Yersinia-triggered arthritis in IL-12p40 deficient mice: relevant antigens and local expression of Toll-like receptor mRNA.

Journal:	*Scandinavian Journal of Rheumatology*
Manuscript ID:	SJR-2006-0024.R1
Manuscript Type:	Article
Date Submitted by the Author:	11-May-2006
Complete List of Authors:	DI GENARO, MARIA; National University of San Luis, Biochemistry and Biological Science Cargnelutti, Diego Esteban; National University of San Luis, Biochemistry and Biological Science Castro, Diego Osvaldo; National University of San Luis, Biochemistry and Biological Science Eliçabe, Ricardo Javier; National University of San Luis, Biochemistry and Biological Science Gutiérrez, Jesica Victoria; National University of San Luis, Biochemistry and Biological Science Correa, Silvia Graciela; National University of Córdoba, Clinical Biochemistry Stefanini de Guzmán, Ana María; National University of San Luis, Biochemistry and Biological Science
Keywords:	Bacterial antibodies, Bacterial antigens, IL-12, Joints, Mice, Oral infection, Reactive arthritis, TLR, Yersinia enterocolitica

Yersinia-triggered arthritis in IL-12p40 deficient mice: relevant antigens and local expression of Toll-like receptor mRNA.

Category submitted: Original article

M. S. Di Genaro[1], D. E. Cargnelutti[1], D. O. Castro[1], R. J. Eliçabe[1], J. V. Gutiérrez[1], S. G. Correa[2] and A. M. S. de Guzmán[1]

[1]Laboratory of Microbiology, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis, 5700, San Luis, Argentina. [2]Immunology, CIBICI (CONICET) Faculty of Chemical Science, National University of Córdoba, 5500, Córdoba, Argentina.

Corresponde to: Dr. Silvia Di Genaro. Laboratory of Microbiology, Chemistry, Biochemistry and Pharmacy Faculty, National University of San Luis, 5700, San Luis, Argentina. FAX: 0054-2652-431301. E-mail address: sdigena@unsl.edu.ar

Short title: IL-12p40 and *Yersinia*-triggered arthritis.

Abstract

Objetives: We studied the role of IL-12p40 at the onset of reactive arthritis (ReA) after *Yersinia enterocolitica* O:3 infection, and analyzed relevant microbial antigens and articular expression of Toll-like receptor (TLR) mRNA. Methods: Wild-type C57BL/6 and IL-12p40 deficient (IL-12p40-/-) mice were orogastrically infected with *Y. enterocolitica* O:3. Early (day 3) and late (day 21) after infection, the number of bacteria was determined in Peyer's patches (PP), mesenteric lymph nodes (MLN), the spleen and joints. Histologic studies of joints were performed. Collagen-specific and anti-*Yersinia* antibodies were measured by ELISA. Presence of *Yersinia* antigens was studied by dot-blot. Induction of articular mRNA of TLR2, TLR4, and tumor necrosis factor (TNF)-α was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). TNF-α protein levels were measured by ELISA. Results: at day 3, bacterial recovery in PP, MLN and spleen was significantly increased in IL-12p40-/- mice. Histopathological changes were observed in IL-12p40-/- mice at day 21 after infection, and correlated with higher antibody response against type II collagen. Although live bacteria could not be isolated at day 21 after infection, articular microbial components, especially from outer membrane (OM), were detected. Moreover, intra-articular immunoglobulins to *Yersinia* antigens were significantly higher in IL-12p40-/- mice. Furthermore, mRNA levels for TLR2, TLR4 and TNF-α, and TNF-α protein were increased in joints from IL-12p40-/- mice. Conclusions: we concluded that IL-12p40 influences the resistance against *Yersinia*-triggered ReA. Bacterial products such as *Yersinia* OM could contribute to the ReA by induction of articular TLR expression that result in inflammatory response in the joint.

Key words: *Yersinia enterocolitica* - Reactive arthritis - IL-12 – Mice - Oral infection - Bacterial antigens – Bacterial antibodies – Joints – TLR.

Introduction

Reactive arthritis (ReA) is a chronic, sterile synovitis that develops after gastrointestinal or urogenital infections, often with some latency, at joints distant from the site of the primary infection [1,2]. *Yersinia enterocolitica*, a Gram-negative bacterium, causes intestinal infection which could be complicated by ReA. Because the pathogenesis of ReA is incompletely understood, appropriate treatments are not available [2]. There is increasing evidence that patients with ReA show a limited T helper (Th)1 cytokine response, which is necessary for the elimination of bacteria [3,4]. Interleukin (IL)-12 is the strongest inducer of interferon (IFN)-γ production in Th1 cells [5], that plays a critical role in protective immunity against *Y. enterocolitica* [6,7]. IL-12 is a heterodimeric cytokine consisting of two subunits, designed p40 and p35 [5]. Interestingly, IL-12p40 knockout mice generally showed a stronger defect in cell-mediated immune response than IL-12p35 knockout mice [5]. The recent discovery that IL-12 shares p40 with IL-23, and that p40 shares homology with EB13 subunit of IL-27, indicates that the family of IL-12-related cytokines (IL-12, IL-23 and IL-27) could determine an effective cell-mediated immune response and may act complimentary [5]. Although the critical role of IL-12p40 in *Y. enterocolitica* O:8 protection has been yet demonstrated [6,7,8], the impact of IL-12p40 deficiency on the *Yersinia*-induced ReA has not been explored. In addition, the most frequently occurring human arthritogenic *Y. enterocolitica* serotype O:3 has demonstrated to be arthritogenic for some strains of mice [9]. However, the arthritogenicity of this serotype in C57BL/6 IL-12p40-/- mice has not been studied. In addition, since abnormality of IL-12p40 gene expression in human has been reported [10], and IL-12 deficiency has

been detected in patients with ReA [4], the results presented here could be extrapolated to human ReA.

In the present work, first we evaluated the role of IL-12p40 at the onset of ReA after oral infection with *Y. enterocolitica* O:3. Second, due to bacterial products could contribute in the ReA induction [2], we analyzed the relevant microbial antigens and bacterial-specific intra-articular antibodies in relation to ReA. Finally, since Toll-like receptors (TLRs) recognize bacterial components involved in the development of this arthritis [2], and TLR mRNA are associated with increased proinflammatory cytokine (*e.g.* TNF-α) [11], we investigated the articular expression of TLR2, TLR4 and TNF-α mRNA in the mice. This study may be helpful to further disclose principles of pathogenesis of ReA and to design strategies for its specific therapeutic treatment.

Materials and methods

Mice

We used IL-12p40-/-mice [12] on a C57BL/6 background and C57BL/6 wild-type mice. Mice were kept under specific-pathogen-free conditions in positive-pressure cabinet (EHRET, Germany) and provided with sterile food and water *ad libitum*. Female and male mice 6 to 8 weeks old were used for the experiments. Three independent experiments were carry out with seven mice per group (total n=21 per group, 11 female and 10 male in both IL-12p40-/- and wild-type cohorts).

Bacteria and infection

Strain MHC 700 of *Y. enterocolitica* serotype O:3 (kindly provided by Dr. Kapperud, Department of Bacteriology, Oslo, Norway) was used for infection. Bacteria were cultured overnight in Luria broth at 26 ºC, harvested during the log phase, and frozen in 1 ml aliquots at –20ºC. Prior to each experiment, an aliquot was thawed, washed, and resuspended in sterile PBS, pH 7.4. Mice were starved for 8 h, and 200 µl of 5 % $NaHCO_3$ in PBS was given prior to infection. Seven mice per group were infected orogastrically with a gastric tube, and 1 x 10^8 to 5 x 10^8 yersiniae in 200 µl was injected. The actual number of bacteria administrated was controlled for each experiment by plating serial dilutions of the inoculated suspension on Mueller-Hinton agar and counting the colony-forming units (CFU) after incubation at 26 ºC for 48 h. According with previous experiences, we selected two time points to study the mice: day 3, as early time of the infection to study the susceptibility of IL-12p40-/- mice to *Y. enterolitica* serotype O:3 and to compare our results with other studies with serotype O:8 [7,8], and day 21, as late time of the infection to evaluate the bacterial clarification and the development of ReA. Thus, after 3 or 21 days after infection, mice were sacrificed and PP, MLN, the spleen, and joints of each mouse were aseptically removed. Three uninfected mice per group were used as controls.

Bacteriologic examination

The number of bacteria present in PP, MLN and the spleen, was determined by homogenization of these organs in phosphate-buffered-saline (PBS, pH 7.4), and plating of serial dilutions of the homogenates on Mueller-Hinton agar or Mac-Conckey-Irgasan [12]. To determine bacterial numbers in joints, two ankles from each mouse were excised by removing the skin and cutting just above and below tibiotarsal joint and placed immediately

in 1 ml of cold PBS. Joint extract was performed using a tissue homogenizer and plated on Mueller-Hinton agar. The detection limit was 25 CFU (log = 1.4) [12].

Assessment of arthritis

Mice were examined for visual appearance of arthritis in peripheral joints and the arthritis score was calculated as previously described [13]. Clinical severity of arthritis was graded on a four-number scale for each paw: 0 normal joint; 1 slight inflammation and redness; 2 severe erythema and swelling affection of the entire paw, with inhibition of the use; and 3 deformed paw of joint, with ankylosis, joint rigidity, and loss of function. The total score for clinical disease activity was based on all four paws, with a maximum score of 12/animal [14].

Histologic evaluation

Histologic examination of joints was performed after routine fixation, decalcification and paraffin embedding. Five-micrometer-thick sections were cut and stained with hematoxylin and eosin. Histopathologic scoring was performed as described by Choe *et al* [14] and Kyo *et al* [2]. Ankle from mice were assigned inflammation scores of 0-5, where 0 = normal, 1 = minimal infiltration of inflammatory cells, 2 = mild infiltration, 3 = moderate infiltration, 4 = marked infiltration, and 5 = severe infiltration. The same ankles were then given a score for cartilage/bone resorption, according to the following criteria: 0 = normal, 1 = minimal (small areas of resorption), 2 = mild (more numerous areas of resorption), 3 = moderate (obvious resorption of trabecular and cortical bone, without full-thickness defects in the cortex; loss of some trabeculae), 4 = marked (full-thickness defects in the cortical bone and marked trabecular loss, without distortion of the profile of the remaining

cortical surface), and 5 = severe (full-thickness defects in the cortical bone and marked trabecular bone loss, with distortion of the profile of the remaining cortical surface). The total score was defined as the sum of the score for inflammation and the score for cartilage/bone destruction. Each slice was scored by 2 independent observers and the average score was used.

Detection of antibodies specific for type I and II collagens

Sera obtained on days 0, 7, 14 and 21 after infection were added with dilution 1:50 into a 96-well plate precoated with 10 µg/ml type I or 5 µg/ml type II collagen. Peroxidase-conjugated goat antibodies specific for mouse IgG (Sigma) were added. This was followed by addition of the enzyme substrate (H_2O_2) and chromogen O-phenylendiamine (Sigma). The optical densities (OD) were determined by an automated microplate reader (Bio-Rad) at 490 nm. Sera from uninfected IL-12p40-/- and C57BL/6 mice were tested as negative controls.

Investigation of bacterial antigens and antibodies in the joint

At 21 days after infection, joint extracts were prepared as described above and centrifugated at 2000 x g for 20 min at 4 ºC. The supernatants were then used for detection of bacterial antigens and antibodies. Antigen preparations of *Y. enterocotilica* O:3 were obtained. Whole bacteria disrupted by sonication (SO) were prepared from washed bacterium pellets [16]. Outer membrane fraction (OM) and cytoplasmic proteins (Cyt) were prepared as described Michiels *et al* [16]. *Yersinia* outer proteins (Yops), which are released in the culture supernatant [17], were precipitated with trichloroacetic acid and

prepared as described by Trček *et al* [18]. The specificity of the intra-articular antibodies against *Yersinia* antigens was studied by dot blot. Similar protein concentration of the *Yersinia* antigen preparations, obtained as described above, were seeded (2µl) on nitrocellulose membrane. After blocking, the membrane was incubated with each clarified joint extract. Peroxidase-conjugated goat antibodies specific for mouse IgG (Sigma) were added. This was followed by addition of the enzyme substrate (H_2O_2) and chromogen enzyme 4-choro-1-naphtol (BioRad). Similarly, the articular presence of OM antigens was studied by dot blot, seeding 2 µl of each joint extract on nitrocellulose membrane. After blocking, the membrane was incubated with a pool of mouse anti-*Yersinia* OM serum previously developed in our laboratory by OM subcutaneous immunization of mice. In addition, the levels of antibodies against the different *Yersinia* antigen preparations were studied by ELISA. A 96-well plate was precoated with 10 µg/ml of SO, OM, Cyt, or Yops. Joint extracts were assayed without dilution. This was followed by addition of peroxidase-conjugated goat anti-mouse IgG, H_2O_2 and O-phenylendiamine (Sigma) as described above. Joint extracts from uninfected mice were used as controls.

TLR2, TLR4 and TNF–α mRNA levels in joints.

At day 21 after infection, anckle joints were excised as described above and immediately frozen in liquid N_2. Each frozen joint was then wrapped in aluminum foil and pulverized with a hammer [19]. The still frozen, pulverized tissue was then placed immediately in 1 ml of TRIzol reagent (Invitrogen). RNA extraction was performed as described the manufacturer. RNA from 4 individual joint samples for each group was pooled for analysis. Synthesis of cDNA was performed using 2 µg of total RNA, SuperScript II reverse

transcriptase (Invitrogen) and oligo-dT primer (Invitrogen). Polymerase chain reaction (PCR) was performed on cDNA for a total of 25 (β-actin), 30 (TNF-α) and 35 (TLR2 and TLR4) cycles at 94ºC for 30 s, 65ºC for 45 s and at 72ºC for 60 s. Reverse transcription (RT)-PCR products were visualized by agarose gel electrophoresis. Semiquantitative mRNA levels were determined by analyzing of the intensity of each band using the program ImageJ (Version 1.34s). Values were expressed in arbitrary units as the ratio of mRNA to the corresponding β-actin mRNA level. The sequences of the sense and antisense primers used in this study are as follows (5' to 3'): β-actin sense, TGGAATCCTGTGGCATCCATGAAAC; antisense, TAAAACGCAGCTCAGTAACAG TCCG (348 bp product); TNF-α sense, GGCAGGTCTACTTTGGAGTCATTGC; antisense, ACATTCGAGGCTCCAGTGAATTCGG (307 bp product); TLR2 sense, ACAGCTACTGTGTGACTCTCCGCC; antisense, GGTCTTGGTGTTCATTATCTTGC GC (602 bp product); TLR4 sense, GACCTCAGCTTCAATGGTGC; antisense, TATCAG AAATGCTACAGTGGATACC (740 bp product).

Measurement of TNF-α protein in joint extracts

At day 21 after infection, levels of TNF-α in joint extracts from four mice per group were quantified with an ELISA kit (Chemikine™ mouse TNF-α sandwich ELISA kit, Chemicon International) according to the manufacture's instructions.

Statistical analysis

Differences between groups were tested for significance by Student's unpaired *t*-test. A *p* value less than 0.05 was considered statistically significant. All experiments were repeated at least three times and revealed comparative results.

Results

Susceptibility of IL-12p40 to *Y. enterocolitica* O:3 infection

The survival of *Y. enterocolitica* O:3 infected IL-12p40-/- mice was impaired compared with C57BL/6 mice (75 % vs 100 %). To determine whether IL-12 deficiency affected mucosal and systemic *Yersinia* clarification, we assessed the bacterial numbers in cultures of PP, MLN and the spleen on day 3 and day 21 after the infection. As shown in Fig. 1, on day 3 the bacterial recovery was significantly increased in PP, MLN and the spleen of IL-12p40-/- compared with wild-type mice ($p < 0.04$, $p< 0.04$ and $p< 0.001$, respectively). At that time we found bacteria in the joints of both wild-type and knockout mice. On day 21, the bacteria were mostly clarified from PP, MLN, the spleen, and joints of both groups of mice (Fig. 1).

Effect of IL-12 deficiency on clinical disease activity and joint histology

IL-12p40-/- mice developed slightly macroscopic signs of arthritis (Score 1-2/paw) with an incidence ranging from 40 to 50 %. The total score for clinical disease activity based on all four paws was significantly higher in IL-12p40-/- mice than in wild-type mice (Fig 2A) on day 16-24 after infection ($p<0.01$- $p<0.03$). Histopathological changes were found in the joints of IL-12p40-/- mice (Fig. 2 A). In these animals, dilation of the joint cavity, luminal disorganization and desquamation of the synovial membrane were observed. Mild proliferation of synovial lining cells and the development of pannus at the junction of

cartilage and synovium were also detected. Trabecular bone loss was observed (Total score 7-9) (Fig 2A). Neither clinical arthritis nor histological changes were observed in wild-type C57BL/6 mice during the course of infection (Fig. 2A). According with the histological alterations in IL-12p40-/- mice, we found a significantly stronger anti-type II collagen specific response ($p<0.02$, $p<0.01$, $p<0.04$ on day 7, 14 and 21 after infection, respectively) with no differences in the levels of anti- type I collagen antibodies compared with the wild-type C57BL/6 mice (Fig. 2B).

Assessment of intra-articular antibodies against *Yersinia* antigens

By dot-blot, among different *Yersinia* antigen preparations, at 21 days after infection, we detected stronger intra-articular antibody response to *Yersinia* SO and OM antigens in IL-12p40-/- mice (Fig 3A). Moreover, significantly higher levels of intra-articular antibodies against SO, OM and Yops ($p<0.005$, $p<0.0007$, $p<0.05$, respectively) were detected by ELISA in IL-12p40-/- mice compared with wild-type C57BL/6 mice (Fig 3B). In addition, OM antigens were detected in joint extracts from infected mice (Fig 3C). Joint extracts from uninfected mice of both groups of mice showed negative reactions (Fig 3A and C).

TLR2, TLR4, and TNF-α mRNA expression in the joint

Since the enhanced expression of receptors, such as TLR2 and TLR4, that recognize specific molecular patters associated with microbes [2], triggers the release of pro-inflammatory cytokines such as TNF-α [11], the expression of TLR2, TLR4 and TNF-α mRNA was investigated. We detected that TLR2, TLR4 and TNF-α mRNA levels were

markedly increased in joints of IL-12p40-/- mice compared with the control (Fig. 3D). This increase was paralleled by a significant increase in TNF-α protein level ($p<0.003$) (Fig 3D).

Discussion

In the present study we analyzed the role of IL-12p40 in *Yersinia*-induced arthritis. We used *Y. enterocolitica* O:3, a serotype frequently associated with ReA in humans [20]. We show here that IL-12 plays a central role in early protection against *Y. enterocolitica* O:3. Our data confirm previous works in IL-12p40-/- mice infected with *Yersinia* [7,8]. Moreover, this study stresses the critical role of IL-12p40 in immunity against serotype O.3 of *Yersinia* at mucosal surfaces, where we detected differences bacterial load of the PP and MLN between wild-type and knockout mice. Since the initial immune response to bacteria or their antigenic components at mucosal surface level could influence the extent of their dissemination to the joints, IL-12p40 deficiency could influence also the susceptibility to *Yersinia*-induced ReA. In addition, the significant higher bacterial load of MLN and the spleen of IL-12p40-/- mice on day 3 after infection suggests that IL-12 restricts the early spread of *Yersinia* [7]. Moreover, the presence of the bacteria in the joint at day 3 after infection in both wild-type and immunodeficient mice suggested an early synovial invasion of the pathogen. This matches clinical reports of direct invasion of *Yersinia* which develop in typical cases of ReA after a short phase of bacterial arthritis [20]. Moreover, other studies have demonstrated an early synovial invasion of *Y. enterocolitica* in rat [21] and hamster models [22]. Late after the infection (day 21) like in clinical observations [20], we observed that bacteria culture from joint extracts resulted negative, and almost bacterial clarification in PP, MLN, and the spleen was detected in the mice.

In the present study IL-12p40-/- mice developed clinical arthritis with slight swelling and thickness in joints. Histologic examination in the affected joints revealed synovial hyperthrophy and mild cartilage damage, resembling human ReA [23]. Type I and type II collagens are the main collagens of synovial membrane and cartilage, respectively [24]. Therefore, a kinetic analysis of the humoral immunity to these collagens was performed. We detected statistically significant higher levels of type II collagen antibodies in IL-12p40-/-mice, which correlated with the histological alterations in the joint. This result suggests reactivity to articular collagens. Probably, bacteria stimulate strong immune responses against several antigens and also work as a trigger for autoimmune reaction against type II collagen [25].

Following the accumulated evidence for the involvement of bacterial products in ReA [2,26,27,28], we investigated bacterial antigens and antibodies in joint extracts of infected mice. The significant increase of anti-*Yersinia* antibodies observed in infected IL-12p40-/- is according with other *Yersinia*-induced arthritis animal model and suggested that arthritis susceptibility correlates with strong antibody response [29]. This enhancement of the anti-microbe antibody response could be due to the absence of IL-12p40 which modulates Th2 induced-humoral immune response by polarization of T cell towards Th1 phenotype [5]. Thus, IL-12p40-/- mice mounted a polarized Th2 cell response after *Salmonella enteritidis* [30] or *Leishmania major* infections [31]. In addition, innate immune response mediated by TLRs to bacterial components could be involved in triggering B cells to produce the increase of anti-*Yersinia* antigen antibodies [32]. Interestedly, by dot-blot with antigens on the nitrocellulose, we found in joints from IL-12p40-/- mice stronger reactivity to the whole bacterial antigens (SO) and especially to *Yersinia* OM antigens. This suggested presence of these antigens in the joint which was confirmed by dot blot with joint

extracts on the nitrocellulose using *Yersinia* OM-specific sera (Fig 3C). These results indicate that OM components could have a role as causative antigen of articular inflammation. In fact, OM proteins of *Salmonella* have demonstrated to be the immunodominant antigens in *Salmonella* ReA [33]. Moreover, OM includes TLR ligands such as LPS [34] and TLR4 ligands have the potential to induce arthritis [2]. In addition, lipoproteins derived from *Borrelia burgdorferi*, a causative bacterium in Lyme disease, can evoke acute inflammatory arthritis depend on TLR2 mediated pathway [35]. In the present study, we also observed higher expression of TLR2, TLR4 and TNF-α mRNA, and TNF-α protein in the joints of infected IL-12p40-/- mice. Since stimulation of TLRs by microbial components triggers expression of several genes of proinflammatory cytokine such as TNF-α [2,11,36], these findings suggest that increased TNF-α mRNA and protein expressions could be associated with the increased expression of TLR mRNA. These results match with clinical observations [37], and suggest that enhanced TLR mRNA expression in IL-12p40-/- mice induced by OM antigens could be associated with the increased expression of TNF-α and the inflammatory response observed in this group of mice. On the other hand, the expression of TLR mRNA could be a consequence of direct exposure to microbial compounds or of the presence of inflammatory mediators such as IL-1β or TNF-α in the joints, as demonstrated by other authors [38,39]. Since in our study both TNF-α mRNA and protein were increased in the joints of IL-12p40-/- mice, this cytokine could be a candidate mediator that enhances TLR expression in inflamed joints.

In conclusion, the present study identifies the potential of IL-12p40 to protect against arthritis induced by *Yersinia* infection. The possible mechanisms underlying the susceptibility of IL-12p40-/- mice to ReA could be their impaired bacterial clearance since

low bactericidal capacity of the host has been suggested as a prerequisite for arthritis development [29]. Although IL-12 but not IL-23 is considered an important mediator of immune response against microbial pathogens [5,10], further studies could elucidate the protective role against the ReA of each IL-12-related cytokines. In addition, we reported that antigens present in the OM and TLRs could play a central role in the pathogenesis of *Yersinia*-induced ReA. This study contributes to a better understanding of the host factors predisposing to ReA, suggests a potential role of innate immunity-mediated inflammation in ReA, and may provide data for treatments for ReA

Acknowledgements

This work was carried out with the financial support of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 2430) and National University of San Luis (Projects 8803 and 0401). María S. Di Genaro and Silvia G Correa are member career of CONICET. The assistance for histological analysis of Lic Claudia Aguilera and Ms Angelina Bernardi, and the critical review of the manuscript by Dr. Nidia Gomez is gratefully acknowledged. The authors acknowledge the institutional animal care of National University of San Luis for reviewing the experimental work

References

1. Leirisalo-Repo. Reactive arthritis. Scand J Rheumatol 2005;34:251-9.
2. Kyo F, Futani H, Matsui K, Terada M, Adachi K, Nagata K, et al. Endogenous interleukin-6, but not tumor necrosis factor α, contributes to the development of Toll-like receptor 4/Myeloid differentiation factor 88-mediated acute arthritis in mice. Arthritis Rheum 2005;52:2530-40.
3. Appel H, Neure L, Kuhne M, Braun J, Rudwaleit M, Sieper J. An elevated level of IL-10 and TGFbeta-secreting T cells, B cells and macrophages in the synovial membrane of patients with reactive arthritis compared to rheumatoid arthritis. Clin Rheumatol 2004;23:435-40.
4. Bas S, Kvien TK, Buchs N, Fulpius T, Gabay C. Lower level of synovial fluid interferon-gamma in HLA-B27-positive than in HLA-B27–negative patients with *Chlamydia trachomatis* reactive arthritis. Rheumatology 2003;42:461-7.
5. Becker C, Wirtz S, Neurath M. Stepwise regulation of Th1 responses in autoimmunity: IL-12-related cytokines and their receptor. Inflamm Bowel Dis 2005;11:755-64.
6. Bohn E, Autenrieth IB. IL-12 is essential for resistance against *Yersinia enterocolitica* by triggering IFN-gamma production in NK cells and CD4+ T cells. J Immunol 1996; 156:1458-68.
7. Hein J, Sing S, Di Genaro S, Autenrieth I. Interleukin-12 and Interleukin-18 are indispensable for protective immunity against enteropathogenic *Yersinia.* Microbial Pathogenesis 2001;31:195-9.
8. Di Genaro MS, Waidmann M, Kramer U, Hitziger N, Bohn E, Autenrieth IB. Attenuated *Yersinia enterocolitica* mutant strains exhibit differential virulence in

cytokine deficient mice: implications for the development of novel live carrier vaccines. Infect Immun 2003;71:1804-12.

9. de los Toyos JR, Vazquez J, Sampedro AM, Hardisson C. *Yersinia enterocolitica* serotype O:3 is arthritogenic for mice. Microb Pathog 1990;8:363-70.
10. Haraguchi S, Day NK, Nelson RP, Emmanuel P, JE Duplantier, Chistodoulou CS, et al. Interleukin 12 deficiency associated with recurrent infections. Proc Natl Acad Sci;95:13125-9.
11. Takeda K, Kaisho T, Akira S. Toll-like receptor. Ann Rev Immunol 2003;21:335-76.
12. Autenrieth I.B, Kempf V, Sprinz T, Preger S, Schnell A. Defence mechanisms in Peyer's patches and mesenteric lymph nodes against *Yersinia enterocolitica* involve integrins and cytokines. Infect Immun 1996;64:1357-68.
13. Banda NK, Vondracek A, Kraus D, Dinarello CA, Kim S-H, Bendele A, et al Mechanisms of inhibition of collagen-induced arthritis by murine IL-18 binding protein. J Immunol 2003;170:2100-5.
14. Banda NK, Kraus D, Vondracek A, Huynh H, Bendele A, Holers VM, et al. Mechanisms of effects of complement inhibition in murine collagen-induced arthritis. Arthritis Rheum 2002;46:3065-75.
15. Choe JY, Sarah BC, Wu SR, Corr M. Interleukin 1 receptor dependence of serum transferred arthritis can be circumvented by Toll-like receptor signalling. J Immunol 2003;197:537-42.
16. Michiels T, Wattieau P, Brasseur R, Ruysschaert JM, Cornelis G. Secretion of Yop proteins by *Yersiniae*. Infect Immun 1990;58:2840-9.

17. Heesemann J, Gross U, Schmidt N, Laufs R. Immunochemical analysis of plasmid-encoded proteins released by enteropathogenic *Yersinia* sp grown in calcium deficient media. Infect Immun 1986;54:561-7.

18. Trček J, Wilharm G, Jacovi C A. Heesemann J. *Yersinia enterocolitica* Yop Q: strain-dependent cytosolic accumulation and post-translational secretion. Microbiology 2002;148:1457-65.

19. Brown CR, Blaho VA, Laiacono CM. Susceptibility to experimental Lyme arthritis correlates with KC and monocyte chemoattractant protein-1 production in joints and requires neutrophil recruitment via CXCR2. J Immunol 2003;171:893-901.

20. Toivanen A, Toivanen P. Epidemiologic aspects, clinical features, and management of ankylosing spondylitis and reactive arthritis. Curr Opin Rheumatol 1994;6:354-9.

21. Grispeng-Lerche C, Skurnik M, Toivanen P. Role of Yad A-mediated collagen binding in arthritogenicity of *Yersinia enterocolitica* serotype O:8: experimental studies with rats. Infect Immun 1995;63:3222-6.

22. Di Genaro MS, Escudero ME, Muñoz E, Aguilera C, Juárez A, Scardapane L, et al. Arthritogenicity of *Yersinia enterocolitica* O:8 in hamsters: analysis of the immune response. Folia Microbiol 1998;43:690-6.

23. Keat A. Reiter's syndrome and reactive arthritis in perspective. N Engl J Med 1983;309:1606-15.

24. Schulze-Koops H, Burkhardt H, Heesemann J, von der Mark K, Emmrich F. Characterization of the binding region for the *Yersinia enterocolitica* adhesin Yad A on types I and II collagen. Arthritis Rheum 1995;38:1283-9.

25. Singh S K, Girschick H J. Lyme borreliosis: from infection to autoimmunity. Clin Microbiol Infect 2004;10:598-614.

26. Hoogkamp-Korstanje JA, de Koning J, Heesemann J. Persistence of *Yersinia enterocolitica* in man. Infection 1988;16:81-5.

27. Granfors K, Jalkanen S, von Essen R, Lahesmaa-Rantala R, Isomaeki O, Pekkola-Heino K, et al. A. *Yersinia* antigens in synovial fluid cells from patients with reactive arthritis. N Eng J Med 1989;322:216-21.

28. Granfors K, Jalkanen S, Linberg AA, Maki-Ikola O, von Essen R, Lahesmaa-Rantala R, et al. *Salmonella* lipopoysaccharide in synovial cells from patients with reactive arthritis Lancet 1990;335:685-8.

29. Gaede KI, Heesemann J. Arthritogenicity of genetically manipulated *Yersinia enterocolitica* serotype O8 for Lewis rats. Infect Immun 1995;63:714-9.

30. Mattner F, Magram J, Ferrante J, Launois P, Di Padova K, Behin R, et al. Genetically resistant mice lacking interleukin-12 are susceptible to infection with *Leishmania major* and mount a polarized Th2 cell response. Eur J Immunol 1996;26:1553-9.

31. Lehmann J, Bellmann S, Werner C, Schroder R, Schutze N, Alber G. IL-12p40-dependent agonistic effects on the development of protective innate and adaptive immunity against *Salmonella enteritidis*. J Immunol 2001;167:5304-15.

32. Leadbetter EA, Rifkin IR, Hohtbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature 2001,416:603-7.

33. Saxena S, Aggarwal A, Misra R. Outer membrane protein of salmonella is the major antigenic target in patients with salmonella induced reactive arthritis. J Rheumatol 2005;32:86-92.

34. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 in the *Lps* gene product. J Immunol 1999;162:3749-52

35. Hirschfeld M, Kirschning CI, Schwandner R, Wesche H, Weis JH, Wooten RM, et al. Inflammatory signaling by *Borrelia burgdorferi* lipoproteins is mediated by Toll-like receptor 2. J Immunol 1999;163:2382-6.

36. Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immun 2005;17:1-14.

37. De Rvcke L, Vandooren B, Kruithof E, De Kevser F, Vevs EM, Baeten D. Tumor necrosis factor alpha blockade treatment down-modulates the increases systemic and local expression of Toll-like receptor 2 and Toll-like receptor 4 in spondyloarthropathy. Arthritis Rheum 2005;2:2146-58.

38. Seibl R, Birchler T, Loeliger S, Hossle JP, Gay RE, Saurenmann T, et al. Expression and regulation of Toll-like receptor in rheumatoid arthritis synovium Am J Pathol 2003;162:1221-7.

39. Raffeiner B, Dejaco C, Duftner C, Kullich W, Goldberger C, Vega SC, et al. Between adaptive and innate immunity: TLR4-mediated perforin production by $CD28^{null}$ T-helper cells in ankylosing spondylitis. Arthritis Res Ther 2005;7:1412-20.

Figure legends

Fig. 1. Bacterial load in wild-type and IL-12p40-/- mice after *Y. enterocolitica* O:3 infection: Bacterial number in PP (Peyer's patches), MLN (mesenteric lymph nodes), the spleen and joints after 3 and 21 days of oral infection with *Y. enterocolitica* O:3. The results are the average of three independent experiments (seven mice per group). Each point on the scatter plot represents log CFU per organ from an individual mouse. Dotted line represents the detection limit (log CFU = 1.4). The median for each data set from mice that survived is indicated with a horizontal bar, and asterisks represent statistically significant. CFU (colony-forming units), ns: not significant.

Fig. 2. A: Clinical disease activity and histological examination of the joints from wild-type C57BL/6 and IL-12p40-/- mice 21 days after infection: The clinical disease activity was determined every day. The data are expressed as the arthritis score for each group vs days after infection. The results are representative of one of three experiments. Joints from wild-type C57BL/6 mice showed no histological changes. IL-12p40-/- mice showed dilation of the joint cavity, luminal disorganization and desquamation of the synovial membrane (original magnification x 40). The score indices were calculated as described materials and methods. Values are the mean ± SD. Representative results from three independent experiments are shown. **B: Kinetics of anti-type I and type II collagen antibody production following infection.** Levels of IgG against type I and type II collagens were evaluated in sera from wild-type C57BL/6 and IL-12p40-/- mice prior to or 7, 14, and 21 days after the infection. Asterisks represent statistically significant.

Fig. 3. A: Articular antibodies to *Yersinia* antigens in wild-type C57BL/6 and IL-12p40-/- mice at 21 days after infection by dot-blot: *Yersinia* antigen preparations were seeded on nitrocellulose and incubated with joint extract from each mouse. Representative results from three mice per group are shown. **B: Articular antibodies to *Yersinia* antigens in wild-type C57BL/6 and IL-12p40-/- mice at 21 days after infection by ELISA**: the plate was coated with *Yersinia* antigen preparations; each joint extract was assayed without dilution. Asterisks represent statistically significant. **C: Articular *Yersinia* OM antigens in joint extracts of wild-type C57BL/6 and IL-12p40-/- mice at 21 days after infection by dot-blot:** joint extracts from wild-type C57BL/6 and IL-12p40-/- mice were seeded on nitrocellulose and incubated with a pool of mouse anti-*Yersinia* OM serum. Representative results from three mice per group are shown. **D: TLR2, TLR4 and TNF-α mRNA, and TNF-α protein expressions in joint.** Total RNA from 4 pooled joints per group of mice was analyzed by RT-PCR at day 21 after infection. Relative mRNA levels are showed normalized with β-actin. TNF-α protein levels in joint extracts were measured by ELISA at day 21 after infection. Data are expressed as mean TNF-α levels ± SD of four mice per group and are representative of one of three independent experiments.

Figure 1

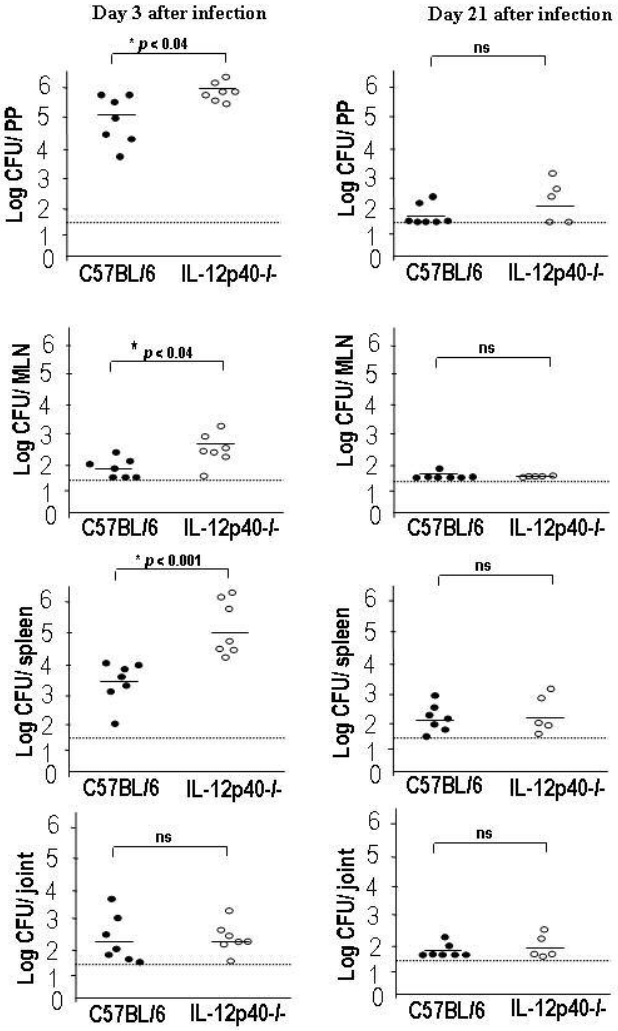

Day 3 after infection
Day 21 after infection
* p < 0.04
ns
Log CFU/ PP
Log CFU/ MLN
Log CFU/ spleen
Log CFU/ joint
* p < 0.001
C57BL/6
IL-12p40-/-

Figure 2

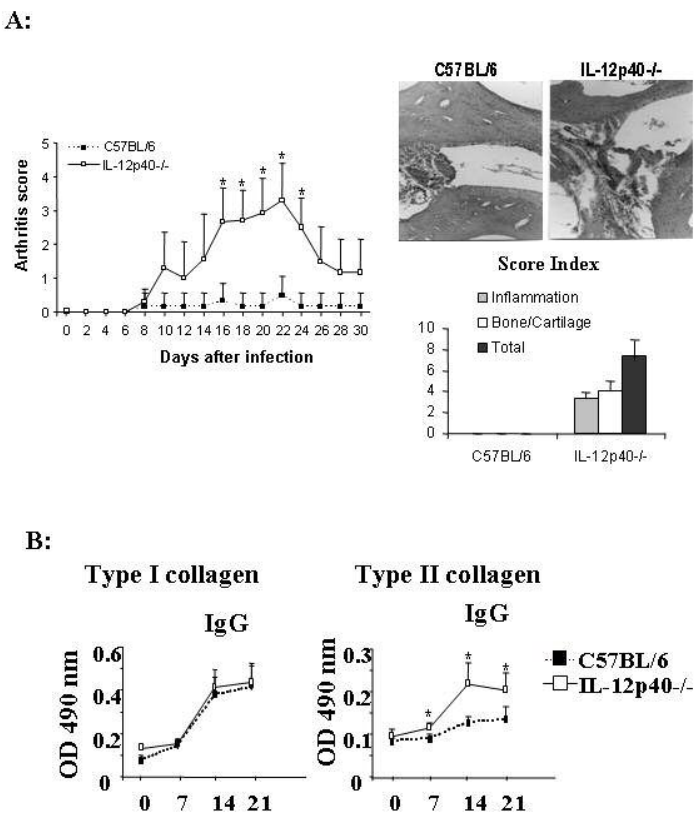
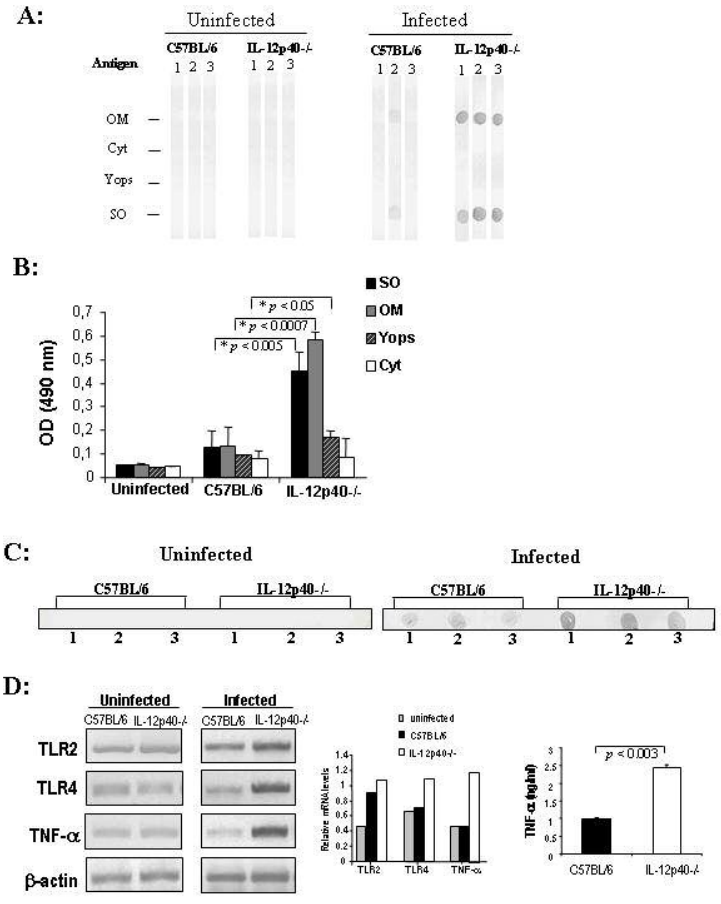

A:
C57BL/6
IL-12p40-/-
Arthritis score
Days after infection
Score Index
Inflammation
Bone/Cartilage
Total
B:
Type I collagen
IgG
Type II collagen
IgG
OD 490 nm
C57BL/6
IL-12p40-/-

Figure 3


A:
Uninfected
Infected
C57BL/6
IL-12p40-/-
Antigen
1 2 3
OM
Cyt
Yops
SO
B:
SO
OM
Yops
Cyt
*p < 0.05
*p < 0.0007
*p < 0.005
OD (490 nm)
Uninfected
C57BL/6
IL-12p40-/-
C:
Uninfected
Infected
D:
TLR2
TLR4
TNF-α
β-actin
uninfected
p < 0.003