

Salmonella enterica Induces Joint Inflammation and Expression of Interleukin-17 in Draining Lymph Nodes Early after Onset of Enterocolitis in Mice

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In developing countries, one-third of reactive arthritis (ReA) cases are associated with *Salmonella* enterocolitis; nevertheless, there is no animal model for studying this pathology. Here we induced a self-limiting *Salmonella enterica* serovar Enteritidis enterocolitis in mice to analyze the onset of ReA. BALB/c mice received orally 20 μ g of streptomycin 24 h before intragastric inoculation of a low dose (3×10^3 to 4×10^3 CFU) of *S. Enteritidis*. In response to *Salmonella* infection, a 30-fold increase in the expression of interleukin-17 (IL-17), measured by quantitative PCR, was observed in mesenteric lymph nodes 5 days postinfection. At this time synovitis was already evident, and concomitantly, a significant increase in joint tumor necrosis factor alpha (TNF- α) was detected by enzyme-linked immunosorbent assay (ELISA). The early development of joint lesions was accompanied by an increased expression of IL-17 in inguinal and popliteal lymph nodes. Infection with 10^7 CFU of an isogenic $\Delta invG$ mutant bearing a defective type III secretion system of *Salmonella* encoded in the pathogenicity island 1 apparatus (TTSS-1) induced enterocolitis histologically similar to that triggered by the wild-type strain. Interestingly, despite the higher infective dose used, the mutant did not trigger intestinal IL-17. Moreover, no synovitis was observed in mice suffering $\Delta invG$ enterocolitis. Neutralization of IL-17 in mice infected with *S. Enteritidis* prevented both synovitis and the increment of TNF- α in the joints, suggesting that IL-17 participates in the generation of *Salmonella*-induced ReA through the induction of TNF- α in the joints.

Reactive arthritis (ReA), an inflammatory arthritic condition that is commonly associated with *Salmonella* infections, represents a significant health burden, yet it is poorly understood. It can be defined as the development of sterile inflammatory arthritis as a sequel to remote infection, often in the gastrointestinal tract (24). This observation is particularly interesting because there is no anatomic link between these two organs. In some patients, symptoms resolve within months, but in others, they may persist for years. The strong genetic association of spondyloarthritis with human leukocyte antigen (HLA) B27 has been extensively documented (3, 44, 46, 54), although the mechanism by which HLA-B27 mediates inflammation remains unclear (47).

One of the theories trying to explain the cooccurrence of gut and joint inflammation points to trafficking of intestinal lymphocytes or mononuclear cells, particularly macrophages. Lymphocyte migration and homing to specific target tissues are mediated by a variety of adhesion molecules, such as integrins and selectins, and by chemokine receptors. The homing of intestinal lymphocytes is determined by a specific set of adhesion molecules, the β_7 integrin subfamily, of which two members are critically involved in lymphocyte homing to the intestine: $\alpha_4\beta_7$ and $\alpha_e\beta_7$ (6). In early spondyloarthritis patients, activated T cells carrying the $\alpha_4\beta_7$ and $\alpha_e\beta_7$ integrins were enriched in inflamed synovial tissue (10), suggesting an intestinal origin of these T cells.

Th17 cells are critical for pathogenesis in several models of arthritis (22, 31, 41). Because activated Th17 cells produce interleukin-17 (IL-17), this inflammatory cytokine is a likely contributor to the pathogenesis of arthritis. IL-17 can induce cell adhesion molecules and proinflammatory cytokines, like tumor necrosis factor alpha (TNF- α) (49, 58, 64), and therefore could play a critical role in the development of joint inflammation. In

fact, IL-17 is considered a key mediator of TNF- α -induced bone loss by closely interacting with IL-1 in blocking bone-protective T-cell responses (67). In rheumatoid arthritis patients, IL-17 is present in the synovium and synovial fluid (7, 34, 65), and it plays a crucial role in the activation of T cells at the sensitization phase in the development of autoimmune arthritis (41). In ReA, IL-17 contributes to the development of joint inflammation (52); in fact, elevated levels of IL-17 have been detected in synovial tissue of these patients (51).

IL-17-producing Th17 cells are present almost exclusively in the small intestinal lamina propria and other mucosal tissues of mice kept under specific-pathogen-free conditions (25). This suggests the involvement of IL-17 in mucosal immune surveillance against the invasion of enteric pathogens such as *Salmonella* (38). In this regard, it has been recently demonstrated that *Salmonella* infection triggers early IL-17 production, which is crucial for host defense and is mediated by CD4⁺ T helper cells (14). Therefore, it is likely that IL-17-mediated joint inflammation during *Salmonella* ReA is triggered by the initial IL-17 response of the intestine to infection. In support of this hypothesis, a linkage between in-

Received 29 March 2012 Accepted 1 April 2012

Published ahead of print 9 April 2012

Editor: A. J. Bäuml

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doi:10.1128/IAI.00324-12

testinal IL-17-producing T helper cells and joint inflammation has been recently shown in K/BxN mice (60).

Despite the coexistence of gut and joint inflammation in ReA, very few models exist in which gut and joint inflammation appear simultaneously. One such model is an HLA-B27 transgenic rat, which develops a spondyloarthritis-like phenotype with colitis and arthritic features (26, 27). These rats, however, remain free from disease when kept in germfree conditions, reflecting the interplay between predisposing genes and bacteria (19). Furthermore, T cells are required for the development of colitis as well as arthritis in these rats, as demonstrated in studies with athymic HLA-B27 transgenic rats (2).

Salmonella enterica serovar Enteritidis is the most common bacterial infectious cause of food-borne disease in the United States (37) and—followed by *Salmonella enterica* serovar Typhimurium—the most frequently isolated from humans worldwide (21). To our knowledge, there is no animal model for studying *Salmonella* enterocolitis-induced ReA. Here we applied a previously described infection protocol to induce synovitis (43) in streptomycin-pretreated mice to achieve a self-limiting *S. Enteritidis* enterocolitis model in which gut and joint inflammation coexist.

MATERIALS AND METHODS

Mice. Female BALB/c mice were obtained from our vivarium, maintained under standard conditions, and provided with food and water *ad libitum*. At the end of the experiments, mice were killed with carbon dioxide. The Animal Ethics Committee of the University of Buenos Aires approved all experimental protocols used in this work.

Bacterial strains and growth conditions. The wild-type (wt) strain of *Salmonella enterica* serovar Enteritidis 5694 and isogenic mutant AC518 ($\Delta invG$) were used to infect mice. *S. Enteritidis* 5694 was kindly given by Anne Morris Hooke, Miami University; it was originally from F. Collins's collection, Trudeau Institute, Saranac Lake, NY. Mutant AC518 was constructed in our laboratory as described before (43). *InvG* protein is a core component of the type III secretion system (TTSS) of *Salmonella* encoded in the pathogenicity island 1 (SPI-1) apparatus (TTSS-1). Bacteria were cultured in Trypticase soy broth at 37°C at 200 cycles per minute up to an optical density (OD) of 0.6. Bacteria were pelleted by centrifugation and suspended to the appropriate density in saline solution. In all cases the number of bacteria was determined by plating appropriate dilutions on Trypticase soy agar plates.

Salmonella infection and generation of enterocolitis. Mice were pretreated with 20 mg of streptomycin (Sigma-Aldrich) given by the oral route (20), and 24 h later they received 3×10^3 to 4×10^3 CFU of the wt or 10^7 CFU of the $\Delta invG$ mutant strain of *S. Enteritidis* by the same route. For intragastric infection, 0.2 ml of the bacterial suspension was introduced into the stomach with a 21-gauge blunt needle on a 1.0-ml plastic syringe.

Bacterial colonization and persistence. At the indicated times postinfection, mice were sacrificed and bacterial loads were analyzed. All Peyer's patches located along the large intestine (six to eight), one-third of the spleen, one knee, and both popliteal lymph nodes were removed aseptically from each animal. Samples were homogenized in 1 ml of sterile saline solution, except for spleens, which were processed in 0.33 ml; thus, in these experiments, the number of CFU/ml equals the number of CFU/organ. Homogenates were diluted properly in saline and plated on *Salmonella-Shigella* (SS) agar. Samples were also cultured for 18 h in selenite broth for enrichment. *Salmonella*-like colonies appearing on SS plates were grown on triple sugar iron agar slants and tested for somatic antigen O9.

Persistence and colonization of the bacteria were also studied by PCR. Tissues were homogenized in 1% peptone-buffered water (PBW). After 24 h of enrichment at 37°C, DNA extraction was performed using the phenol-chloroform technique (8). A standard PCR of 45 cycles was car-

ried out using selective primers to amplify *invA* and *sopA* genes with 285-bp and 113-bp products, respectively. The primers used were as follows: for *invA*, forward 5'-CTGAAATTATCGCCACGTTCCGGCAA-3' and reverse 5'-CATCGCACCGTCAAAGGAACC-3' (8); for *sopA*, forward 5'-TCCACCGTGAAGTTGATTG-3' and reverse 3'-GCACTGAGGATGTGCTGGTA-5' (15). The cycling program was 95°C for 10 s, 55°C for 10 s, and 72°C for 15 s and one cycle of 40°C for 30 s. Aliquots (20 μ l) of the reaction mixtures were electrophoresed through a 2.0% agarose gel, and fragments were revealed by staining with ethidium bromide.

Anti-cytokine-blocking experiments. IL-17 was neutralized using 2.5 mg/kg of body weight of rat anti-mouse IL-17 monoclonal antibody (MAB) (MAB421; R&D Systems, Minneapolis, MN) intraperitoneally. As a control, the same dose of isotype-control Ab was injected. Briefly, 20 mice were treated 24 h before infection and at days 1 and 3 postinfection with an anti-IL-17 MAB or the isotype control Ab. On day 5, animals were sacrificed. Left knees obtained were used for histological analysis, and right knees were utilized for TNF- α detection.

Histological analysis of intestine and joints. Intestinal samples were fixed in formalin and processed by standard procedures for paraffin embedding. Knee joints were dissected, fixed in formalin for 2 days, decalcified in EDTA for 30 to 40 days, and then embedded in paraffin. Standard sections of 5 μ m were prepared and stained with hematoxylin-eosin (HE) using routine histology techniques. An experienced pathologist blinded to the experimental protocol evaluated findings of intestine and joint abnormalities. Synovial alterations were scored as follows: 0, no changes; 1, slight thickening of synovial cell layer (up to 3 layers of synoviocytes) accompanied by congestion and edema of the external membrane; 2, moderate thickening of synovial cell layer (3 to 5 layers of synoviocytes) accompanied by congestion and edema of the external membrane; 3, severe thickening of synovial lining (more than 5 layers) accompanied by congestion and edema of the external membrane.

qPCR. Total RNA was extracted from the midportion of the cecum (approximately 0.5 cm), knee joints, and popliteal and mesenteric lymph nodes using TRIzol reagent (Life Technologies, Inc., Carlsbad, CA) at different time points according to the experiment. Total RNA (1 μ g per sample) was reverse transcribed with oligo(dT) as a primer using Expand reverse transcriptase (Promega Corporation, Madison, WI) according to the manufacturer's protocol. Quantitative real-time reverse transcriptase PCR (qPCR) was performed using a Sybr green PCR kit (Applied Biosystems Inc., Foster City, CA) in an Applied Biosystems 7500 sequence detector. Primer sequences were as follows: for TNF- α , forward 5'-ATGAGCACAGAAAGCATGATC-3' and reverse 5'-TACAGGCTTGTCCTCGAATT-3' (23); for IL-17, forward 5'-GCTCCAGAGGCCCTCAGA-3' and reverse 5'-AGCTTCCCTCCGCATTGA-3' (17). All genes were analyzed in the same run for 18S expression for normalization: forward 5'-AACACGGGAAACCTCACCC-3' and reverse 5'-CCACCACTAAGAACGGCCA-3'. PCR parameters were 50°C for 2 min, 94°C for 2 min, and 40 cycles of 94°C for 30 s and 60°C for 1 min. Quantification of gene expression was calculated using the comparative threshold cycle (C_T) method, normalized to the 18S control and efficiency of the reverse transcriptase reaction (relative quantity, $2^{-\Delta\Delta C_T}$). The replicates were then averaged, and the fold induction was determined, with the value in the control group as 1 (23).

Cytokine analysis. Knee samples were obtained 5 days after oral inoculation with 3×10^3 to 4×10^3 CFU of *Salmonella enterica*. Tissue homogenates from five animals were pooled and subjected to centrifugation (12,000 rpm, 1 min) to pellet all cell debris prior to concentration using an Amicon Ultra-4 centrifugal filter unit (Merck Millipore). Supernatants were stored at -20°C until further use. Samples were analyzed for TNF- α and IL-17 using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Cytokine levels were expressed as picograms per ml.

Statistical analysis. Unless otherwise stated, all results are the average \pm standard deviation (SD) of results from at least three separate ex-

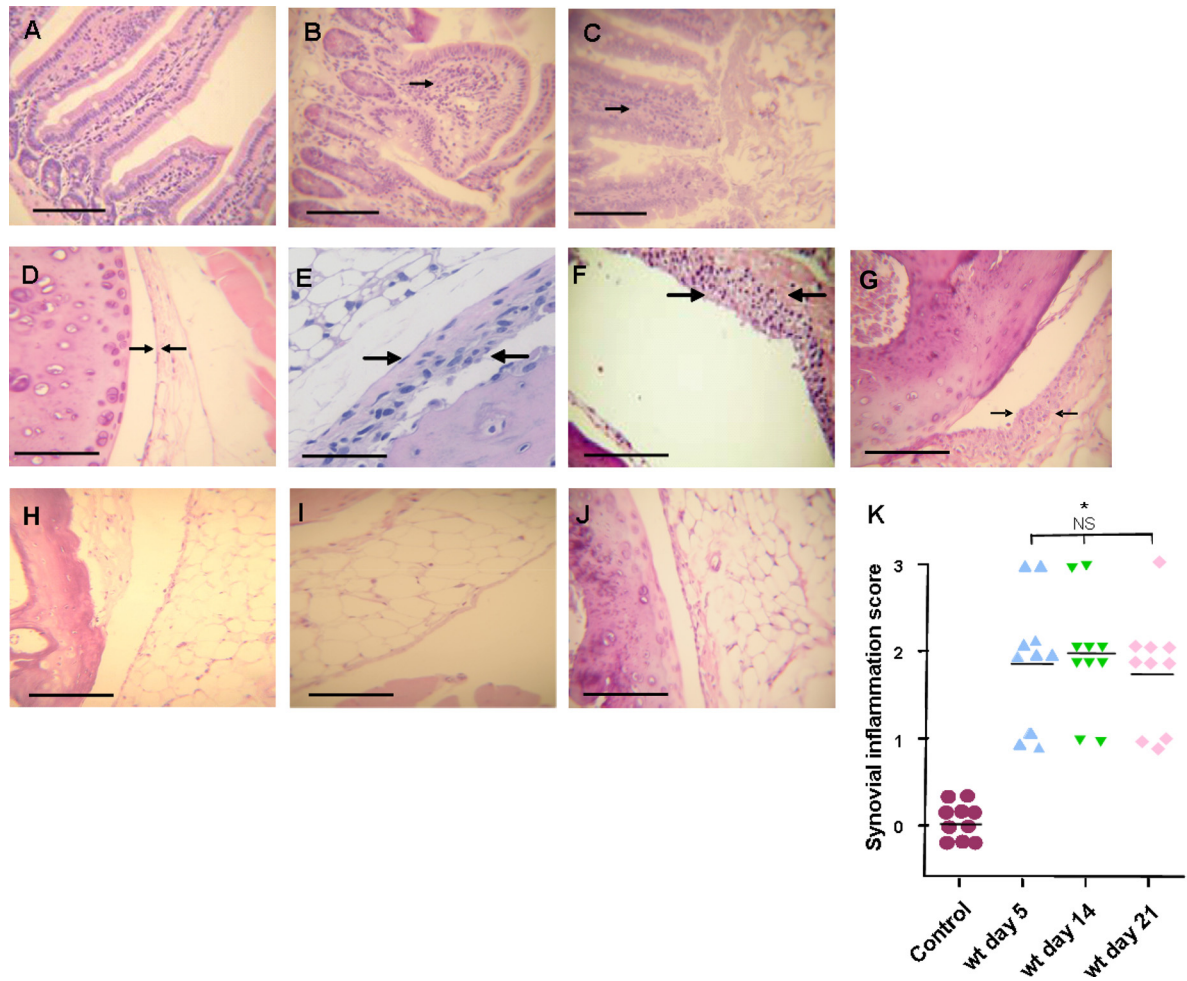


FIG 1 Histological changes in large intestine and joints after *S. Enteritidis* enterocolitis. Histology studies after intragastric inoculation with 3×10^3 to 4×10^3 CFU of the wild type (wt) or 10^7 CFU of the *invG* mutant of *S. Enteritidis* ($\Delta invG$). (A, B, and C) Large intestine. (A) Control animals: normal display of intestinal mucosa; (B) Enterocolitis in wt infected mice, note the loss of normal villi display and the infiltration of mononuclear cells (arrow); (C) enterocolitis in mice inoculated with the $\Delta invG$ strain, changes are similar to those observed in the wt group. HE stain. Bar: 100 μ m. (D to J) Joints. (D) Control animals: normal synovial capsule (arrows). (E, F, and G) Mice infected with the wt strain: moderate hyperplasia, with 3 to 5 layers of synoviocytes (arrows) at days 5, 14, and 21, respectively. (H, I, and J) Mice infected with the $\Delta invG$ strain: no differences were found with respect to control mice (D) at any time point studied (days 5, 14, and 21, respectively). HE stain. Bar, 1 mm (A, C, D, E, F and G) or 100 μ m (B). (K) Synovial inflammation scores. *, significant differences ($P < 0.01$) between wt-infected mice and control group mice were found at all time points assessed. NS, no significant differences among wt-infected mice. Data were collected from three independent experiments.

periments. P values were determined using the Mann-Whitney test for nonparametric values. A P value of <0.05 was considered to be statistically significant.

RESULTS

A *Salmonella* enterocolitis model useful for studying reactive arthritis. In developing countries, one-third of reactive arthritis cases are associated with *Salmonella* enterocolitis (53). Here we describe an experimental model useful for studying the pathogenesis of *Salmonella* reactive arthritis and its treatment or prevention. Mice received 20 μ g of streptomycin 24 h before intragastric infection. The experimental group was inoculated with a low dose (3×10^3 to 4×10^3 CFU) of the wild-type (wt) strain of *S. Enteritidis*. A second group of mice received 10^7 CFU of the isogenic $\Delta invG$ mutant unable to secrete SPI-1 effectors. We have shown previously in the typhoid model of infection that a functional

SSTT-1 is necessary to trigger joint inflammation (43). A third group of untreated mice were included as a control.

Regardless of the infecting bacterial strain, 48 h after inoculation, all animals showed signs of disease, including diarrhea, rough hair coat, and lethargy. Histology studies revealed that wt *S. Enteritidis* induces diffuse enterocolitis, characterized by an epithelium diminished in height, mononuclear infiltration of the mucosa and submucosa, and loss of normal villus architecture (Fig. 1B). As seen in Fig. 1C, the enteritis generated by 10^7 CFU of the $\Delta invG$ mutant is indistinguishable from that induced by 10^3 CFU of the wt strain. Twenty percent of the animals infected with the wt strain died by day 7 postinoculation (Table 1); the remaining 80% overcame the infection and survived for at least 70 days (data not shown). No deaths occurred among mice infected with the $\Delta invG$ mutant (Table 1).

Colonization and persistence of *Salmonella* strains after en-

TABLE 1 Colonization and persistence of *S. Enteritidis* after enterocolitis onset

Dose (CFU) and infecting strain	Days p.i.	CFU/ml (range)		DNA in knees & lymph nodes ^a	Survival ^c
		Peyer's patches	Spleen		
3×10^3 to 4×10^3 , wild type	2	58 (16–85)	57 (20–97)	Negative	5/5
	4	77 (13–105)	94 (32–104)	Negative	5/5
	14	Negative	72 (23–97)	Negative	4/5
	21	Negative	Positive ^b	Negative	4/5
10^7 , $\Delta invG$ mutant	2	112 (47–124)	135 (73–252)	Negative	5/5
	4	97 (38–112)	126 (57–139)	Negative	5/5
	14	Negative	Negative	Negative	5/5
	21	Negative	Negative	Negative	5/5

^a Negative, no bacterial DNA was detected by PCR assay.

^b Detected by PCR assay only.

^c Number of survivors/total number of mice. On day 7 two animals died, one from the group to be sacrificed at day 14 and one from the group to be sacrificed at day 21. Survival rate was 80% for mice infected with the wild-type strain and 100% for animals receiving the *invG* mutant.

terocolitis onset were analyzed at days 2, 4, 14, and 21 postinfection. The presence of bacteria was assessed in Peyer's patches, spleen, knee joints, and popliteal lymph nodes (Table 1). Bacterial loads recovered from Peyer's patches of animals infected with the $\Delta invG$ mutant were higher than those found in mice infected with the wt strain, although these differences were not statistically significant. Cultures were positive for at least 4 days. By day 14, the wt and the mutant strains were cleared from Peyer's patches. The spleen was also colonized by both strains; again, at days 2 and 4 bacterial loads of the $\Delta invG$ mutant were higher than those of the wt strain but not significantly different. At day 14 only the wt strain could be quantified in spleen, and by day 21 the wt strain was detected in spleen homogenates only by PCR. Neither live *Salmonella* bacteria nor their DNA were detected in the joints or draining lymph nodes at any time tested for any of the bacterial strains studied.

Clinical studies showed that the median delay between intestinal infection and the onset of reactive arthritis symptoms is about 15 days (39). With this in mind, we investigated whether joint inflammation was evident early upon *Salmonella* infection. To this purpose, animals were sacrificed at days 5, 14, and 21 after infection to evaluate joint histological changes. We found that alterations of the synovial membrane were already present at day 5 postinfection with the wt strain. The synovial membrane presented moderate hyperplasia with mononuclear infiltration (Fig.

1E); in addition, the external membrane showed congestion and edema (not shown). As shown in Fig. 1F and G, the observed lesions were similar at all time points assessed and persisted for at least 21 days. The average score of these lesions was 2 according to our scale (Fig. 1K). On the other hand, mice infected with the $\Delta invG$ mutant did not present synovial changes at any of the time points assessed (Fig. 1H to J).

Different intestinal TNF- α and IL-17 expression between animals infected with the wild type or the $\Delta invG$ mutant of *S. Enteritidis*. Gut inflammation plays a crucial role in the development of reactive arthritis following intestinal infection (28). TNF- α and IL-17 are among the most prominent cytokines induced soon after *Salmonella* infection (17, 35), and they are also linked to the generation of arthritis (39). With this in mind, we analyzed the expression of TNF- α and IL-17 in the large intestine by qPCR 48 h after *Salmonella* inoculation. The results are depicted in Fig. 2. Soon after infection with 3×10^3 to 4×10^3 CFU of wt *Salmonella*, mice showed an 8-fold increase in the intestinal expression of TNF- α with respect to the control group ($P < 0.01$). IL-17 expression was also significantly elevated in mice infected with the wt strain. A 24-fold increase in the expression of this inflammatory cytokine was detected in mice suffering enterocolitis compared to that in untreated animals ($P < 0.01$). In contrast, in mice infected with 10^7 CFU of the $\Delta invG$ mutant, neither the

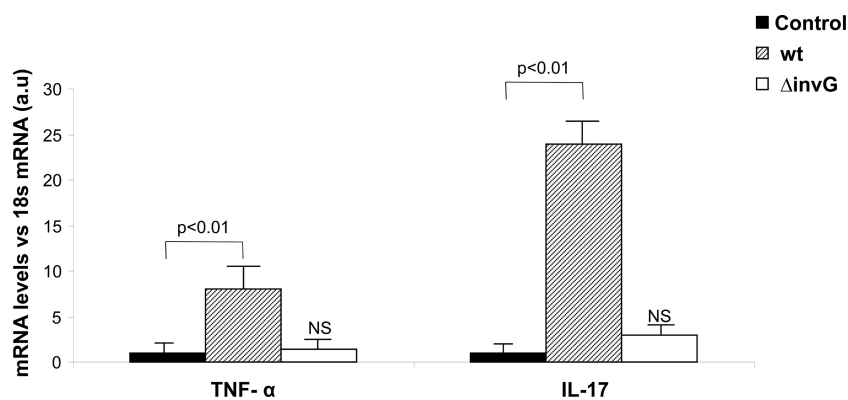


FIG 2 Cytokine expression in the large intestine after *S. Enteritidis* enterocolitis. TNF- α and IL-17 expression was evaluated by qPCR 48 h after intragastric inoculation with 3×10^3 to 4×10^3 CFU of the wild-type (wt) or 10^7 CFU of the *invG* mutant of *S. Enteritidis* ($\Delta invG$). Results are expressed as mean \pm SD ($n = 7$). a.u., arbitrary units; NS, no significant differences compared to the control group. Representative data from three independent experiments.

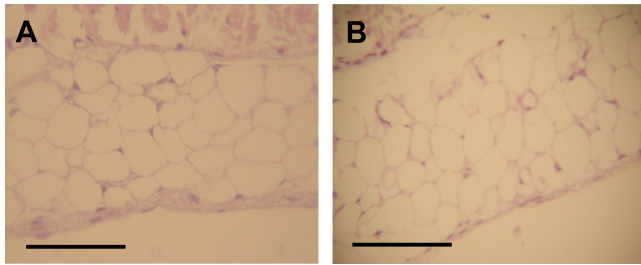


FIG 3 Neutralization of IL-17 suppresses *Salmonella*-induced synovitis. Histology of knee joints in mice treated with anti-IL-17 MAb at day 5 after intragastric inoculation with 3×10^3 to 4×10^3 CFU of wild-type *S. Enteritidis*. (A) Control animals: normal display of the synovial layer. (B) Mice received anti-IL-17 MAb intraperitoneally 24 h before and at day 1 and 3 after infection. Histology was similar to that in the uninfected animals. HE stain. Bar, 1 mm.

expression of TNF- α nor that of IL-17 was augmented (Fig. 2). Altogether, our results suggest a relation between intestinal inflammatory cytokines induced by *Salmonella* with a functional SSTT-1 and the appearance of synovitis.

Blocking IL-17 prevents synovitis induced by *Salmonella* enterocolitis. In order to investigate the involvement of IL-17 in the *Salmonella*-induced synovitis, blocking experiments were conducted in mice infected with the wt strain of *S. Enteritidis* by neutralizing IL-17 *in vivo*. Remarkably, blockade of IL-17 successfully suppressed synovitis in knee joints analyzed on day 5 postinfection (Fig. 3). These findings support the essential role of IL-17 in *Salmonella* enterocolitis-induced ReA.

TNF- α and IL-17 in the joints during *Salmonella* enterocolitis-induced synovitis. Next, we analyzed whether synovitis observed in mice infected with the wt strain of *Salmonella* was accompanied by the local expression of TNF- α and IL-17. To this purpose, qPCR was assessed in joint homogenates at days 5, 14, and 21 postinfection. No significant differences were found in the expression of articular TNF- α of mice suffering *Salmonella* enterocolitis compared to that of control animals for any time point evaluated (Fig. 4). Likewise, no significant differences in the relative amounts of IL-17 mRNA were found between infected and untreated mice (Fig. 4).

In view of these results, and keeping in mind that the number

of cytokine-producing cells in the synovial tissue may be low (55), we decided to investigate the presence of TNF- α and IL-17 in $5 \times$ concentrated joint homogenates. The production of these cytokines was measured by ELISA (Fig. 5). Five days after the onset of *Salmonella* enterocolitis, the amount of local TNF- α was significantly higher ($P < 0.05$) than that in untreated animals. As shown in Fig. 5, the increase in TNF- α did not go along with an increase in local IL-17. Additional experiments showed that blocking of IL-17 prevents the increment in local TNF- α observed in infected mice (Fig. 5). These results suggest that local production of TNF- α (induced by IL-17) may be responsible for the early inflammation of the synovial membrane triggered by *Salmonella* enterocolitis.

Cytokine profile resemblance between joint and intestinal draining lymph nodes during enterocolitis induced by *S. Enteritidis*. The involvement of lymph nodes in the pathogenesis of arthritis was proposed decades ago; moreover, it was shown that during acute inflammatory arthritis, Th17 cell differentiation is initiated in draining lymph nodes (9). Therefore, we decided to investigate whether the early onset of synovitis triggered by *Salmonella* enterocolitis is accompanied by an increase in the expression of cytokines in the joint-draining lymph nodes. To this end, qPCR was performed on RNA collected from popliteal and inguinal lymph nodes of mice infected with *S. Enteritidis*. We found that whereas the expression of TNF- α was not significantly augmented 5 days after infection, the expression of IL-17 showed a 15-fold increase ($P < 0.01$) compared to that of the control (Fig. 6A). Concomitantly, a similar cytokine profile was found in mesenteric lymph nodes. As shown in Fig. 6B, a significant 30-fold increase in the expression of IL-17 ($P < 0.01$) compared to that in controls was observed at day 5 postinfection. On the other hand, the expression of TNF- α in mesenteric lymph nodes of infected mice was not different from that found in the control group (Fig. 6B). These results show that *S. Enteritidis* enterocolitis triggers a similar IL-17 expression profile in both intestinal and joint-draining lymph nodes.

On the whole, our studies demonstrate that *Salmonella* enterocolitis triggers early synovitis, increased expression of IL-17 in intestinal and joint-draining lymph nodes, and an augmented amount of TNF- α in the inflamed joints.

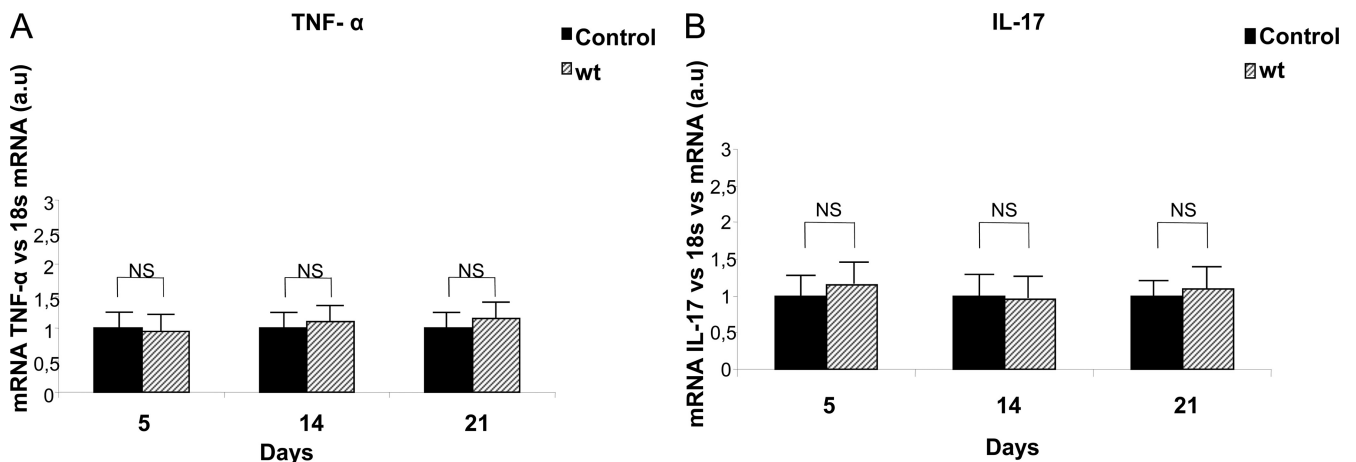


FIG 4 Cytokine expression in joints after *S. Enteritidis* enterocolitis. TNF- α (A) and IL-17 (B) expression were evaluated by qPCR at different time points after infection of mice with 3×10^3 to 4×10^3 CFU of wild-type *S. Enteritidis* (wt). Results are expressed as mean \pm SD ($n = 5$). a.u., arbitrary units. Representative data from three independent experiments. NS, no significant differences.

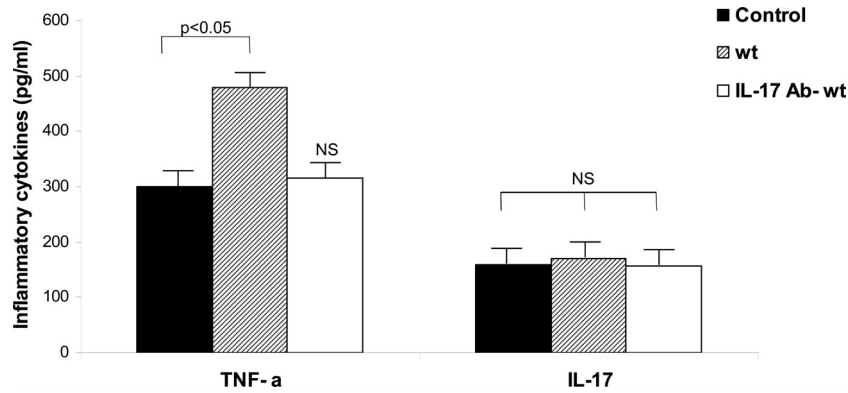


FIG 5 TNF- α and IL-17 in the inflamed joints early after *S. Enteritidis* enterocolitis. The presence of TNF- α and IL-17 was evaluated by ELISA 5 days after oral infection with 3×10^3 to 4×10^3 CFU of wild-type *S. Enteritidis*. wt, mice received only the pathogen. IL-17 Ab- wt, Mice received anti-IL-17 MAb intraperitoneally 24 h before and at day 1 and 3 after infection. Results are expressed as mean \pm SD ($n = 5$). Data were collected from three to five independent experiments. NS, no significant differences with the control group.

DISCUSSION

Oral *Salmonella* infection of streptomycin-pretreated mice has been widely utilized to study the early stages of enterocolitis (20), yet the high dose of the pathogen used renders the model unsuitable for long-term experiments. Here we show that a reduction of the bacterial burden induces self-limiting enterocolitis in 80% of

the animals pretreated with the antibiotic. An oral dose of circa 10^3 CFU of *S. Enteritidis* was sufficient to increase expression of IL-17 in intestinal and joint-draining lymph nodes, to augment TNF- α in the inflamed joints, and to trigger early synovitis.

A recent clinical study reported that the median delay between enterocolitis and the beginning of ReA symptoms is about 15 days

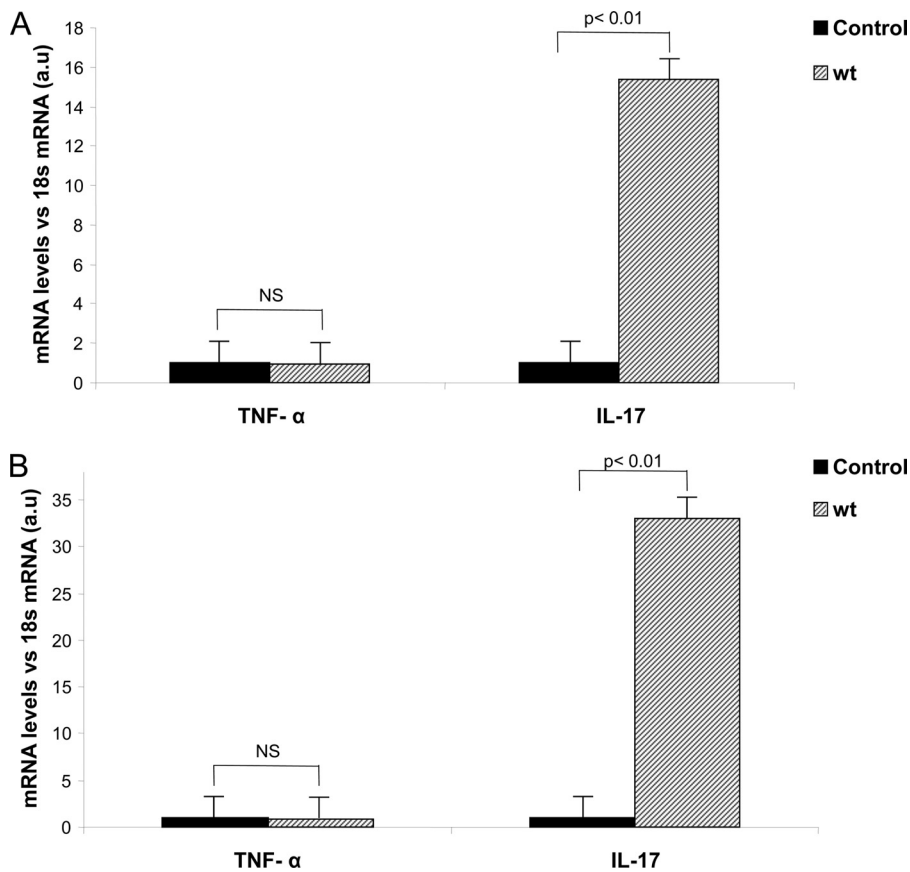


FIG 6 Cytokine profile resemblance between joint and intestinal draining lymph nodes early after *S. Enteritidis* enterocolitis. The expression of IL-17 was evaluated in popliteal and inguinal lymph nodes (A) and mesenteric lymph nodes (B) by qPCR 5 days after oral infection with 3×10^3 to 4×10^3 CFU of wild-type *S. Enteritidis* (wt). Results are expressed as mean \pm SD ($n = 4$). a.u., arbitrary units. Data were collected from three independent experiments. NS, no significant differences.

(39). These data suggest that joint damage triggered by intestinal infection could be an early event. In fact, taking advantage of our animal model, we demonstrated here that *Salmonella* induces aseptic joint inflammation within 5 days after enterocolitis onset. Altogether, our results indicate that the animal model used in this work is useful for studying the pathogenesis of ReA induced by *Salmonella* enterocolitis.

In a previous work, we demonstrated that a functional TTSS-1 in the infecting *Salmonella* strain is essential for the development of synovitis; it was suggested that proinflammatory signaling mediated by *Salmonella* TTSS-1 in the gut is required for the induction of histological changes in the joints (43). In this work, the observation that the $\Delta invG$ mutant is unable to induce IL-17 expression in the large intestine allowed us to speculate that this cytokine is crucial in the generation of *Salmonella*-induced synovitis. This hypothesis was further confirmed by the neutralization of IL-17 *in vivo*.

Clinical evidence indicates that Th17 cells may play a role in the induction and maintenance of gut inflammation in human diseases such as Crohn's disease and ulcerative colitis. Indeed, IL-17A and IL-17F are highly expressed in the gut of these patients. Analogously, high expression of IL-17 was found in the synovial fluids of spondyloarthritis-affected patients, and an increased number of circulating Th17 memory-like T cells has been recently reported in these patients (29, 59). In our model, while the neutralization of IL-17 prevented joint inflammation, neither IL-17 protein nor IL-17 expression could be detected in inflamed joints. These results are in line with some clinical and experimental findings. A detailed work by Steiner and coworkers (55) showed that although important in the generation of synovitis, the number of cytokine-expressing cells in the synovium of arthritis patients is low, about 0.1 to 0.3% of all T cells investigated. In concordance, T-cell cytokines have proven difficult to detect in synovial samples of arthritis patients (66). Moreover, it was reported that in experimental adjuvant arthritis, the expression of synovial IL-17 is transient and restricted to day 13 after the induction of the disease (4). Therefore, we cannot rule out the presence of IL-17 or the expression of IL-17 mRNA in the joints of mice suffering *S. Enteritidis* enterocolitis.

Conflicting data have been reported on the production of cytokines in ReA patients. Some studies revealed low levels of Th1 cytokines in peripheral blood and synovium (1, 62, 63); the impaired production of these cytokines has been suggested as being part of the pathogenesis of ReA (5) by causing the failure of bacterial elimination at the early stages of the disease. On the other hand, it was reported that in T-cell clones derived from synovial fluid of ReA patients, antigens from disease-related bacteria induce Th1 cytokine secretion (36, 48, 50) and the presence of Th17 cytokines in synovial fluid of ReA patients was also reported (51, 52). We found that in *S. Enteritidis* enterocolitis, early synovitis is accompanied by a moderate increase in the amount of TNF- α in the inflamed joints. This result could be in agreement with the slight increment in circulating TNF- α observed in ReA patients compared to that in healthy controls (56). Whether ReA patients experience slight or substantial increases in synovial TNF- α , compared to that in controls, is uncertain because of the ethical restriction to obtain synovial samples from healthy individuals. This fact certainly stresses the importance of developing animal models to study ReA. Live *Salmonella* can induce TNF- α production in synovial fibroblasts, as shown *in vitro* (40). It is unlikely, however,

that this is the source of synovial TNF- α in our animal model because neither live *Salmonella* bacteria nor their DNA was found in the inflamed joints. Many attempts to isolate living pathogens from synovial fluid during ReA failed, although the presence of *Salmonella*-related antigens has been detected in the joints of these patients (18). Therefore, it is possible that recirculation of antigen-loaded macrophages may provide the antigenic stimulus necessary to sustain T cell activation and inflammation of the joints.

Increasing evidence points to IL-17 as a key mediator of TNF- α -induced joint damage in arthritis (32, 67). Our results support this proposition; in addition to preventing synovitis, the neutralization of IL-17 reduces the amount of TNF- α in the joints of *S. Enteritidis*-infected mice. A study on the mechanism of joint damage during collagen-induced arthritis suggests that IL-17, produced by T cells, acts on monocytes, resulting in TNF- α production; in turn, TNF- α can induce the differentiation of monocytes into mature osteoclasts (61). The induction of TNF- α by IL-17 could also be explained because synoviocytes and fibroblasts express receptors for this interleukin (30), which, once stimulated, induce the production of inflammatory molecules (13, 45), including TNF- α (33). Moreover, a reciprocal stimulation of synoviocytes and IL-17-producing cells has been proposed to explain the persistent inflammatory cytokine milieu (i.e., TNF- α , IL-6, IL-15) in the joints of rheumatoid arthritis patients (12). Furthermore, IL-17 and TNF- α have additive effects on the expression of synoviolin, a novel E3 ubiquitin ligase implicated in the pathogenesis of rheumatoid arthritis. It has been demonstrated that the induction of synoviolin by IL-17 prolongs the survival of fibroblast-like synoviocytes contributing to synovial hyperplasia (57).

Early works on adjuvant arthritis suggested the role of draining lymph nodes in the development of joint lesions (4, 42). More recently, investigations conducted in the B27-transgenic rat model for spondyloarthritis showed that Th17 cells contribute to both intestinal and joint inflammation; IL-17-producing cells were expanded in mesenteric as well as in popliteal lymph nodes (16). In agreement with this, we found early expression of IL-17 in popliteal and inguinal lymph nodes of mice undergoing *S. Enteritidis* enterocolitis. Our findings are also in line with data obtained from an IL-17-dependent model of acute inflammatory arthritis. It was found that following intra-articular inoculation of bovine serum albumin, Th17 cell differentiation is initiated in draining lymph nodes (9).

Our results, together with previously published data (43), provide evidence in favor of the gut-joint axis proposed to explain the pathogenesis of ReA (11, 28). Several lines of evidence indicate that ReA may originate from the relocation to the joints of the immune process primarily induced in the gut (11). The transfer of the intestinal inflammatory process into the joints implies that immune cells activated in the gut-draining lymph nodes can localize, at a certain point of the intestinal disease, either into the gut or into the joints (11). The concept that immune cells, activated in the inflamed gut and migrating into the joints, may be able to reproduce in this tissue a similar immune response is sustained by the observation that common immunological processes operate at both these sites. Moreover, a linkage between intestinal IL-17-producing T helper cells and joint inflammation has been recently shown in K/BxN mice (60). In agreement with these results, we found that—in *S. Enteritidis* enterocolitis—the inflammatory cytokine profile of mesenteric lymph nodes resembles that of popliteal and inguinal lymph nodes.

In summary, a low dose of *S. Enteritidis* inoculated to streptomycin-pretreated mice generates a self-limiting enterocolitis model useful for studying ReA. Soon after infection, animals develop synovitis with the presence of TNF- α in the joints and the expression of IL-17 in the large intestine and in mesenteric, inguinal, and popliteal lymph nodes. Neutralization of IL-17 abrogated synovitis as well as the increase of TNF- α in the joints. To our knowledge, this is the first report showing a rapid response to *S. Enteritidis* enterocolitis in an extraintestinal tissue. Altogether, the mouse model presented here closely resembles human *Salmonella* ReA.

ACKNOWLEDGMENTS

We thank María Isabel Bernal, Lida Suligoy, and Roberto Caccuri for their superb technical assistance.

This work was supported in part by grants from Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina (PIP-O992/08), and Secretaría de Ciencia y Técnica de la Universidad de Buenos Aires, Argentina (UBACyT M009 and 20020100100541).

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