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Arthritis induced with cartilage-specific antibodies is IL-4 dependent

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Key Words: IL-4, Th1/Th2, collagen type II, mAb and arthritis

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Abbreviations: CAIA, Collagen antibody induced arthritis; CII, Collagen type II; CIA, Collagen induced arthritis; RA, Rheumatoid arthritis

Summary

It is widely believed that IL-4 exerts its influence by profiling the immune response during priming and expansion of immune cells and thereby modulates the outcome of chronic inflammation. In the present investigation, collagen antibody induced arthritis (CAIA) was used to delineate the role of IL-4 in a T cell independent inflammatory phase. Mice predisposed to Th2 cytokines (BALB/c and Stat4 deficient mice) developed a more severe arthritis than mice biased towards Th1 cytokines (C57BL/6 and Stat6 deficient mice). Reduced incidence of CAIA was observed in IL-4 deficient mice compared to control littermates. Infiltrating cells in the paws of IL-4 sufficient mice had increased osteoclast activity, tumor necrosis factor α (TNF-α) and Interleukin-1β (IL-1β) secretion. Massive infiltration of granulocytes, joint and cartilage damage were present in arthritic paws. Depletion of IL-4 suppressed CAIA, which was ameliorated by IFN-γ neutralization. IL-1R and IL-1RTNFR deficient mice were completely resistant to CAIA. Thus, IL-4 promotes an antibody-mediated and TNF-α/IL-1β dependent inflammation in vivo.

Introduction

Rheumatoid arthritis (RA) is a chronic debilitating inflammatory autoimmune disease involving articular cartilage. Recent observations suggest that RA may not be a single disease but a collection of syndromes. Many cellular and molecular factors contribute to the pathogenesis of this complex disease. Cytokines play a prominent role in RA and their complex interactions in RA development are only just starting to be discovered. The proinflammatory cytokines TNF-α and IL-1 are critical mediators in the inflammatory process of arthritis [1, 2] and neutralizing these effector cytokines have proven successful in treatment of RA.

The CD4+ T helper cell response is classically compartmentalized into Th1 and Th2 responses [3]. Th1 cells produce IL-2, IFN-γ and IL-12 and contribute mainly to cellular immunity. Th2 cells produce cytokines such as IL-4, IL-5, and IL-13 and support IgG1 and IgE antibody production, and the underlying humoral immunity. The classification of Th1/ Th2 dichotomy has provided a framework in which to compare types of immune responses. However, this segregation of immune responses has also lead to oversimplification of the autoimmune diseases into either Th1 or Th2 type. The inflammatory process in RA has been suggested to be promoted by Th1 cytokines whereas Th2 cytokines might suppress the disease [4]. This assumption was supported by the regulatory effect of Th2 cytokines on Th1 activity [5]. Furthermore, Th2 cytokines modulated macrophage function and protected subchondral bone erosion and cartilage damage by delaying differentiation of osteoclasts [6], possibly through osteoprotegrin ligand-mediated mechanisms [7]. Therapeutic strategies by blocking Th1 cytokine IFN-γ have also been initiated in RA patients [8]. However, these assumptions have been challenged because disease mechanisms in reality are very complex [9]. Hence, there is a need for animal models that are able to delineate the role of these cytokines in the arthritis disease process.

Th2 cytokine, IL-4 is multifunctional [10] and has been shown to induce expression of MHC class II molecules on B cells, activate mast cells, mediate Th2 cell commitment and survival, and induce Ig class switching to IgG1 and IgE. IL-4 is believed to exhibit its anti-inflammatory effects by suppression of macrophage functions such as IL-1, TNF-α and NO production [11, 12] and could suppress synoviocyte proliferation [13]. Moreover, IL-4 stimulates the synthesis of several cytokine inhibitors such as interleukin-1 receptor antagonist (IL-1Ra), IL-1-receptor type II and TNF receptors [11, 12, 14]. In addition, IL-4 inhibits both osteoclast activity and survival, and thereby blocks bone resorption *in vitro* [6].

Using recombinant IL-4 and adenovirus gene therapy, suppression of early events of collagen induced arthritis and proteoglycan induced arthritis were observed [15-18]. But, the suppressive effect is not substantial, however, in some cases the effect could be enhanced by a combination strategy [15, 19]. Most of the studies involving amelioration of arthritis with IL-4 showed that this cytokine reduced the level of anti-CII antibody production [16, 17, 20, 21] suggesting that the arthritis suppressing effect of IL-4 could possibly be explained at the level of antibody synthesis in these studies. Alternatively, IL-4 could operate on several levels in the arthritis process [19]. Earlier, we demonstrated that IL-4 actually plays different roles depending on the type of adjuvant used and the phase (acute vs chronic) of the clinical disease. Our results, in a variant of CIA mouse model, indeed suggested that IL-4 deficiency had a promoting effect on development of disease relapses [22], although we also noted a pronounced protection from arthritis during the early severe stages. Hence, it is of interest to understand whether IL-4 deficiency can actually promote antibody mediated inflammation in vivo, which is believed to be the major pathogenic inflammatory pathway during acute CIA.

Collagen antibody induced arthritis (CAIA) is now extensively used to study the inflammatory phase of arthritis. Antibody-mediated arthritis is dependent on FcγR [23] and complement [24] but not B or T cells [25], with neutrophils and macrophages being the major mediators of this inflammation [26]. The CAIA mouse model provides an opportunity to study the inflammation phase without involving the priming phase of the immune response. Hence, we used this model to gain an understanding how IL-4 regulates the inflammatory process, which may prove essential for designing effective therapeutic interventions for RA.

Results

BALB/c mice developed more severe arthritis than C57BL/6 mice

BALB/c mice are believed to have a genetic predisposition towards Th2 responses [27] compared to the Th1 cytokine milieu prevalent in C57BL/6 mice. Arthritis induced with CII specific monoclonal antibodies in various mouse strains demonstrated genetic heterogeneity in the development of inflammatory phase of the joint disease [26]. As shown in Table 1, BALB/c mice developed more severe CAIA (32.9±3.0) with higher incidence (100%) than the C57BL/6 mice (2.3±0.3; 21%). This observation raised the possibility for a pro-inflammatory role of the Th2 cytokines.

Stat6 but not Stat4 deficient mice are relatively resistant to CAIA

In order to confirm our hypothesis that the anti-inflammatory Th2 cytokines might actually promote arthritis at the effector phase, we compared CAIA susceptibility between Stat6 and Stat4 deficient mice. Stat6 deficient mice are known to be unable to mount a Th2 response whereas Stat4 deficient mice are unable to mount a Th1 response. In concordance to our hypothesis, Stat 6 mice had less severe arthritis than the Stat 4 mice both before and after LPS injection (LPS injection promotes incidence and severity of antibody initiated arthritis) (Figure 1). However, during the later phase of the disease, arthritis score declined rapidly in Stat4 but not in Stat6 deficient mice.

IL-4 deficient mice are less prone to collagen antibody mediated arthritis

To directly address the question, whether the Th2 cytokine IL-4 has arthritis promoting effect, we tested CAIA in both the IL-4 deficient and sufficient mice on the B10.RIII background. In the two similar experiments, with several animals in each group, 63-67% of IL-4 sufficient and 13-20% of IL-4 deficient animals developed arthritis ($P \leq 0.0275$). We consistently found that IL-4 deficiency significantly suppressed the incidence of the antibody-induced arthritis (Figure 2). However, the used IL-4 gene deficient animals also had linked genes originating from the embryonic stem cells. Thus, although the mice were backcrossed to C57Bl/10 for 13 generations, they still had a 12.7 cM of 129-linked fragment on chromosome 11. Nevertheless, there were no IL-4 spots observed in the Con A-stimulated lymph node cultures from IL-4 deficient animals in the ELISPOT assay [22]. On the other hand, this does not exclude the influence of another 129 gene; therefore to confirm and clarify our findings, we decided to deplete IL-4 using 11B11 antibodies in the naïve B10.RIII mice in the CAIA experiment. We found that the IL-4 depleted mice had significantly reduced incidence of arthritis induced by the anti-CII antibodies (Figure 3), thereby confirming our observation that IL-4 deficiency is indeed suppressing CAIA. Histology of the IL-4 deficient non-arthritic mice was almost normal whereas the arthritic IL-4 deficient mice (3 out of 10) did not differ from the IL-4 sufficient mice (Figure 4 I, J and N). Similarly, in the IL-4 antibody depleted mice, proteoglycan depletion was prominent in arthritic compared to non-arthritic mice (Figure 4 O & P),

Several lines of evidence support a role for osteoclasts in focal bone erosion in RA and in animal models of arthritis [28]. IL-4 has been shown to inhibit osteoclast formation by acting on osteoclast precursors via peroxisome proliferator-activated receptor [29] and stat6 dependent inhibition of NF-kB [30]. However, a possible role of IL-4 on osteoclastogenesis in an acute inflammatory condition *in vivo* has not been investigated. In this study, we found that the anti-CII antibody injected IL-4 sufficient mice had in fact more pronounced osteoclast activity than the IL-4 deficient ones (Figure 4C & D). Whether the observed increase in osteoclast activity is due to IL-4 or to other secondarily elevated pro-inflammatory cytokines present in the arthritic joint was not however ascertained.

Pro-inflammatory cytokines TNF-α and IL-1 were predominant in the arthritic mouse joints [31] and IL-4 is believed to exhibit its anti-inflammatory effects by suppression of these pro-inflammatory cytokines [11, 12]. However, in these studies it is not possible to dissect the priming and inflammatory phase of arthritis separately. Since we observed that IL-4 deficient mice were protected from the inflammatory phase of arthritis induced with anti-CII antibodies, we stained for TNF-α and IL-β producing cells in the paws of IL-4 deficient and sufficient mice. In concordance with our clinical observations, joint sections from IL-4 sufficient mice had more cells producing TNF-α (Figure 4E & F) and IL-1β (Figure 4G & H) than IL-4 deficient mice.

IFN-γ neutralization abrogates anti-IL-4 mediated suppression

IFN-γ has been demonstrated as one of the major macrophage activation factors and it is known to oppose IL-4 effects. Interestingly, as shown in Figure 3, IFN-γ depletion by DB1 antibodies enhanced the development of arthritis induced by CII specific monoclonal antibodies. To understand whether IFN-γ has any role in the anti-IL-4 mediated suppression of arthritis, we co-transferred the IL-4 and IFN-γ depleting antibodies. Surprisingly, neutralization of IFN-γ abrogated the anti-IL-4 mediated arthritis suppression (Figure 3) suggesting an important ameliorating role for IFN-γ at the effector phase of arthritis pathogenesis. Histology of the affected joints correlated well with the clinical observations (Figure 4 K, L and M). In the cumulative incidence of arthritis, the p value is highly significant ($p = 0.001$) between the groups in which IFN- γ relieved the suppression induced by anti-IL-4 treatment (both) and anti-IL-4 alone treated mice.

IL-1R and IL-1RTNF-R deficient mice are resistant to CAIA

Previous studies demonstrated that IFN-γ deficient mice have up regulated levels of interleukin-1β but not TNF-α and IL-6. Furthermore, TNF-α and IL-1 but not IL-6 have been shown to be important in CAIA [32]. Here, we confirmed these findings using IL1-R and IL-1RTNFR deficient mice, which were totally resistant to CAIA induction using our set of anti-CII monoclonal antibodies (Figure 5).

Discussion

IL-4 is produced by T cells, mast cells and bone marrow stromal cells and is perceived as an anti-inflammatory cytokine; however, under certain circumstances IL-4 may function as a pro-inflammatory cytokine. In the present investigation, for the first time we show that genetic deletion or antibody neutralization of the anti-inflammatory Th2 cytokine IL-4 increased the resistance to develop collagen antibody induced arthritis (CAIA). Infiltrating cells in the paws of IL-4 sufficient but not deficient mice showed increased osteoclast activity and TNF-α secretion. Massive infiltration of granulocytes, joint and cartilage damage were observed in the arthritic paws. Anti-IL-4 mediated suppression of the inflammatory arthritis was abrogated by IFN-γ depletion. Similarly, stat6 deficient mice that are unable to mount Th2 responses were resistant to arthritis after antibody transfer compared to Stat4 deficient mice. Even after the secondary stimulus LPS injection that enhances the arthritis incidence and severity [26], stat6 deficient mice showed significantly less severe arthritis compared to stat4 deficient mice. Interestingly, IL-1R and IL-1RTNFR deficient mice were completely resistant to CAIA.

Similar to our present observations, when the function of IL4 was neutralized either by antibody administration or genetic ablation the onset and severity of CIA were greatly reduced [22, 33]. Furthermore, DBA/1 mice expressing an IL-2Rβ/IL-4R chimeric transgene showed similar results. In these mice, IL-2 binding of the receptor transmits a signal via the IL-4 pathway [34]. Arthritis developed in these chimeric transgenic mice at an accelerated rate with increased severity. The autoimmune disease was associated with an increase in type 2 cytokines (IL-4, IL-5, IL-10). Earlier, we also found that IL-4 plays different roles depending on the type of adjuvant used in CIA and the phase (acute or chronic) of the clinical disease [22]. Despite the protective effect against cartilage erosions, local delivery of adenoviral vector containing IL-4 actually increased the inflammation in collagen-induced arthritis [35].

On the other hand, several studies have demonstrated a therapeutic role of IL-4 in inflammatory arthritis using antigen induced arthritis mouse models [15-18, 20, 21]. However, in these models, the therapeutic effect of IL-4 could very well be at the level of antibody synthesis. Here, we addressed the role of IL-4 at the effector level of arthritis without involving the priming phase by injecting pre-formed CII specific antibodies and found that IL-4 could have a pro-inflammatory role at the end stage effector phase of arthritis. The possible contrasting results as compared with earlier studies on CIA model could possibly be explained by the effect that the effector phase of CIA and CAIA are not identical, as the CAIA isolates the antibody-mediated component of the pathogenesis. RA is a chronic disease and many cellular components and humoral mediators interact and aid in the disease progression. Antibodies to several antigens and the presence of rheumatoid factor in RA patients have been well documented. Hence, it is possible that the antibody initiated/mediated mechanisms studied using CAIA are more relevant and useful not only in the initiation phase of arthritis but also during relapses in the chronic phase of RA.

Antibody induced arthritis develops also in the absence of B and T cells [25], however we do not exclude a modulatory role of T cells in CAIA. In this inflammatory process, it is possible that the effect of anti-IL-4 treatment and /or the anti-IFN-γ co-treatment could be due to an effect on neutrophils, macrophages or other effector cells [26]. Alternatively, regulation of pro-inflammatory cytokines like TNF-α and IL-1β which are known to be involved in the antibody–mediated disease [32] could also be important in this situation. Interestingly, the suppression of arthritis mediated by neutralization of IL-4 is dependent on IFN-γ, since co-treatment of these mice with neutralizing anti-IFN-γ mAb abolished the suppressive effect of anti-IL-4. It has long been recognized that IFN-γ is one of the major macrophage activation factors. However, it is possible that macrophage activation factors other than IFN-γ or contact mediated macrophage activation might also be involved in the anti-IL4 mediated inhibition of antibody mediated inflammation.

Like RA, the inflammatory response in the arthritic mouse joint is predominantly mediated by the pro-inflammatory cytokines TNF-α and IL-1 [31], all of which have been successfully targeted to down-regulate the disease

[36]. Up regulation of IL-1β but not TNFα and IL-6 expression has been observed in the IFN-γ deficient C57BL/6 mice enabling the CIA induction in the non-susceptible mouse strain [37]. Similar results have been reported using IFN-γ deficient mice in other models of autoimmunity in which susceptibility to autoimmunity was enhanced by preventing the effects of IFN-γ [38]. Furthermore, mice lacking IFN-γ receptors were also found to be highly susceptible to CIA [39, 40]. This concept is supported by the observations that IFN-γ has been shown to be efficient in the treatment of RA [8] and that IFN-γ production is diminished in peripheral blood and in synovial fluid lymphocytes of RA patients [41]. Hence, in the present study it is possible that the depletion of IFN-γ might have increased pro-inflammatory cytokines such as IL-1β, which plays a prominent role in the inflammation of both RA patients as well as in animal models of autoimmune arthritis. Recent studies also suggest a distinct role for IL-17 in the absence of IL-4 and IFN-γ [42]. However, since the role of IFN-γ is very complex under inflammatory conditions, several reproducible studies are needed to evaluate the efficacy of IFN-γ before treating RA patients with anti-IFN-γ therapy.

In the mouse model, administration of IL-1-β readily accelerates the development of CIA in genetically susceptible strains [43], and treatment with anti–IL1-β or IL-1Ra ameliorates the disease [36, 44]. Similarly, anti-TNF-α treatment inhibited CIA development [45]. Complete resistance to CAIA induced by our set of monoclonal antibodies by IL-1R and IL-1R TNFR deficient animals confirmed the earlier observations using IL-1R and TNFR deficient animals by Kagari et al. [32], emphasizing the importance of these pro-inflammatory cytokines in the inflammatory phase of arthritis. Hence, the elucidation of the cytokine-mediated mechanisms involved in the joint inflammation should be studied in detail to uncover novel redundant systems in the inflammatory processes.

Contrary to the present findings, IL-4 did not affect the effector phase of arthritis induced by the GPI specific polyclonal sera, though IL-4 was found to be crucial for the development of arthritis in KBN model of inflammatory arthritis [46]. The observed difference between the present study and the anti-GPI system might be due to several reasons such as the genetic differences of the mice used, the predominance of γ1 antibodies present in the sera of KBN mouse model or the nature of antigen recognized by these antibodies [46-48]. Hence, it is possible that there could be subtle differences between these two antibody mediated arthritis models. For example, FcγRIIb deficient mice are resistant to KBN sera induced arthritis but are highly susceptible to CAIA [23, 47].

In the present study, we have observed that the arthritis developed in IL-4 gene deleted or IL-4 neutralized mice had the same severity as that of IL-4 sufficient mice (Figure 2 C and D, and 3B) suggesting that the genetic background of the mice might have influenced the pro-inflammatory role of IL-4. However, IL-4 mediated pathway is not the only pathway that is genetically controlled. A more relevant issue is whether there are genetic effects interacting with the IL-4 mediated mechanisms. Thus, we cannot formally exclude the possibility that IL-4 might operate differently in another genetic background. However, we have kept the genetic background of the mice under control in the present study and IL-4 clearly operates in the proposed way using the C57 black and the BALB/c backgrounds. Hence, we have no reason to believe that this is different using other backgrounds although this is possible and remains to be investigated.

It is also of interest to note that the level of IL-4 production is often low in the synovial fluid and tissue of RA patients [4]. It has been suggested that this lack of IL-4 may contribute to the uneven balance between destructive (Th1 cytokines) and regulatory mediators (Th2 cytokines) in the synovium of the RA process. On the contrary, results from the present study argue that the actual lack of IL-4 observed in RA patients could possibly be protecting the joints from the antibody–mediated inflammatory attack. In conclusion, the application of a simple Th1/Th2 paradigm to autoimmune diseases can mislead the effort to understand the pathogenic mechanisms. Furthermore, efforts to deviate the pre-existing inflammatory cytokines in RA patients by inducing anti-inflammatory cytokines or neutralizing pro-inflammatory cytokines should be critically evaluated before considering them for therapy.

Materials and Methods

Animals

BALB/c, C57BL/6, Stat6 -/- and Stat4-/- mice in BALB/c genetic background, and IL-1R-/- and IL-1RTNFR-/- in B6.129 background and appropriate control mice were obtained from Jackson laboratories (Bar Harbor, ME). B10.RIII strain originated from Professor Jan Klein (Tubingen, Germany) stock. The IL-4 deficient mice of mixed C57BL/6x129 background was kindly provided by Dr.Werner Müller (Cologne, Germany). The IL-4 deficient gene was backcrossed to the C57BL/10 background for 13 generations (B10.Q for 10 generations and further backcrossed for 3 generations to B10.RIII). To provide homozygous IL-4 deficient mice, heterozygous mice were intercrossed. These mice were bred in the Medical Inflammation Research animal house facility in Lund. Four to six month old male mice were used in all the experiments. All the animals were kept in a conventional but barrier animal facility with a climate controlled environment having 12-h light/dark cycles in polystyrene cages containing wood shavings, fed standard rodent chow and water ad libitum. Local animal welfare authorities permitted the animal experiments.

Purification of CII specific monoclonal antibodies

The CII specific hybridomas were generated and characterized as described in detail elsewhere [49, 50]. The anti-CII antibody producing hybridomas, M2139 and CIIC1 were cultured in ultra low bovine IgG containing DMEM Glutamax-I culture medium (Gibco BRL, Invitrogen AB, Sweden) with 100 mg/l of Kanamycin monosulfate (Sigma, USA). Monoclonal antibodies were generated in large scale as culture supernatant (SN) using integra cell line 1000 (CL-1000) flasks (Integra biosciences, Switzerland). Antibodies were purified using γ-bind plus affinity gel matrix (Pharmacia, Sweden) and Äkta purification system (Amersham Pharmacia Biotec AB, Uppsala, Sweden). Briefly, culture SN was centrifuged at 12500 rpm for 30 min, filtered and degassed before applying to the gel matrix. Antibodies were eluted using acetic acid buffer at pH 3.0 and neutralized with 1 M Tris-Hcl, pH 9.0. The peak fractions were pooled and dialyzed extensively against PBS, pH 7.0 with or without azide. The IgG content was determined by freeze-drying and weighing the samples. The antibody solutions were filter sterilized using 0.2 μm syringe filters (Dynagard, Spectrum Laboratories, CA, USA), aliquoted and stored at – 70° C until used. Amount of endotoxin content in the antibody solutions prepared was found to be in the range of 0.02 – 0.08 EU/ mg of protein as analyzed by limulus amebocyte lysate (Pyrochrome) method (Cape Cod Inc., MA, USA).

Passive transfer of antibodies

Arthritis was induced using CII specific monoclonal antibody cocktail as described earlier [26]. Briefly, the cocktail of CIIC1 and M2139 monoclonal antibodies was prepared by mixing equal concentrations of each of the sterile filtered antibody solutions. Mice were injected i.v. with 0.3 - 0.4 ml volumes of antibody solutions as a single dose. As internal controls, mice received equal volumes of PBS. Where indicated, LPS (25 or 50 μg/mouse) was injected i.p. on day 5 to all the mice. None of the control mice that received PBS with or without LPS developed arthritis.

Clinical evaluation of arthritis

Mice were examined daily for the arthritis development for a minimum of 17 days or until the inflammation subsided. Scoring of animals was done blindly using a scoring system based on the number of inflamed joints in each paw, inflammation being defined by swelling and redness as described previously [51]. Scoring was recorded in the phalangeal joints (maximum of 1 point per digit, 5 points per paw), the metacarpus or metatarsus (5 points), and in the wrist and ankle joints (5 points). Thus, the maximum score was 15/paw resulting in a peak of 60 for the total joint count.

Anti-IL-4 and anti-IFN-γ treatment

Rat monoclonal antibodies anti-IL-4 (11B11) and anti-IFN-γ (DB1) were purified from culture SN. Groups of mice were treated with either 1 mg of anti-IL-4, anti-IFN-γ, purified rat IgG, both anti-IL-4 and anti-IFN-γ or untreated. Antibody treatment was done i.p. twice per week, starting from day -1 until day 13 (days -1, 2, 6, 9 and 13). All the mice were transferred with anti-CII mAb cocktail on day 0 i.v. and treated with 25 μg of LPS i.p. on day 5. Mice were monitored for arthritis development for 17 days and paw samples for histology were collected at the end of experiment.

Histological Preparations

Paws were dissected on the indicated days from each group of mice (3 -5 mice per group), fixed in 4% phosphate buffered paraformaldehyde solution (pH 7.0) for 24 hours, decalcified for 3-4 weeks in an solution containing EDTA, Polyvinylpyrrolidone and Tris-HCl, pH 6.95 followed by dehydration and embedding in paraffin. Sections of 6 μm were stained with safranin for detection of mast cells and newly formed cartilage. Proteoglycans were stained using toluidine blue.

For immunohistochemistry, paws were immediately frozen in OCT compound using isopentane on dry ice. The samples were stored at -70°C until cryosectioned at 10 μm at -30°C. Sections were stained with hematoxylin and eosin to visualize joint and cartilage destruction. Osteoclasts were detected using histochemical staining for tartarate–resistant acid phosphatase (TRAP) activity. Sections were counterstained with 0.1% aniline blue. Rat anti-mouse TNF-α (MP6-XT22) from BD pharmingen, CA, USA and biotinylated goat anti-rat IgG (DAKO A/S, Denmark), and goat anti-mouse IL-1β (R&D systems, Minneapolis, USA) and biotinylated horse anti-goat IgG (Vector Laboratories, CA, USA) were used as primary reagents for TNF-α and IL-1β respectively. Avidin peroxidase was used for detection. Diaminobenzidine staining was performed as previously described [52] and the slides were counter stained with Hematoxylin.

Statistical analyses

All the mice were included for calculation of arthritis susceptibility and arthritic animals were used for severity calculations. Mann Whitney or Kruskal Wallis and Chi Square tests were used for analyzing the severity and incidence of arthritis respectively.

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Table 1: Comparison of CAIA in BALB/c and C57BL/6 mice

Mice	Incidence		Mean max score (mean ± SEM)	
	A	L	A	L
BALB/c	89.3	100	5.0±0.9	32.9±3.0
C57BL/6	0	21.4	0	2.3±0.3

Male BALB/c (n=46) and C57BL/6 (n=14) mice were injected i.v. with the arthritogenic anti-CII monoclonal antibodies, M2139 and CIIC1. All the mice received LPS (50 μg/i.p./mouse) on day 5. Development of arthritis was monitored for a minimum of 21 days. n = number of mice in each group. The severity of arthritis was calculated only on those mice that were arthritic in the experiments. A = arthritis susceptibility at day 5 (before LPS injection) and L = maximal arthritis after LPS injection.

Figure legends

Figure 1

Antibody mediated inflammation in stat 4/6 deficient mice. Arthritis incidence (a) and mean arthritis score (b) are given. Groups of six-month old male stat 4 deficient (number of mice (n)=9) and stat 6 deficient (n=9) mice were injected i.v. with 9 mg of anti-CII antibodies on day 0. All the mice received LPS (25 μg/i.p.) in PBS on day 5. Arthritis development was monitored for one month. 100% of arthritis incidence was observed in WT BALB/c mice (data not shown). The severity of arthritis was calculated only on those mice that were arthritic in the experiments. * = $p \leq .05$; ** = $p \leq .01$ and *** = $p \leq .005$. Error bars indicate SEM.

Figure 2

IL-4 deficiency protects against joint inflammation. Four to six months old male IL-4 deficient B10.RIII mice (number of mice (n)=24) and IL-4 sufficient mice (n=30) were injected with CII specific monoclonal antibodies on day 0 and animals were monitored for arthritis on indicated days. Arthritis incidence (A and B) and severity (C and D) from two similar experiments are shown. The severity of arthritis was calculated only on those mice that were arthritic in the experiments. * = $p \leq .05$. Error bar indicates SEM.

Figure 3

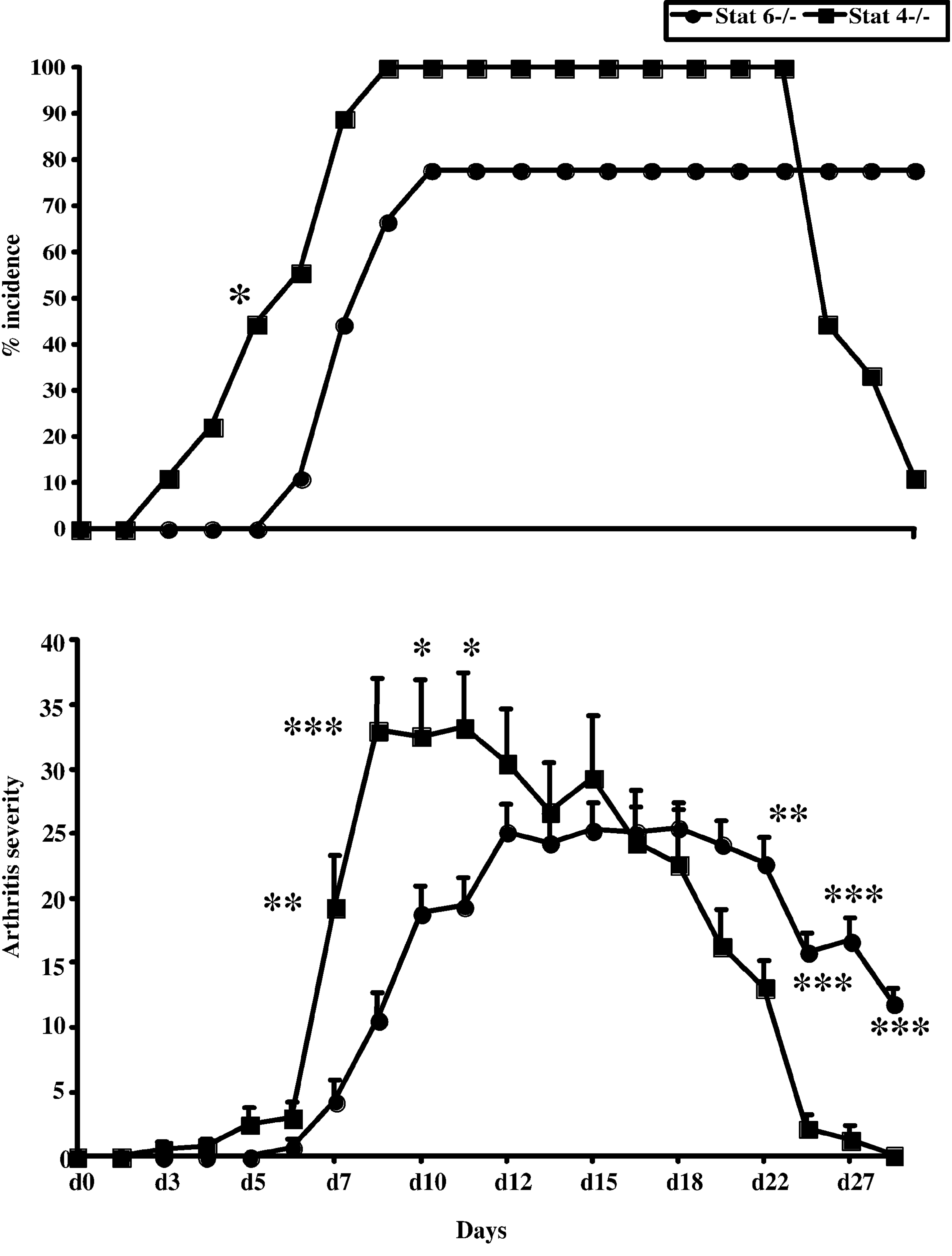
Cytokine depletion modulates antibody-mediated disease. Arthritis incidence (a) and mean arthritis score (b) are depicted. Groups of mice were treated with either 1 mg of anti-IL-4 (number of mice (n)=10), anti-IFN-γ (n=10), purified rat IgG (n=10), both anti-IL-4 and anti-IFN-γ (n=10) or untreated (n=20). Antibody treatment was done i.p. two times per week, starting from day -1 until day 13 (days -1, 2, 6, 9 and 13). All the mice were transferred with mAb cocktail on day 0 i.v. and treated with 25 μg of LPS i.p. on day 5. Mice were monitored for arthritis development for 17 days. The severity of arthritis was calculated only on those mice that were arthritic in the experiments. * = $p \leq .05$; ** = $p \leq .01$ and *** = $p \leq .005$. Error bar indicates SEM.

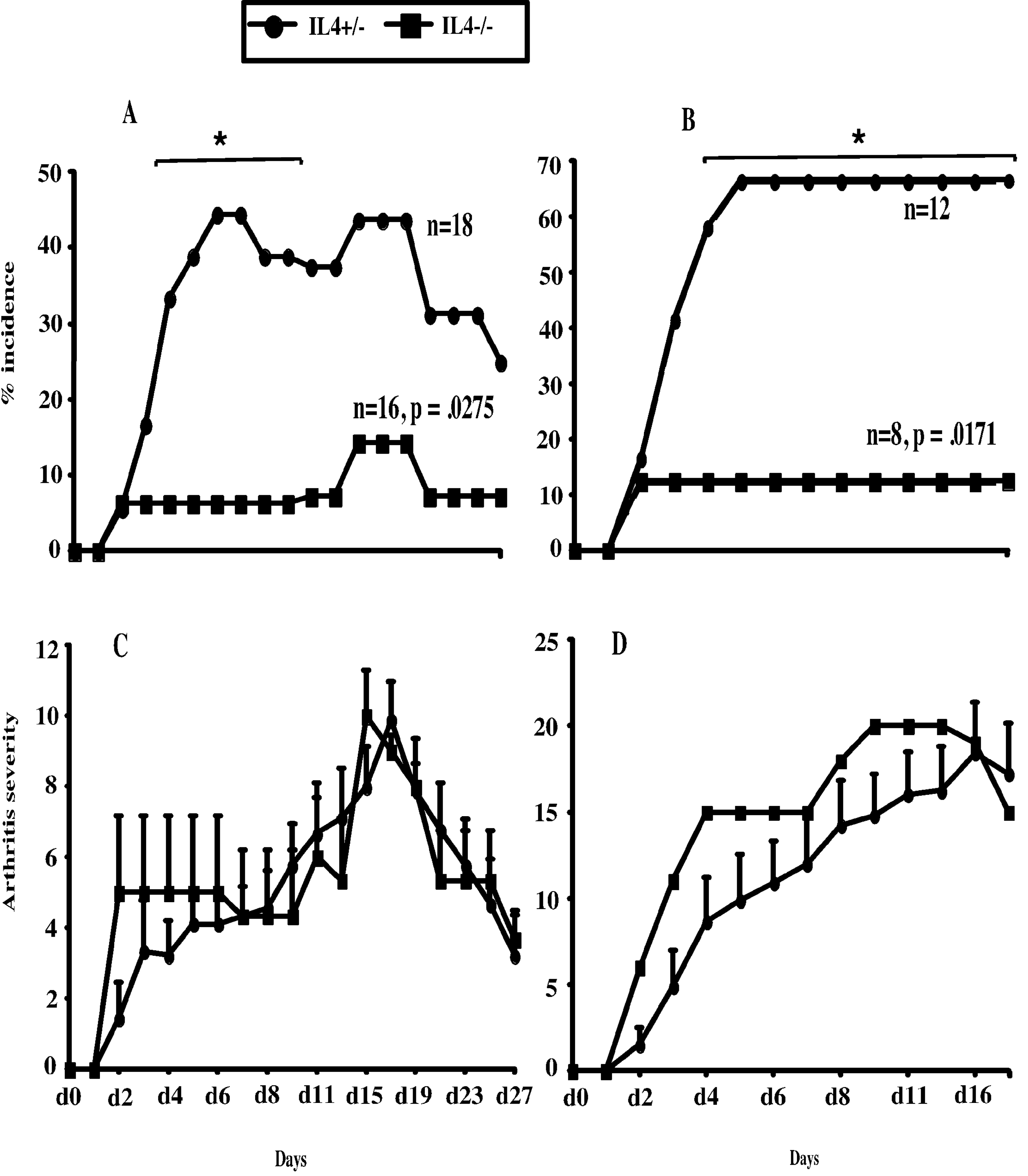
Figure 4

Histology of paws from anti-CII antibody injected IL-4 deficient and sufficient, and cytokine depleted mice. Paws (number mice (n)=3-4) were taken from anti-CII antibody injected IL-4 deficient and sufficient mice for on day 18. Hematoxylin-eosin (A and B X10) and staining for TRAP (C and D X20), and TNF-α (E and F x20) and IL-1 β (G and H X20) secreting cells were shown. Similarly, paws from cytokine-depleted mice (n=4-5) were taken for paraffin embedding on day 17. Safranin staining of mouse paws treated with anti-IL-4 non-arthritic (I x10) and arthritic (J X10), and anti-IFN-γ (K X10), purified rat IgG (L X10), both anti-IL-4 and anti-IFN-γ (M X10) or untreated (N X10) are shown. Toluidine blue staining of anti-IL-4 treated non-arthritic (O X40) and arthritic (P X40) joints. Original magnifications X 10-40. IL-4 is produced by T cells, mast cells and bone marrow stromal cells.

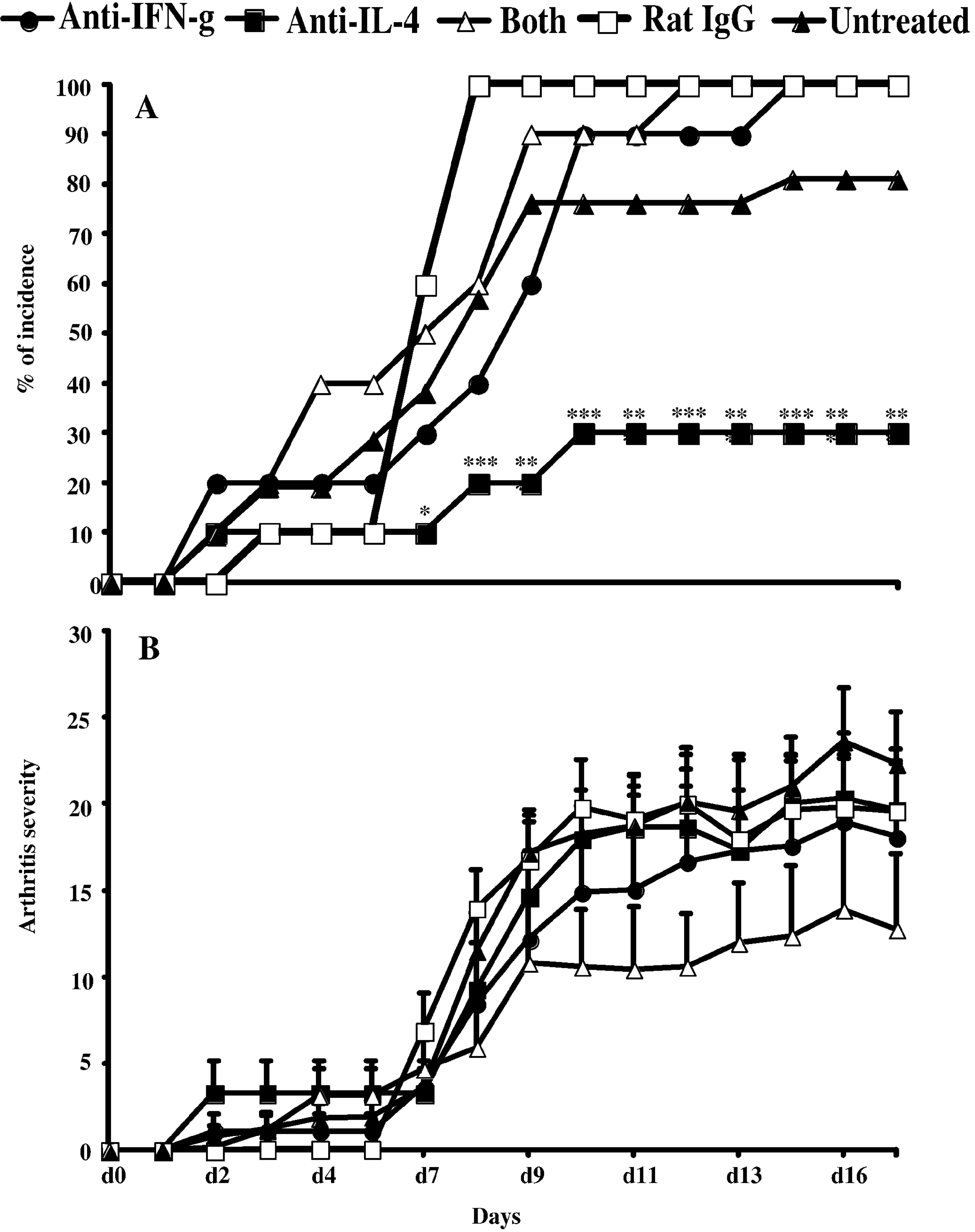
Figure 5

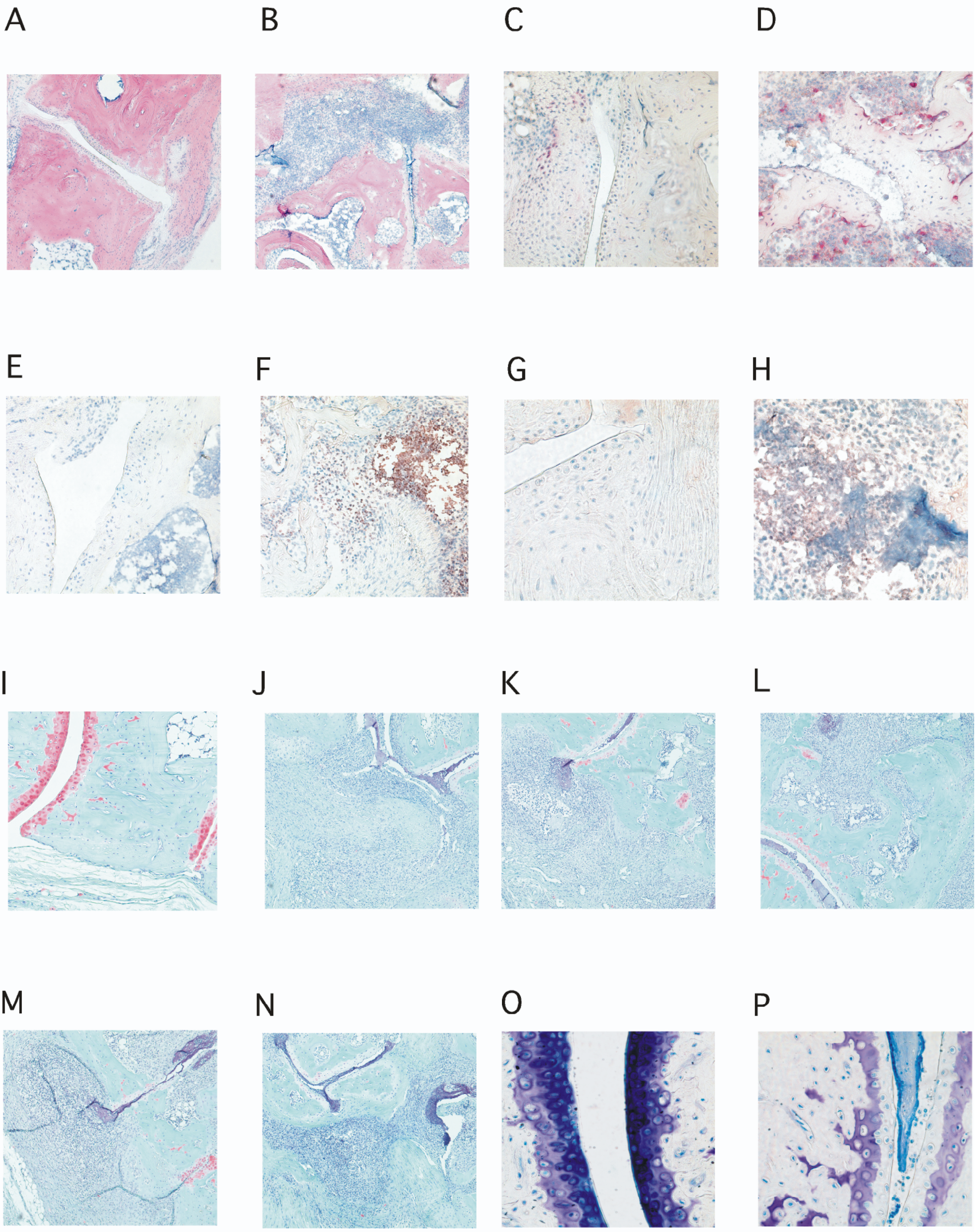
CAIA in IL1-R and IL-1RTNFR deficient mice. Incidence (a) and mean max score (b) are indicated. Groups (number mice (n)=7-9) of six-month old male IL1-R, IL-1RTNFR double deficient and control B6.129 mice were injected i.v. with 9 mg of anti-CII antibodies on day 0. All the mice received LPS (25 μg/i.p.) in PBS on day 5. Mice were monitored for arthritis development for 18 days. The severity of arthritis was calculated only on those mice that were arthritic in the experiments. Error bar indicates SEM.

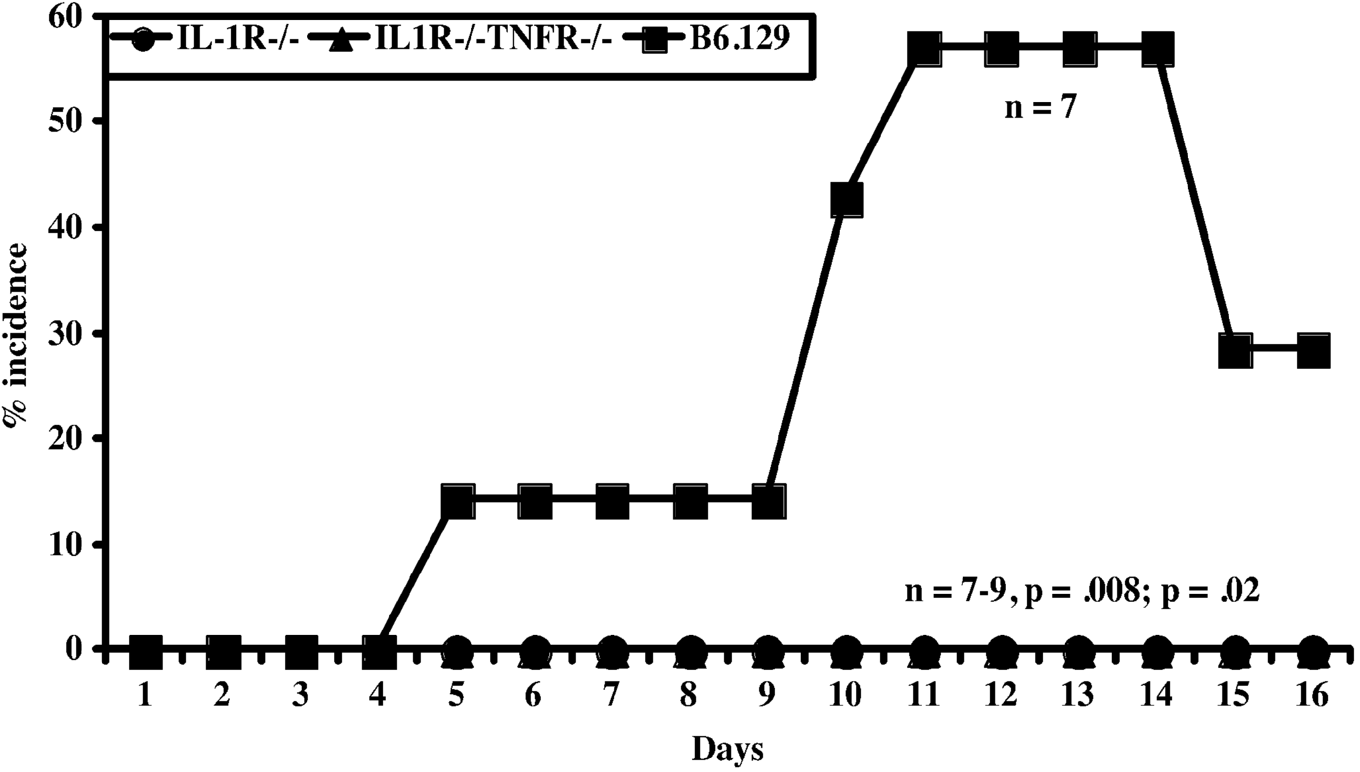

Stat 6-/-
Stat 4-/-
% incidence
100
90
80
70
60
50
40
30
20
10
0
*
Arthritis severity
40
35
30
25
20
15
10
5
0
*
*
***
**
**
***
***
***
d0
d3
d5
d7
d10
d12
d15
d18
d22
d27
Days



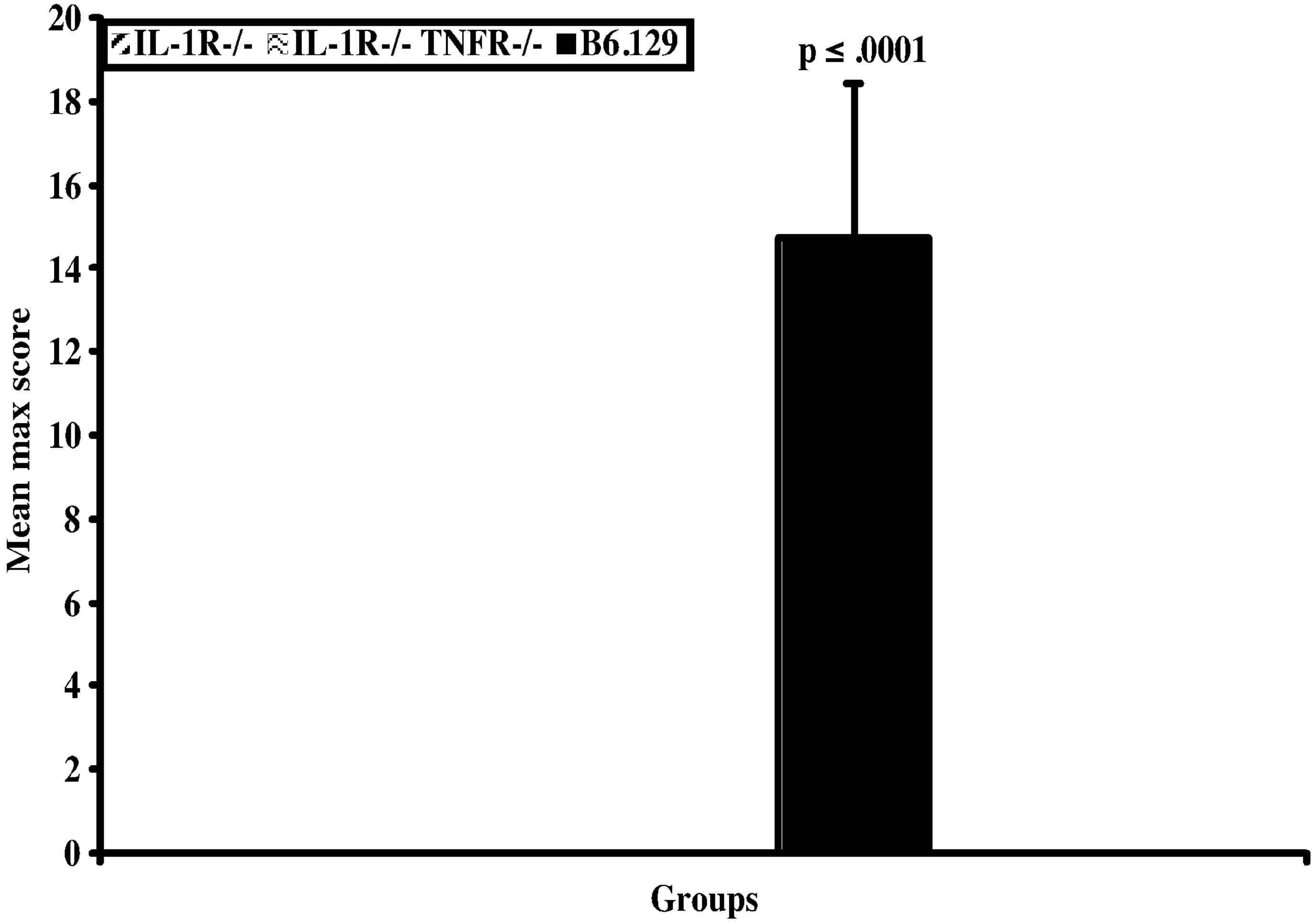
IL4+/-
IL4-/-
A
B
*
*
% incidence
n=18
n=16, p = .0275
n=12
n=8, p = .0171
0
10
20
30
40
50
60
70
C
D
Arthritis severity
0
2
4
6
8
10
12
5
15
20
25
d0
d2
d4
d6
d8
d11
d15
d19
d23
d27
d16
Days
Days


Anti-IFN-g
Anti-IL-4
Both
Rat IgG
Untreated
A
% of incidence
100
90
80
70
60
50
40
30
20
10
0
*
*** **
*** ** *** ** *** ** **
B
Arthritis severity
30
25
20
15
10
5
0
d0
d2
d4
d7
d9
d11
d13
d16
Days


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P



IL-1R-/-
IL1R-/-TNFR-/-
B6.129
60
50
40
30
20
10
0
% incidence
n = 7
n = 7-9, p = .008; p = .02
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Days



IL-1R-/-
IL-1R-/- TNFR-/-
B6.129
p ≤ .0001
20
18
16
14
12
10
8
6
4
2
0
Mean max score
Groups