1 (2 μg/ml). Naive CD62LCD4+ T cells were stimulated with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) for 3 d and supernatants were harvested. T cells depleted from spleen or LNs of immunized mice using positive selection of CD90.2 cells by MACS and then mitomycin treated and incubated with 5/4E8 T cells. Cells were cultured in RPMI 1640 medium containing 5% FBS and supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine for 5 d. Supernatants were assayed for cytokines by ELISA, including IFN-γ, IL-17, and TNF-α (R&D Systems, Minneapolis, MN).

Flow cytometry

Spleen, LNs, and joint cells were extracted from immunized mice at the time of sacrifice and analyzed by flow cytometry. Joint tissues were minced and treated with collagenase (0.25 mg/ml) and cell populations in the joints were assessed using anti–CD4, anti–CD8, anti–Gr-1, anti–CD11b, and anti–B220. Intracellular cytokine staining, cells were stimulated with PMA (25 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich) and treated with GolgiPlug (BD Pharminogen) for 4 h. After cell surface staining, cells were permeabilized using the Cytofix/Cytoperm Plus kit (BD Pharmingen) and stained for anti–CD44–allophycocyanin–C7.7, anti–IFN-γ–allophycocyanin, and anti–IL-17a–FITC. Chemokine receptors were measured on joints tissue CD4+IFN-γ or CD4+IL-17+ T cells using anti–CXCR3-PE, anti–CCR4-PE–Cy7, and anti–CCR6-PerC–Cy5.5. Alexa Fluor 488 (AF-488)-g1-associated APCs were identified using anti–CD11c-PE–Cy7. CD11c+ cells were gated on CD8 and B220+ cells. CD11c+CD8–B220– cells were further gated on CD205+ and CD130+ cells. DC cells that were Ag positive were then examined for C11b expression. Similar gating strategy was used for spleen and inguinal LNs. The Abs were anti–CD11c-PE–Cy7, anti–CD11b-PerCP–Cy5.5, and anti–CD8–allophycocyanin–Cy7, anti–B220–PE–allophycocyanin, and anti–CD103–Pacific Blue. Cells were analyzed using a FACS Canto II instrument (BD Flow Cytometry Systems, San Jose, CA).

Detection of APCs

To detect which APCs were associated with Ag after immunization we labeled rG1 with Alexa Fluor. AF488–labeled rG1 (2mg/ml in PBS) was prepared using an AF488 protein labeling kit (Invitrogen). Briefly, 0.5 ml protein and 50 μl 1 M bicarbonate were mixed and then incubated with AF488 for 1 h at room temperature. The labeled protein was separated from free dye using BioGel P-30 fine size exclusion purification resin (Bio-Rad). Mice were immunized with AF488-G1/DDA by either the i. p. or s.c. route. Spleen and inguinal LN tissues were harvested 24 h later and treated with collagenase (0.25 mg/ml) to release tissue-adherent APCs. AF488-G1–labeled cells were detected and phenotyped by flow cytometry.
Histology
Hind ankle joints of immunized mice were isolated. Joints were fixed in 10% neutralized formalin, decalcified in 5% formic acid, and embedded in paraffin. At least three sections per paw (~200 μm apart) were stained with H&E. Cellular infiltration and bone erosion were measured on a scale of 0–4 by a blinded observer and values represent mean ± SD of four mice.

Measurement of rheumatoid factor in serum
Mouse IgG-type rheumatoid factor (RF) was measured in mouse IgG2a-Fc–coated plates as previously described (42). IgG-type RF was measured in serially diluted (1:2000 to 1:8000) serum samples. IgG-type RF was detected using polyclonal Abs to mouse G1.

Quantitative RT-PCR
Spleen and inguinal LNs were harvested 6 h after immunization i.p. or s.c. T cells were depleted using Thy1.2 magnetic beads and RNA isolated using TRI-Reagent (Molecular Research Center, Cincinnati, OH). Reverse transcription was performed with random hexamers for priming and Superscript II reverse transcriptase (Invitrogen). Gene-specific amplification was performed using QuantitFast SYBR Green PCR master mix (Qiagen) and normalized to β-actin levels for each sample. All samples were run in triplicate on a Bio-Rad iQ5 machine using Bio-Rad proprietary iQ5 software. To confirm that the same amount of RNA was added to each PCR reaction, murine β-actin amplification was performed on each sample. Relative fold induction was calculated using the 2−ΔΔCT method where ΔΔCT = ΔCTtreatment − ΔCTcontrol. ΔCT is ΔCTtarget gene − CTβ-actin and CT is the cycle at which the threshold is crossed. PCR product quality was monitored using post-PCR melt curve analysis. Controls were from naive nonimmunized spleen and inguinal LNs. Fold increases were normalized to naive cell populations.

Statistical analysis
All significance was determined using computer-based statistics (PC statistical software from SPSS, Chicago, IL). Statistical differences in disease severity among more than two groups were determined using two-way ANOVA. All data are presented as the mean ± SD of four mice. Additional calculations were performed with the post-hoc Bonferroni test. Differences were considered significant when p value <0.05 was considered significant.

Results
Th1-dependent PGIA correlates with the production of IFN-γ and not IL-17
We previously reported that PGIA induced by immunization i.p. with PG in CFA is inhibited in IFN-γ−/− mice, whereas arthritis in WT and IL-17−/− mice is similar (17, 19). Likewise, using the rG1 domain of PG in DDA, PGIA was suppressed in IFN-γ−/− mice immunized i.p., and arthritis developed in WT and IL-17−/− mice in a similar manner (Fig. 1A, 1B). Splenocytes from arthritic WT mice stimulated in vitro with rG1 produced a robust IFN-γ response as well as a substantial number of IFN-γ–producing CD4+ T cells in comparison with limited IL-17 secretion and few IL-17–producing T cells (Fig. 1C–F). Because arthritis develops during many weeks, alternation in the cytokine profile may occur over time. We therefore assessed whether at an earlier time point after immunization IL-17–producing cells could be identified. Splenic CD4+ T cells isolated 9 d after immunization i.p. with rG1 and stimulated in vitro with rG1 produced a strong IFN-γ response but very little IL-17 (Fig. 1G). Analogous results were observed after i.p. immunization with rG1/CFA (Fig. 1H). These data demonstrate a correlation between the ability of Th cells to produce IFN-γ and their requirements in arthritis. Additionally, immunization by the i.p. route skewed the response toward an IFN-γ–producing T cell. This is not due to the adjuvant used for immunization, as neither rG1/DDA nor rG1/CFA induced an IL-17 response after i.p. immunization.

Subcutaneous immunization induces an IL-17 response
CIA, EAE, and EAU are classically induced by i.d. or s.c. administration of Ag, in contrast to PGIA, which has historically been stimulated by the i.p. route. To determine whether the immunization route, i.p. versus s.c., influences the differentiation of cytokine-producing T cells, we first assessed the location of T cell priming after i.p. and s.c. immunization. CFSE-labeled Thy1.2 CD4+ T cells from TCR transgenic mice, 5/4E8 specific for a peptide in the human G1 domain of PG, were transferred into Thy1.1 congenic WT mice and immunized 24 h later, a time point when the percentage of 5/4E8 T cells was similar in spleen and peripheral LNs. Twenty-four hours after 5/4E8 T cell transfer, mice were immunized with rG1/DDA or as controls with either saline, DDA, or rG1 alone. 5/4E8 T cells did not proliferate in saline- or DDA alone–treated mice (data not shown). 5/4E8 T cell proliferation to rG1 alone was earlier than for rG1/DDA, but the proliferation was not sustained (data not shown). We focused on rG1/DDA, as rG1 alone does not induce arthritis. Proliferation of 5/4E8 T cells was tracked over a 24- to 72-h period. In the rG1/DDA i.p. immunized mice, 5/4E8 T cell proliferation was first detected at 36 h in the spleen, mesenteric LNs, and mediastinal LNs (Fig. 2A), followed by the lumbar LNs at 48 h and inguinal and brachial LNs at 72 h. In s.c. immunized mice, 5/4E8 T cell proliferation was initially detected in inguinal LNs at 36 h but not in other LNs until after 48 h (Fig. 2A, 2C). In general there was evidence of 5/4E8 T cell proliferation in spleen and all LNs by 72 h. We next assessed whether there was a difference in the cytokine-producing cells after i.p. versus s.c. immunization. IFN-γ–producing 5/4E8 T cells were found in spleen on day 5 after 5/4E8 T cell transfer irrespective of the route of immunization. In the LNs, IFN-γ–producing 5/4E8 T cells were found at sites of T cell priming. IFN-γ was produced by T cells after i.p. immunization in mediastinal, mesenteric, and lumbar LNs and after s.c. immunization in inguinal and lumbar LNs (Fig. 2B, 2D). The reduced IFN-γ in some of the LNs from either i.p. or s.c. immunization correlated with reduced T cell numbers at these sites. We found a significant increase in the number of IL-17–producing 5/4E8 T cells after immunization by the s.c. route in spleen, inguinal, and lumbar LNs in comparison with the i.p. route of immunization (Fig. 2B, 2E). Additionally, the percentage of IL-17–secreting CD4+ T cells relative to the total IFN-γ– and IL-17–producing T cells increased significantly in spleen and in mesenteric, inguinal, and lumbar LNs after immunization by the s.c. route (Fig. 2F). In the mesenteric LNs there was a population of IFN-γ/IL-17 double-positive 5/4E8 T cells in both the i.p. and s.c. immunized mice. The number of IFN-γ/IL-17 double-positive 5/4E8 T cells in the i.p. route (36.12 ± 5.74) was not significantly different from the s.c. route (29.90 ± 10.11) (n = 5–6). The results presented in this study indicate that immunization by the s.c. route preferentially generates a CD4+ IL-17 response.

T cell priming in the draining LN after s.c. immunization induces Th17 differentiation
IL-17–producing T cells contribute to the pathology of inflammatory skin disorders (43). We hypothesized that disruption of the skin barrier in the absence of overt immunization could lead to an increase in memory IL-17–producing cells in the draining LNs. Factors that contribute to an increase in memory Th17 cells could potentially influence Th17 priming in the LN. We investigated whether there was an increase in the CD62L−CD44hi memory T cells in nonimmunized mice in the inguinal LN versus the spleen. Despite a greater proportion of CD4+ T cells, the inguinal LN had fewer memory T cells than did the spleen, and the ratio of naive to memory cells was higher in the spleen (data not shown). Additionally, the naive CD62Lhi T cells from the spleen stimulated with anti-CD3/CD28 produced abundant IL-17, indicating that the T cells in the spleen did not have an intrinsic defect in differen-
Figure 1. Th1-dependent PGIA correlates with the production of IFN-γ and not IL-17. Groups of age-matched female mice were immunized i.p. with rG1/DDA three times at 3-wk intervals and evaluated for arthritis severity and incidence. (A) WT (n = 9) and IFN-γ−/− (n = 9) and (B) WT (n = 11) and IL-17−/− (n = 11). (C) Spleen cells from arthritic WT mice were stimulated ex vivo with rG1 and assayed for IFN-γ and IL-17 by ELISA cells. (D) Representative FACS plot of intracellular IFN-γ and IL-17 from spleen cells of arthritic WT mice stimulated with PMA and ionomycin and (E) the percentage of CD4+ cells that express intracellular IFN-γ and IL-17. (F) Ratio of the IFN-γ to IL-17 percentage for the samples in (E). CD4+T cells from WT mice 9 d after immunization with rG1/DDA (G) or with rG1/CFA (H) were stimulated with rG1 and assayed for IFN-γ and IL-17. Arthritis, in vitro cytokines, and the FACS results are presented as means ± SEM of 5-10 mice from two to three independent experiments.
LNs in IL-6– and IL-12p40–deficient mice, whereas IL-17 in IL-12p35–deficient mice was enhanced. IFN-γ levels were unaffected by a deficiency in IL-6 but were inhibited in IL-12p40– and IL-12p35–deficient mice (Fig. 4F–H). Because IL-1 signaling, IL-6, and IL-23p19 are all important for Th17 differentiation, the low level of IL-23p19 and IL-1b in the spleen after i.p. immunization may account for the deficiency in Th17-primed cells.

**Different DC populations take up Ag after i.p. versus s.c. immunization**

To determine whether different DC subsets play a role in Th1 versus Th17 differentiation in the spleen and inguinal LN following either i.p. or s.c. immunization, we examined the uptake of Ag in different DC populations after immunization by flow cytometry. WT mice were immunized with AF-488–labeled rG1 (AF-488-rG1)/DDA by either the i.p. or s.c. route and spleen and inguinal LNs were harvested 24 h later. AF-488-rG1 expression (mean fluorescence intensity) was compared in the spleen after i.p. and s.c. immunization on CD11c+ DCs expressing either CD8 or B220 and on CD8–CD205+ DCs expressing either CD103+ or CD103+CD205+.

**FIGURE 2.** Subcutaneous immunization induces an IL-17 response. (A) CFSE-labeled Thy1.2 CD4+ 5/4E8 T cells were transferred into Thy1.1 congenic WT mice and immunized 24 h later with rG1/DDA either i.p. or s.c. Proliferation of CFSE-labeled T cells was monitored from 24 to 72 h in spleen and peripheral LNs by flow cytometry. (B) Representative FACS of transferred 5/4E8 T cells in spleen and LNs 5 d after immunization and (C) the percentage of CD4+ 5/4E8 T cells that have undergone one round of proliferation at 36 h. The number of CD4+ T cells is shown that express intracellular IFN-γ (D) and IL-17 (E) after stimulation with PMA and ionomycin for 4 h. (F) Percentage of CD4+ T cells producing IL-17 relative to the total number of IL-17 and IFN-γ producers in i.p. versus s.c. immunized mice. FACS results are representative of three experiments and T cell cytokines for three to four individual mice per group with five experiments. Values are the means ± SEM. *p < 0.05.
were CD11b+ (Fig. 5E, 5F).

s.c. route. In comparison with WT mice, IFN-γ and IL-17 production were suppressed after s.c. immunization (Fig. 6C, 6D, left and middle panels) in contrast to i.p. immunization (Fig. 1). The ratio of the number of IFN-γ- to IL-17–producing cells was dramatically lower in the spleen s.c. (4.64 ± 0.63) and inguinal LN (1.72 ± 0.35) following s.c. immunization (Fig. 6C, 6D, right panel) in comparison with the spleen following i.p. immunization (22.09 ± 1.88) (Fig. 1F).

Similarly, there was a robust production of IL-17 from spleen and inguinal LN CD4+ T cells from arthritic mice stimulated in vitro with rG1 (Fig. 6E). These CD4+ T cells from WT mice were activated early after priming by the s.c. route and produced substantial IL-17 (Fig. 6F). These data demonstrate that PGIA can develop after immunization by either the i.p. or s.c. route. However, altering the route of immunization from i.p. to s.c. changes the Th cytokine requirement for the induction of disease.

Joint tissue histology is similar in i.p. and s.c. immunized mice despite the presence of T cell IL-17 in joint tissues of s.c. immunized mice

To determine whether the similarity in paw erythema and swelling in i.p. and s.c. immunized mice corresponded to comparable cellular infiltration and joint damage, we examined joint histology from hind ankle joints of mice that were maximally swollen (i.e., a score of 4).

The histological picture in i.p. and s.c. immunized mice was characteristic of acute arthritis (Fig. 7A–D). There was a similar degree of cellular infiltration and bone erosion that scored as a maximum score of 4 for all ankle joints from both i.p. (n = 4) and s.c. (n = 4) mice. The infiltration of mononuclear and polymorphonuclear cells in the synovial cavity and adjacent tissue, edema of the synovial and periartricular tissues, and synovial hyperplasia were similar in i.p. and s.c. immunized mice. Inflammation started in the small joints beginning with the distal interphalangeal, followed by the proximal interphalangeal, metatarsophalangeal, and carpometacarpal joints, and then spread to other larger joints over time. No clinical histologic differences can be seen in i.p. versus s.c. immunized mice. These data indicate that despite the difference in requirement for proinflammatory cytokine in the development of arthritis the resulting infiltration and bone erosion were similar.

The predominant population of cells isolated from the joint of arthritic mice was Gr-1+CD11b+ neutrophils (Fig. 7E, right panel). There was no difference in the percentage neutrophils in the joints of mice immunized by the i.p. versus the s.c. route. Lymphocytes and macrophages comprise a minor population of joint cells, and there was a significant increase in CD4+ T cells, B220+ B cells, and F4/80+ macrophages with a significant decrease in CD11c+ DC in s.c. compared with i.p. immunized mice (Fig. 7E, left panel).

Remarkably, CD4+ T cells from the joint tissues of i.p. and s.c. immunized mice stained for intracellular IFN-γ, but only the CD4+ T cells from the joint tissues of s.c. immunized mice stained for intracellular IL-17 (Fig. 7F–H). Chemokine receptor expression on the infiltrating IFN-γ–secreting CD4+ T cells were CXCR3+CCR6+, with a large portion of this population expressing CCR4 (Fig. 7G, left panel) from either i.p. or s.c. immunized mice. However, there was a significant reduction in the CCR4 expression of CXCR3+ and CCR6+ cells, which did not reach significance in CXCR3+CCR6+CCR4+ cells. Despite very few IL-17–producing CD4+ T cells in the joint after i.p. immunization, these T cells expressed chemokine receptors significantly higher than did IL-17–producing CD4+ T cells after s.c. immunization.

To determine whether there were any other unique features that could separate these two routes of inducing arthritis, we examined the levels of RF. We measured the levels of anti-mouse IgG2α-Fc

Conversion of PGIA from a Th1- to a Th17-mediated disease

Based on the difference in the ability of CD4+ T cells to produce IL-17 after priming by the i.p. versus the s.c. route, we hypothesize that the route of immunization correlates with susceptibility/resistance to arthritis in the IL-17−/− mice. To assess whether PGIA could be converted to an IL-17–mediated arthritis, we immunized WT, IFN-γ−/−, and IL-17−/− mice with G1/DDA by the s.c. route. In comparison with WT mice, IFN-γ−/− mice developed exacerbated disease (Fig. 6A). In IL-17−/− mice both the severity and the incidence of arthritis were suppressed compared with WT mice (Fig. 6B). In WT mice there was an increase in the percentage of IL-17–producing cells in spleen and inguinal LNs after s.c. immunization (Fig. 6C, 6D, left and middle panels) in contrast to i.p. immunization (Fig. 1). The ratio of the number of IFN-γ- to IL-17–producing cells was dramatically lower in the spleen s.c. (4.64 ± 0.63) and inguinal LN (1.72 ± 0.35) following s.c. immunization (Fig. 6C, 6D, right panel) in comparison with the spleen following i.p. immunization (22.09 ± 1.88) (Fig. 1F).

Similarly, there was a robust production of IL-17 from spleen and inguinal LN CD4+ T cells from arthritic mice stimulated in vitro with rG1 (Fig. 6E). These CD4+ T cells from WT mice were activated early after priming by the s.c. route and produced substantial IL-17 (Fig. 6F). These data demonstrate that PGIA can develop after immunization by either the i.p. or s.c. route. However, altering the route of immunization from i.p. to s.c. changes the Th cytokine requirement for the induction of disease.

Joint tissue histology is similar in i.p. and s.c. immunized mice despite the presence of T cell IL-17 in joint tissues of s.c. immunized mice

To determine whether the similarity in paw erythema and swelling in i.p. and s.c. immunized mice corresponded to comparable cellular infiltration and joint damage, we examined joint histology from hind ankle joints of mice that were maximally swollen (i.e., a score of 4).

The histological picture in i.p. and s.c. immunized mice was characteristic of acute arthritis (Fig. 7A–D). There was a similar degree of cellular infiltration and bone erosion that scored as a maximum score of 4 for all ankle joints from both i.p. (n = 4) and s.c. (n = 4) mice. The infiltration of mononuclear and polymorphonuclear cells in the synovial cavity and adjacent tissue, edema of the synovial and periartricular tissues, and synovial hyperplasia were similar in i.p. and s.c. immunized mice. Inflammation started in the small joints beginning with the distal interphalangeal, followed by the proximal interphalangeal, metatarsophalangeal, and carpometacarpal joints, and then spread to other larger joints over time. No clinical histologic differences can be seen in i.p. versus s.c. immunized mice. These data indicate that despite the difference in requirement for proinflammatory cytokine in the development of arthritis the resulting infiltration and bone erosion were similar.

The predominant population of cells isolated from the joint of arthritic mice was Gr-1+CD11b+ neutrophils (Fig. 7E, right panel). There was no difference in the percentage neutrophils in the joints of mice immunized by the i.p. versus the s.c. route. Lymphocytes and macrophages comprise a minor population of joint cells, and there was a significant increase in CD4+ T cells, B220+ B cells, and F4/80+ macrophages with a significant decrease in CD11c+ DC in s.c. compared with i.p. immunized mice (Fig. 7E, left panel).

Remarkably, CD4+ T cells from the joint tissues of i.p. and s.c. immunized mice stained for intracellular IFN-γ, but only the CD4+ T cells from the joint tissues of s.c. immunized mice stained for intracellular IL-17 (Fig. 7F–H). Chemokine receptor expression on the infiltrating IFN-γ–secreting CD4+ T cells were CXCR3+CCR6+, with a large portion of this population expressing CCR4 (Fig. 7G, left panel) from either i.p. or s.c. immunized mice. However, there was a significant reduction in the CCR4 expression of CXCR3+ and CCR6+ cells, which did not reach significance in CXCR3+CCR6+CCR4+ cells. Despite very few IL-17–producing CD4+ T cells in the joint after i.p. immunization, these T cells expressed chemokine receptors significantly higher than did IL-17–producing CD4+ T cells after s.c. immunization.

To determine whether there were any other unique features that could separate these two routes of inducing arthritis, we examined the levels of RF. We measured the levels of anti-mouse IgG2α-Fc...
Abs and found that the levels were similar in mice immunized with PG by either route (Fig. 7I). Another potential difference might be in the level of TNF-α. Cells from the spleen and inguinal LN were stimulated in vitro with rG1, and supernatants were assayed for TNF-α. There was no difference between TNF-α levels in spleen after i.p. immunization (274 ± 6.3) and in inguinal LN after s.c. immunization (318.47 ± 124.1).

Discussion

This study was designed to determine whether the route of Ag exposure alters the proinflammatory cytokines required for development of arthritis. We have previously reported that PGIA is dependent on IFN-γ, as neutralization of IFN-γ or in mice deficient in IFN-γ arthritis is inhibited (16, 17). Additionally, IL-17 is not required for the development of PGIA (19). These findings are in contrast to CIA in which reduction in IFN-γ leads to exacerbation in disease and loss of IL-17 leads to inhibition of arthritis (20, 21, 31). What accounts for the difference between these two arthritis models? One possibility is the difference in mouse strain susceptibility. BALB/c and C3H mice are susceptible to PGIA, whereas DBA/1 and to some extent C57BL/6 mice are susceptible to CIA (44–46). However, although WT BALB/c mice are not susceptible to CIA, a deficiency in IFN-γ renders BALB/c mice susceptible, indicating that BALB/c mice are not innately resistant to developing CIA (47). The difference in the requirements for IL-17 in PGIA and CIA was also not dependent on the adjuvant use to induce arthritis, as CFA induces a Th1-dependent response in PG-immunized mice (16). We hypothesized that the difference between these models is the route of immunization. PGIA has traditionally been induced by i.p. immunization whereas CIA has been induced by i.d. immunization. Similar to CIA, EAE and EAU are IL-17–dependent and are induced by the s.c. immunization (24, 32).

We first assessed the sites of T cell activation after i.p. versus s.c. immunization. As expected, transferred T cells proliferated in the spleen, mediastinal LNs, and mesenteric LNs 36 h after i.p. immunization with G1/DDA. There was no difference between TNF-α levels in spleen after i.p. immunization (274 ± 6.3) and in inguinal LN after s.c. immunization (318.47 ± 124.1).
inguinal LNs, and lumbar LNs after s.c. immunization. The proportion of IL-17–producing cells relative to the total number of IFN-γ– and IL-17–producing cells increased in spleen and several peripheral LNs after s.c. immunization. The reduced splenic CD4+ IL-17 response after i.p. immunization was also observed early (9 d) or late (arthritic mice) after immunization.

We speculated that because IL-17 plays an important role in inflammatory skin disease there could be an increase in IL-17–producing cells due to disruption of the skin barrier, even in the absence of explicit immunization. These memory Th17 T cells might influence the differentiation of Th17 cells in the draining LN. However, there was no increase in memory T cells in the inguinal LN compared with the spleen. Additionally, we found that the reduced IL-17 after i.p. immunization was not a defect in the ability of T cells from the spleen to produce IL-17, as in vitro–activated naive T cells were capable of secreting IL-17. However, T cells activated by the i.p. route generated minimal IL-17. These data indicate that the site of T cell priming determines whether T cells produce IL-17.

What is the evidence that the route of Ag exposure determines T cell cytokine phenotype? Before the advent of Th17 cells the most striking examples of T cell polarization occurred for Th1 and Th2 cells. In models where T cell priming was successfully initiated via the pulmonary tract, the outcome was a predominant Th2 response (48–50). This occurred even to an infectious pathogen (*Leishmania major*) delivered nasally, whereas delivery of *L. major* by other routes of immunization is associated with priming Th1 cells. These data suggest that the lung environment intrinsically favors priming the differentiation of Th2 cells (50).

With the discovery of Th17 cells, it was found that intranasal immunization leads to Th17-biased immune response and IL-17 is critically important in protection after infection with *Mycobacterium tuberculosis* and *Klebsiella pneumoniae* (51–53). Additionally, epicutaneous Ag application, but not i.p. exposure to OVA, induces IL-17 (34). These data raise the possibility that CIA, EAE, and EAU are Th17-mediated autoimmune diseases because they are induced by s.c. or i.d. immunization. In support of this possibility, we show in PGIA that arthritis was induced by either i.p.

**FIGURE 5.** Different DC populations take up Ag after i.p. versus s.c. immunization. AF-488–labeled rG1 was used to immunize WT mice by the i.p. and the s.c. route and spleen and inguinal LNs were harvested 24 h later. (A) Uptake of AF-488–rG1 on spleen CD11c+ DCs expressing either CD8 or B220 and on CD8−B220−DCs expressing either CD103 or Dec205. (B) Uptake of AF-488–rG1 was assessed on inguinal LN CD11c+ DCs expressing either CD8 or B220 and on CD8−B220−DCs expressing either CD103 or Dec205. (C) Spleen and (D) inguinal LN histogram of AF-488–rG1 uptake on different populations of CD11c+ DCs. (E) Assessment of AF-488–rG1+ cells for CD11b expression on spleen CD11c+CD103−CD205−CD8− DCs and (F) inguinal LN CD11c+CD103−CD205−CD8− DCs. Data are presented as means ± SEM of three independent experiments. *p < 0.05.
or s.c. route, but that the i.p. route stimulated a Th1-dependent arthritis whereas the s.c. route provoked a Th17-dependent arthritis. Our findings that either Th1 or Th17 cells stimulated PGIA are supported by data in EAU where either Th17 or Th1 effector cells generated in vitro can drive uveitis (54). We assessed several of the cytokines that are known to drive Th17 differentiation in the spleen after i.p. immunization and in the inguinal LN after s.c. immunization. We observed a significant increase in IL-1\(\beta\) and IL-23p19 cytokine mRNA in inguinal LNs after s.c. immunization in comparison with the spleen after i.p. immunization. Thus, when the APCs in the spleen cannot produce or are not activated to produce IL-1\(\beta\) or IL-23p19, naive T cells will not differentiate into Th17 cells. Considerable in vitro and in vivo evidence demonstrates that IL-6, IL-1\(\beta\), and IL-23 contribute to the differentiation and maintenance of Th17. We confirm these findings showing that mice deficient in IL-6 or IL-23p19 (IL-12p40\(^{-/-}\) mice) exhibited reduced T cell IL-17 after s.c. immunization with rG1. Another alternative possibility is that there is a more rapid activation of Th1 cells in the spleen, and IFN-\(\gamma\) could inhibit the differentiation of Th17 cells. We have previously reported in IFN-\(\gamma^{-/-}\) mice that T cells produce IL-17 after i.p. immunization (55). Additionally, the ability of the IFN-\(\gamma^{-/-}\) mice to develop arthritis late is due to IL-17, as elevated IL-17 contributes to arthritis. We showed that arthritis is inhibited more effectively in IFN-\(\gamma/-/IL-17^{-/-}\) double knockout mice than in IFN-\(\gamma^{-/-}\) mice (55).

Experiments assessing the uptake of labeled rG1 Ag suggest that different populations of DCs present Ag in the spleen after i.p. immunization and in the inguinal LN after s.c. immunization. We observed a significant increase in IL-1\(\beta\) and IL-23p19 cytokine mRNA in inguinal LNs after s.c. immunization in comparison with the spleen after i.p. immunization. Thus, when the APCs in the spleen cannot produce or are not activated to produce IL-1\(\beta\) or IL-23p19, naive T cells will not differentiate into Th17 cells. Considerable in vitro and in vivo evidence demonstrates that IL-6, IL-1\(\beta\), and IL-23 contribute to the differentiation and maintenance of Th17. We confirm these findings showing that mice deficient in IL-6 or IL-23p19 (IL-12p40\(^{-/-}\) mice) exhibited reduced T cell IL-17 after s.c. immunization with rG1. Another alternative possibility is that there is a more rapid activation of Th1 cells in the spleen, and IFN-\(\gamma\) could inhibit the differentiation of Th17 cells. We have previously reported in IFN-\(\gamma^{-/-}\) mice that T cells produce IL-17 after i.p. immunization (55). Additionally, the ability of the IFN-\(\gamma^{-/-}\) mice to develop arthritis late is due to IL-17, as elevated IL-17 contributes to arthritis. We showed that arthritis is inhibited more effectively in IFN-\(\gamma/-/IL-17^{-/-}\) double knockout mice than in IFN-\(\gamma^{-/-}\) mice (55).

Experiments assessing the uptake of labeled rG1 Ag suggest that different populations of DCs present Ag in the spleen after i.p. immunization and in the inguinal LN after s.c. immunization. The peripheral LNs possess several migratory populations of DCs that are absent in the spleen. These migratory DCs such as the CD11c\(^{+}\)CD103\(^{+}\) cells, which take up labeled Ag, may have the capacity to prime Th17 cells, as similar IL-17–inducing DCs are found in the intestine (56). In the spleen, conventional CD8\(^{+}\) DCs produce IL-12, which is essential for Th1 cell activation in the resistance against Toxoplasma gondii infection (57). It has recently been shown that monocyte-derived DCs that express CD11b are also a source of IL-12 in Listeria-infected mice (58). Although different DC populations producing different cytokine may be re-
sponsible for the differentiation of Th1 cells in the spleen after i.p. immunization and Th17 after s.c. immunization, it is also possible that other microenvironment signals contribute to this process.

In assessing joints of arthritic mice the most outstanding feature was the finding that CD4+ T cells in the joint of s.c. immunized mice produced both IFN-\(\gamma\) and IL-17, whereas those from i.p. immunized mice only produced IFN-\(\gamma\). Moreover, there was no histological difference in arthritis induced by either the i.p. or s.c. route; the cellular infiltration and bone erosion in the hind paw joint with the same degree of swelling and erythema were similar. Neutrophils are the major population infiltrating the joint, and there was no difference between the i.p. and s.c. immunized mice. There was an increase in CD4+ T cells, B cells, and macrophages and a decrease in CD11c DCs in s.c. immunized mice. These data suggest that the same end point, arthritis, can be arrived at by different pathways.

**FIGURE 7.** Joint histology and joint tissue T cell expression of cytokines and chemokine receptors. Hind limbs of mice were dissected, fixed in formalin, decalcified, and embedded in paraffin. The tissue sections were stained with H&E. Sections are representative ankle joints from i.p. immunized (A, B) and s.c. immunized (C, D) (\(n = 4\)) mice. Original magnification ×4 (A, C) and ×10 (B, D). (E) Joint tissues were minced and treated with collagenase and cell populations were determined using specific Abs. (F) Cells were stimulated with PMA (25 ng/ml) and ionomycin (500 ng/ml) for 4 h for intracellular IFN-\(\gamma\) and IL-17 staining. Chemokine receptors were measured on joint tissue CD4+ T cells producing either IFN-\(\gamma\) or IL-17 using Abs specific for CXCR3, CCR4, and CCR6. (G) Representative histogram mean of chemokine receptor expression on CD4+ T cells expressing IFN-\(\gamma\) or IL-17. Data are presented as means ± SEM of four mice from two independent experiments. (H) Histogram of joint tissues expressing IFN-\(\gamma\) or IL-17. (I) Histogram of RF units in serum of i.p. and s.c. immunized arthritic mice. *\(p < 0.05\).
In assessing chemokine receptor expression on joint IFN-γ+ and IL-17+ CD4+ T cells, we found that most of the IFN-γ-producing T cells in the joint express low levels of both CXCR3 and CCR6 whereas a portion were positive for CXCR3, CCR6, and CCR4. There was very little difference between i.p. versus s.c. immunized mice except that there were fewer CCR4-expressing cells. Although there were very few IL-17-expressing cells in the joints after i.p. immunization, these T cells expressed similar chemokine receptors as did the IFN-γ-producing cells. However, there was a major reduction in the number of chemokine receptor–expressing IL-17+ cells after s.c. immunization. Because CXCR3 is reported to be expressed mainly on Th1 cells (59) whereas Th17 cells express CCR6 (60), it was unexpected to find these dual expressing CXCR3 CCR6 cells. There are reports of CCR4 expression on both Th1 and Th17 cells (61–63). It is not clear why cytokine-secreting cells do not discrimination between the chemokine receptor expression as reported. One possibility is that because the T cells have already migrated to the joint tissues, their chemokine receptor may be downregulated. Further studies will be needed to address this issue.

In addition to T cells, innate cells rapidly produce IL-17 after infection, injury, or stress (64). These innate IL-17–producing cells include γδ T cells, CD3+ invariant NK T cells, lymphoid-tissue inducer cells, neutrophils, and Paneth cells that are most prominently present in lungs, intestinal mucosa, and skin. In particular the γδ T cells are an important source of IL-17 in CIA, uveitis, and EAE where they augment CD4+ αβ T cell production of IL-17 and enhance disease (65–67). It will be important to determine whether innate IL-17–producing cells are not activated in spleen after i.p. immunization and whether the inability to activate these innate cells contributes to the skewing away from IL-17 production after i.p. immunization.

The respective roles of Th1 and Th17 cells in RA have yielded conflicting data. Elevated levels of IFN-γ protein and RNA transcripts are present in synovial tissues and fluid in RA patients (68–70). In studies looking at IL-17– and IL-17–producing T cells there is a significant increase in RA patients in comparison with osteoarthritis (71–73); however, the relationship to IFN-γ was not measured in these studies. Where IL-17 and IFN-γ were measured in the same patient, there was a higher level of IFN-γ to IL-17 (60, 74–76), although in Yamada et al. (74) and Church et al. (76) there was no significant difference in the frequency of IL-17 and IFN-γ T cells in the blood between RA and control. Yamada et al. and Church et al. also found a higher frequency of IFN-γ– to IL-17–producing T cells in the synovial fluid of patients with RA. In several studies there is a significant correlation between IL-17 levels and progression to arthritis (77) or a more aggressive form of disease (78, 79) or C-reactive protein levels (79, 80); however, despite this correlation many of the patients did not produce IL-17. It is unclear what accounts for the major differences between these studies, but it is possible that differential activation of Th1 and Th17 phenotypes may in part be responsible for the clinical subtypes in RA patients or related to differences in individual responses to therapies. In this regard we were able to show that T cells isolated from arthritis joints produced cytokines that correlated with those produced in the spleen and LN; that is, immunization by the i.p. route T cells produced IFN-γ and not IL-17, whereas immunization by the s.c. route T cells produced both IFN-γ and IL-17. Importantly, the histological manifestations of arthritis, cellular infiltration and bone erosion, were very similar. These data suggest diverse pathways to the same disease. The different animal models may represent unique subtypes or may help us understand disparate clinical responses to better individualize therapy choices. It is unclear how RA is initiated, but it is clear that T cells play an important role in disease. Infection and injury have been associated with disease flares in RA; therefore, it is possible that different compartments or routes of Ag exposure could affect the dominance of a Th1 versus a Th17 response.

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References


46. Inglis, J. J., G. Criado, M. Medghalchi, M. Andrews, A. Sandison, M. Feldmann, and R. O. Williams. 2007. Collagen-induced arthritis in C57BL/6 mice is as...


