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Arthritis in Mice That Are Deficient in Interleukin-1 Receptor Antagonist Is Dependent on Genetic Background

Fang Zhou,[1] Xiaowen He,[2] Yoichiro Iwakura,[3] Reiko Horai,[3] and John M. Stuart[1]

Objective. **To determine the effect of deletion of interleukin-1 receptor antagonist (IL-1Ra) protein in an animal model of rheumatoid arthritis.**

Methods. **BALB/c mice deficient in IL-1Ra ($IL\text{-}1Ra^{-/-}$) were bred with collagen-induced arthritis (CIA)–susceptible DBA/1 mice and B10 mice transgenic for HLA–DRB1*0101 (B10.DR1). After generation of $IL\text{-}1Ra^{-/-}$ mice on the DBA/1 and B10.DR1 backgrounds, the mice were observed for the development of spontaneous arthritis and immunized for induction of CIA.**

Results. **We found that although BALB/c mice deficient in IL-1Ra ($BALB/c^{-/-}$) spontaneously developed chronic inflammatory arthritis, DBA/1 IL-1Ra–deficient ($DBA/1^{-/-}$) and B10.DR1 IL-1Ra–deficient ($B10.DR1^{-/-}$) mice did not. Splenocytes from $BALB/c^{-/-}$ mice produced elevated levels of IL-2, IL-4, IL-6, IL-10, IL-17, and granulocyte–macrophage colony-stimulating factor in response to anti-CD3 stimulation. After immunization with type II collagen (CII), $DBA/1^{-/-}$ and $B10.DR1^{-/-}$ mice had a significantly earlier onset of CIA, and with increased severity compared with $IL\text{-}1Ra^{+/+}$ mice. Immunization of $BALB/c^{-/-}$ mice with CII did not aggravate spontaneous arthritis. All of the immunized mice developed antibodies to CII that correlated with arthritis severity. Levels of antibody to CII in the $BALB/c^{-/-}$ strain were relatively low.**

Conclusion. **These data indicate that the spontaneous arthritis of IL-1Ra deficiency is highly dependent on non–major histocompatibility complex genes and that autoimmunity to CII is not the major disease-inducing event. Class II immune response genes are more important for the regulation of CIA, and although these 2 models of arthritis share many pathogenic mechanisms, they also have significant differences.**

Rheumatoid arthritis (RA) is a chronic, inflammatory disorder that may affect as many as 1% of people worldwide. The etiology of RA is only partially understood, but is likely to involve both environmental and genetic factors. The major genetic contribution to susceptibility is linked to class II immune response genes, specifically HLA–DRB1 (1). This locus accounts for ~50% of the genetic component. Other loci have also been identified in some patients, but there is little agreement on their precise identity or on the population groups that may be involved. It is likely that many genes make small contributions (2).

Because of the difficulty of mapping genetic susceptibility in the diverse human population, many investigators have used animal models to identify homologous genes that may be involved in the pathogenesis of arthritis. One of the most extensively studied models is collagen-induced arthritis (CIA). CIA bears many similarities to RA (3). Like RA, CIA is linked to major histocompatibility complex (MHC) class II immune response genes, and these genes account for ~50% of susceptibility (4,5). Several other loci have been associated with arthritis in both rats and mice (6). Some of these are specific to particular strains, whereas others appear to be common to different species (7). Few specific genes have been precisely identified. Among

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[1]Fang Zhou, MD, John M. Stuart, MD: Veterans Affairs Medical Center, and University of Tennessee Health Science Center, Memphis, Tennessee; [2]Xiaowen He, MD: Emory University School of Medicine, Atlanta, Georgia; [3]Yoichiro Iwakura, PhD, Reiko Horai, PhD: University of Tokyo, Tokyo, Japan (current address: NIH, Bethesda, MD).

Address correspondence and reprint requests to John M. Stuart, MD, Research SVC 151, 1030 Jefferson Avenue, Memphis, TN 38104. E-mail: jstuart@utmem.edu.

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genes that are likely to be involved are those that control the cytokine response.

The proinflammatory cytokine interleukin-1 (IL-1) appears to be an important mediator of inflammation and arthritis, both in humans and in other species. IL-1 is present in the synovial fluid of patients with RA (8). In animal studies, direct administration of IL-1 into normal rabbit joints has been shown to induce severe arthritis (9). Treating CIA-susceptible mice with IL-1 enhances the development of arthritis (10). Systemic administration of antibody to IL-1 ameliorates arthritis (11). These data demonstrate the importance of IL-1 as a mediator of arthritis in both human RA and murine CIA.

IL-1 receptor antagonist (IL-1Ra) is an endogenous inhibitor of IL-1. It functions by binding to IL-1 receptors and preventing binding of IL-1, but has no stimulatory activity itself (12). IL-1Ra has been developed as a therapy for RA (13). In studies of CIA, it has been shown that IL-1Ra prevents the development of disease (14). Horai and coworkers have shown that IL-1Ra$^{-/-}$ BALB/c mice develop spontaneous arthritis (15). The basis for arthritis in these mice is not clearly understood, but T cells are important and IL-17 is essential for disease expression (16).

We backcrossed BALB/c IL-1Ra–deficient mice (BALB/c$^{-/-}$) to 2 strains of mice of different genetic backgrounds, DBA/1 and B10.DR1, and determined their susceptibility to spontaneous disease. We found that although BALB/c$^{-/-}$ mice spontaneously developed chronic inflammatory arthritis, DBA/1$^{-/-}$ and B10.DR1$^{-/-}$ mice were resistant. (Throughout, mice designated $^{-/-}$ are IL-1Ra$^{-/-}$.) Elevated levels of IL-2, IL-4, IL-6, IL-10, IL-17, and granulocyte–macrophage colony-stimulating factor (GM-CSF), induced by anti-CD3 stimulation, were also increased in BALB/c$^{-/-}$ mice compared with the other strains. After immunization with type II collagen (CII), both DBA/1$^{-/-}$ and B10.DR1$^{-/-}$ mice had a significantly earlier onset of CIA, and with increased severity compared with the parental strains. Antibody to CII was correlated with disease severity in CIA.

MATERIALS AND METHODS

Mice. BALB/c$^{-/-}$ mice were obtained from the Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo (Tokyo, Japan). DBA/1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice transgenic for HLA–DRB1*0101 (DR1) were generated as previously described (17). To obtain IL-1Ra$^{-/-}$ mice in the DBA/1 and B10.DR1 strains, BALB/c$^{-/-}$ mice were backcrossed to each of them for 6 or 7 generations and then intercrossed to obtain homozygous deficient mice. Thus, we generated DBA/1$^{-/-}$ and B10.DR1$^{-/-}$ strains. The latter mice were double transgenic in that they contained the DR1 transgene that made them susceptible to CIA and were IL-1Ra$^{-/-}$. The DBA and B10 backgrounds differ substantially both from one another and from BALB/c mice. All mice were bred and maintained at the Veterans Affairs Medical Center (Memphis, TN) in a specific pathogen–free environment, and sentinel mice were tested routinely for the presence of mouse hepatitis and Sendai viruses. The experiments were conducted according to the institutional ethics guidelines for animal experiments.

Screening for the transgene. Mice were screened using polymerase chain reaction to select individuals with the desired genotype. To detect the mutant mouse IL-1Ra locus, genomic DNA was isolated and amplified with the following primers, as published in detail elsewhere (15): P4 (5′-GCT-GTG-ATA-GCA-ACA-GTT-TGT-ACC-3′) and P5 (5′-GAC-TGC-CTT-GGG-AAA-AGC-GCC-TCC-3′). To detect the normal locus, we used Rag-S2 (5′-GCC-TTG-TGA-GCT-TTT-GTG-CCT-CTG-3′) and Rag-A2 (5′-GAA-TGA-GAA-ACC-ACC-TTG-GAC-ACC-C-3′). Mice that were P4 and P5 positive and Rag negative were IL-1Ra$^{-/-}$.

Preparation of CII and immunization. The preparation of native CII and its use as an immunogen to induce CIA has been previously described (18). Briefly, CII was isolated from bovine articular cartilage by limited pepsin digestion, purified, dissolved in 0.01*N* acetic acid, and emulsified with an equal volume of Freund's complete adjuvant. The resulting emulsion was injected intradermally into the base of the tail. Each mouse received a total volume of 0.05 ml containing 100 μg of *Mycobacterium tuberculosis* and 100 μg of antigen.

For proliferation assays, the component α-chains of CII (α1[II]) were purified by heat denaturation and ion-exchange chromatography on carboxymethyl cellulose, as previously described (19).

Arthritis evaluation. All mice were examined 3 times weekly by an independent observer for the development of arthritis. Each limb was graded on a scale of 0–4 for degree of redness and swelling (0 = no evidence of erythema and swelling, 1 = mild redness and swelling of joint and ankle, 2 = definite swelling, 3 = severe swelling of entire limb, and 4 = limb burned out and deformed). A severity score was calculated for the 4 limbs (maximum 16 points for an individual mouse).

Proliferation assay. Mice were immunized with CII intradermally in the footpads. Ten days later, the draining lymph nodes were harvested, minced into single-cell suspensions, washed, and the cells were cultured in the absence or presence of α1(II) or anti-CD3 monoclonal antibody for 72 hours. Proliferative responses were measured by the incorporation of tritium-labeled thymidine during the last 8 hours of culture.

Measurement of cytokines by enzyme-linked immunosorbent assay (ELISA). Quantitative measurement of cytokines was performed using commercially available kits. Briefly, spleens were harvested, individually minced into single-cell suspensions in medium, and washed 3 times. Splenocytes were then adjusted to a concentration of 5×10^6 cells/ml and cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum

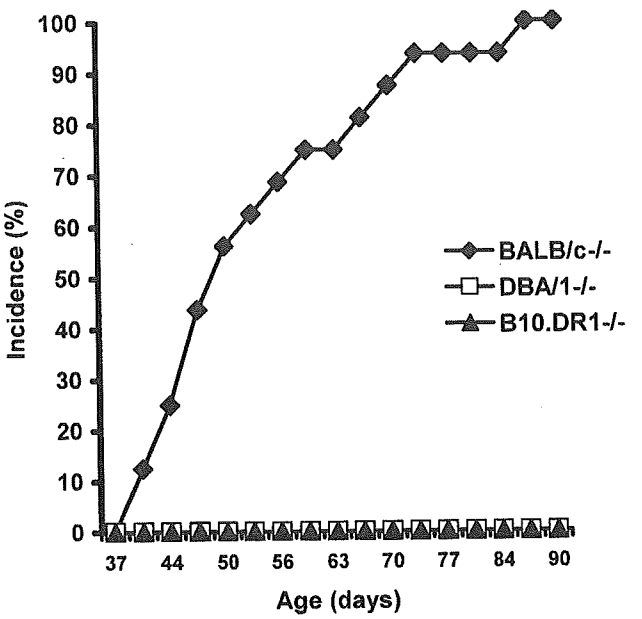

Figure 1. Incidence of spontaneous arthritis in interleukin-1 receptor antagonist (IL-1Ra)–deficient mice with different genetic backgrounds (n = 16 mice in each group). $^{-/-}$ = IL-1Ra$^{-/-}$.

(Hyclone, Logan, UT). Cells were added to plates coated with anti-CD3 for 24 hours, and supernatants were harvested to measure cytokines. IL-2, IL-4, IL-5, IL-10, IL-12, GM-CSF, and tumor necrosis factor α (TNFα) were quantified using a cytokine reagent kit (Bio-Rad, Richmond, CA) and a mouse cytokine 8-plex B assay (Bio-Rad) that permits simultaneous measurement of each of these cytokines in a single supernatant fluid. Interferon-γ and IL-6 were measured using DuoSets (R&D Systems, Minneapolis, MN), and IL-17 was measured using an OptEIA ELISA kit (PharMingen, San Diego, CA). A standard curve was obtained by plotting the absorbance versus the corresponding concentration of the standards. Values are expressed in pg/ml.

Detection of CII antibodies. Mice were bled 43 days after immunization. Serum levels of anti-CII IgG and IgG subclasses were determined by ELISA, as previously described (20). Serial dilutions of a standard serum were added to each plate. From these values, a standard curve was derived by computer analysis using a 4-parameter logistic curve. The total collagen-specific IgG values were calculated in μg/ml.

Statistical analysis. Comparisons were examined by analysis of variance with Bonferroni adjustment for multiple comparisons. Incidence was compared using Fisher's exact test. *P* values less than 0.05 were considered significant.

RESULTS

Development of arthritis in IL-1Ra$^{-/-}$ mice. BALB/c$^{-/-}$ mice were backcrossed to the DBA/1 and B10.DR1 strains for 6 and 7 generations, respectively. Then DBA/1$^{-/-}$ and B10.DR1$^{-/-}$ strains were generated by intercrossing to produce homozygous IL-1Ra$^{-/-}$ mice. Individual animals from each of the strains were observed for the appearance of arthritis. All of the BALB/c$^{-/-}$ mice spontaneously developed arthritis beginning at 5–6 weeks of age. The incidence gradually increased, with 100% becoming arthritic by 12 weeks of age (Figure 1). The swelling and redness were most prominent at the ankle joint of hind limbs and appeared less frequently in the digit joints. The incidence of arthritis in the fore limbs was low. In contrast, none of the DBA/1$^{-/-}$ or B10.DR1$^{-/-}$ mice developed arthritis by the age of 6 months. These observations indicated that additional genetic factors other than IL-1Ra deficiency were involved in arthritis susceptibility.

CD3-induced T cell response. It has previously been shown that T cells are involved in the development of arthritis and that, in BALB/c mice, IL-17 is a critical factor (16). To determine whether differences in the intrinsic ability of T cells to produce cytokine responses affected susceptibility, we examined the CD3-induced T cell response in each strain. For these experiments, spleen cells were harvested from 10-week-old mice and cultured with anti-CD3 for 48 hours. The levels of various cytokines in the culture supernatant fluids were measured. IL-2, IL-4, IL-6, IL-10, IL-17, TNFα, and GM-CSF were all produced in greater quantities by BALB/c$^{-/-}$ mice as compared with either of the other strains (Figure 2). These data demonstrate that BALB/c$^{-/-}$ mice have a higher cytokine response to nonspecific T cell stimulation than either of the other strains.

To determine if the differences were due to higher generalized T cell responsiveness in the BALB/c strain, we measured the relative production of each cytokine in IL-1Ra$^{-/-}$ mice compared with the parental strains. Spleen cells from each strain were cultured with anti-CD3, and cytokine levels were measured. The levels of cytokines in the respective parental and IL-1Ra$^{-/-}$ strains are shown in Figure 3. The data are expressed as the ratio of each cytokine produced by IL-1Ra$^{-/-}$ cells relative to wild-type cells. These data show that the relative increase in cytokine production by the different strains was variable, but in general the increases were no greater in the BALB/c strain than either the B10.DR1 or the DBA/1 strain. A notable exception was in the production of IL-17. This particular cytokine was produced in BALB/c$^{-/-}$ mice at levels 12-fold greater than in wild-type BALB/c mice.

Susceptibility of IL-1Ra$^{-/-}$ mice to CIA. CIA can be induced in mice that have the H-2^q or H-2^r MHC haplotype or in mice that are transgenic for human

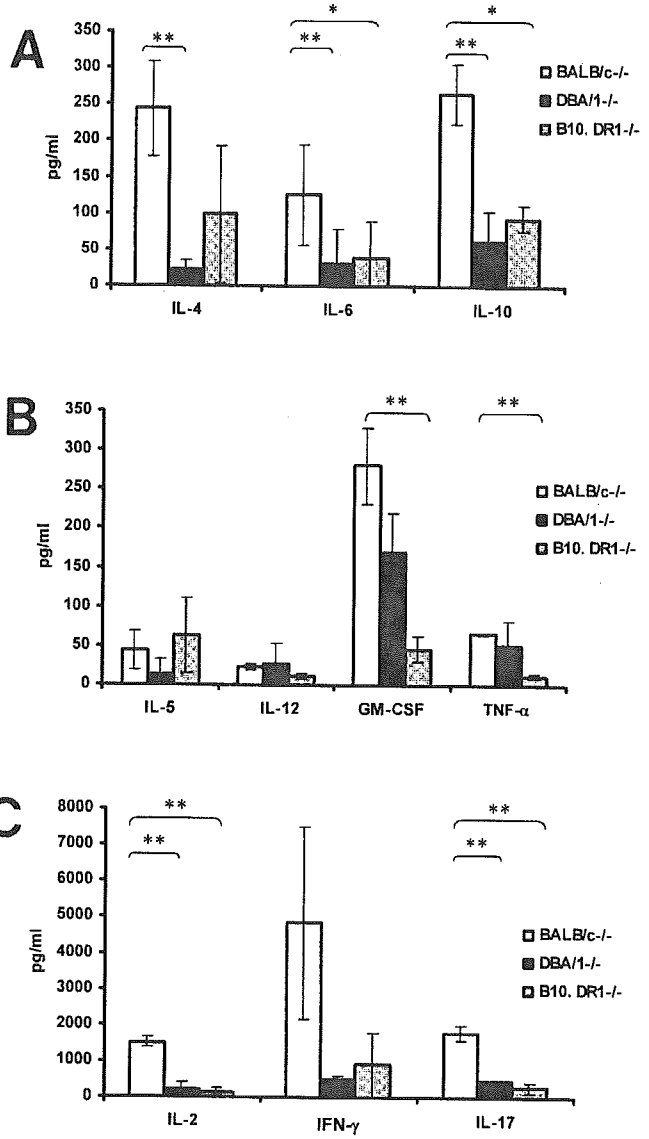

Figure 2. Cytokine production by spleen cells from interleukin-1 receptor antagonist (IL-1Ra)–deficient mice with different genetic backgrounds. Spleens were harvested and cells isolated and cultured on plates coated with anti-CD3. After 24 hours, supernatant fluids were harvested and analyzed for cytokine expression, as described in Materials and Methods. **A,** Levels of IL-6 and IL-10 in BALB/c$^{-/-}$ mice were higher than those in either of the other strains, while the level of IL-4 was higher in BALB/c$^{-/-}$ than in DBA/1$^{-/-}$ mice but not B10.DR1$^{-/-}$ mice ($^{-/-}$ = IL-1Ra$^{-/-}$). **B,** Levels of IL-5 and IL-12 were not different among the groups, whereas levels of granulocyte–macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor α (TNFα) differed between BALB/c$^{-/-}$ and B10.DR1$^{-/-}$ mice but not between BALB/c$^{-/-}$ and DBA/1$^{-/-}$ mice. **C,** Levels of IL-2 and IL-17 were significantly higher in BALB/c$^{-/-}$ mice than in either DBA/1$^{-/-}$ or B10.DR1$^{-/-}$ mice, while interferon-γ (IFNγ) levels did not differ. Values are the mean ± SD (n = 3 mice per group). * = $P < 0.05$; ** = $P < 0.01$, by analysis of variance with Bonferroni adjustment for multiple comparisons.

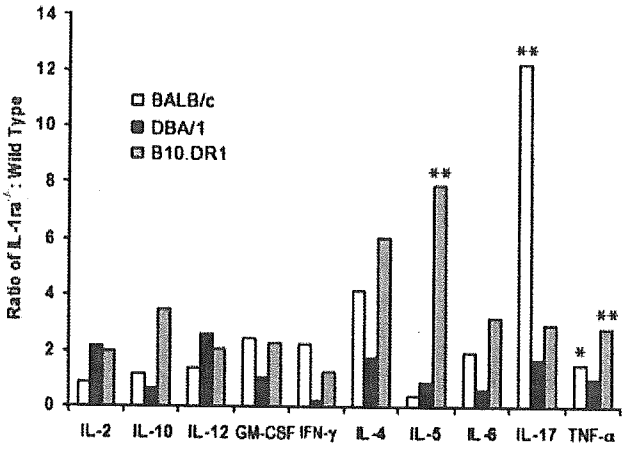

Figure 3. Anti-CD3–induced cytokine production in IL-1Ra$^{-/-}$ mice compared with parental IL-1Ra$^{+/+}$ mice. Mouse spleen cells were cultured on anti-CD3–coated plates for 24 hours, and supernatant cytokine levels were measured as described in Materials and Methods. IL-17 was increased 12-fold in BALB/c$^{-/-}$ mice compared with parental BALB/c$^{+/+}$ mice, TNFα was increased ~2–3-fold in BALB/c$^{-/-}$ and B10.DR1$^{-/-}$ mice compared with parental IL-1Ra$^{+/+}$ mice, and IL-5 was increased ~8-fold in B10.DR1$^{-/-}$ mice compared with parental IL-1Ra$^{+/+}$ mice. * = $P < 0.05$; ** = $P < 0.01$, by Student's *t*-test. See Figure 2 for definitions.

HLA–DRB1*0101 or HLA–DRB1*0401. To determine the effect of elimination of endogenously produced IL-1Ra on CIA, DBA/1 (H-2^q) and B10.DR1 (transgenic for DRB1*0101) mice, with or without IL-1Ra decifiency, were immunized with CII and observed for the development of CIA. DBA/1 mice are highly susceptible to CIA and develop arthritis with 80–100% efficiency beginning ~3 weeks post-CII immunization. B10.DR1 mice are also susceptible, but develop less severe arthritis than DBA/1 mice. In this experiment, increased susceptibility to and severity of CIA were seen in both DBA/1$^{-/-}$ and B10.DR1$^{-/-}$ mice as compared with their IL-1Ra$^{+/+}$ counterparts (Figure 4).

BALB/c mice are resistant to CIA. It has been hypothesized, however, that autoimmunity to CII may develop in BALB/c$^{-/-}$ mice and contributes to the spontaneous arthritis seen in this strain. To determine if immunization with CII would augment the CII autoimmune response and contribute to increased severity of arthritis, we immunized BALB/c$^{-/-}$ mice with CII and observed them for arthritis severity. In these experiments, there was no evidence that arthritis severity was affected by CII immunization (Figure 5).

Immune response to CII. For these experiments, we compared the response of DBA/1 and BALB/c mice with and without IL-1Ra deficiency. Mice were immu-

nized with CII and the draining popliteal lymph nodes were harvested 10 days later. Cells were isolated and enumerated. The total number of cells isolated from the lymph nodes of BALB/c$^{-/-}$ and DBA/1$^{-/-}$ mice was higher than from the respective parental strains. In addition, the number of cells per lymph node in BALB/c$^{-/-}$ mice was higher than in DBA/1$^{-/-}$ mice. The percentage of CD3 cells measured by flow cytometry was not different between the IL-1Ra$^{-/-}$ stains and the parental strains (data not shown), which indicated that IL-1Ra deficiency did not selectively increase the numbers of T cells relative to other cell types. Cells from each strain were cultured with purified α1 chains of CII. As expected, the CIA-susceptible DBA/1 mice had a much higher response than the BALB/c mice. Unexpectedly, the proliferative response of cells from the IL-1Ra$^{-/-}$ strains was lower than was observed in cells from their parental counterparts at all doses of CII tested (Figure 6). These data indicate that although the

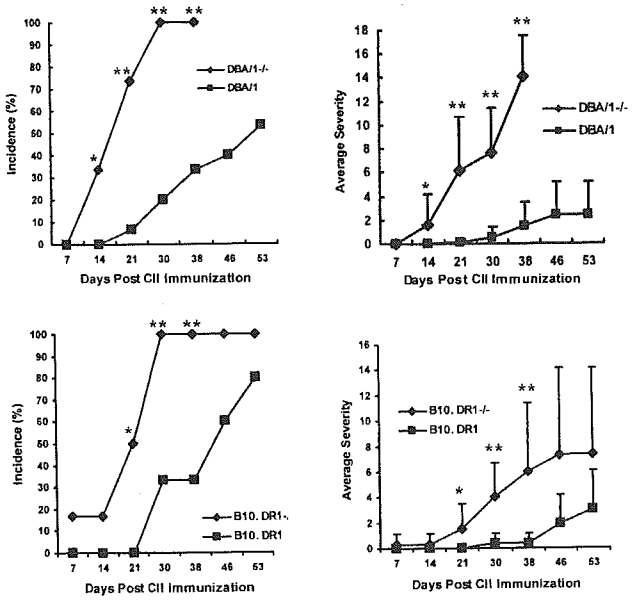

Figure 4. Incidence and severity of collagen-induced arthritis in DBA/1 and B10.DR1 mice after immunization with type II collagen (CII). Severity is the total score for each mouse (maximum 16) summed and divided by the total number of mice. In both groups of mice, arthritis in the interleukin-1 receptor antagonist–deficient (IL-1Ra$^{-/-}$) animals was more severe than in the parental strains. DBA/1 IL-1Ra$^{-/-}$ mice were killed 38 days after immunization because of the severity of the arthritis ($^{-/-}$ = IL-1Ra$^{-/-}$). Data were obtained from 2 independent experiments. Severity data are the mean and SD of each group (n = 15 each for DBA/1 and DBA/1$^{-/-}$ mice, n = 9 each for B10.DR1 and B10.DR1$^{-/-}$ mice). * = $P < 0.05$; ** = $P < 0.01$, by Fisher's exact test for incidence and by analysis of variance for severity.

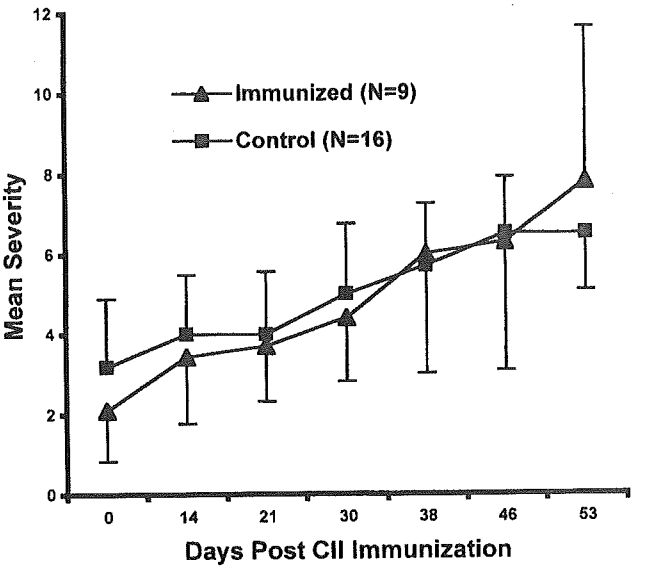

Figure 5. Lack of effect of type II collagen (CII) administration on the severity of spontaneous arthritis in interleukin-1 receptor antagonist–deficient BALB/c mice. Nine mice were immunized with CII and 16 with Freund's complete adjuvant only, at age 6 weeks. All were observed for arthritis over the next 8 weeks. Severity is the total score for each mouse (maximum 16) summed and divided by the number of mice. Values are the mean and SD.

cells from IL-1Ra$^{-/-}$ mice produce relatively high levels of a variety of cytokines, their proliferative responses are actually decreased compared with the parental strains.

Antibody levels. Among strains of mice that are susceptible to CIA, the severity of arthritis is generally correlated with antibody levels. To determine the antibody response to CII, DBA/1 and BALB/c mice were bled 6 weeks after immunization, when arthritis was at maximal severity. The level of anti-CII IgG in BALB/c mice was 35 μg/ml compared with 53 μg/ml in BALB/c$^{-/-}$ mice, whereas in DBA/1 mice it was 995 μg/ml and in DBA/1$^{-/-}$ mice it was 1,116 μg/ml. Among the IgG anti-CII antibodies, the IgG2b subclass level was predominant in all strains, but was increased more in DBA/1$^{-/-}$ mice (data not shown).

DISCUSSION

Deficiency of IL-1Ra caused spontaneous arthritis in BALB/c$^{-/-}$ mice but not in the other strains tested. Although the precise etiology of the arthritis in this recently described model is not fully understood, it has been suggested that autoimmune mechanisms may be involved and there is substantial evidence in support of that possibility. Affected mice have autoantibodies

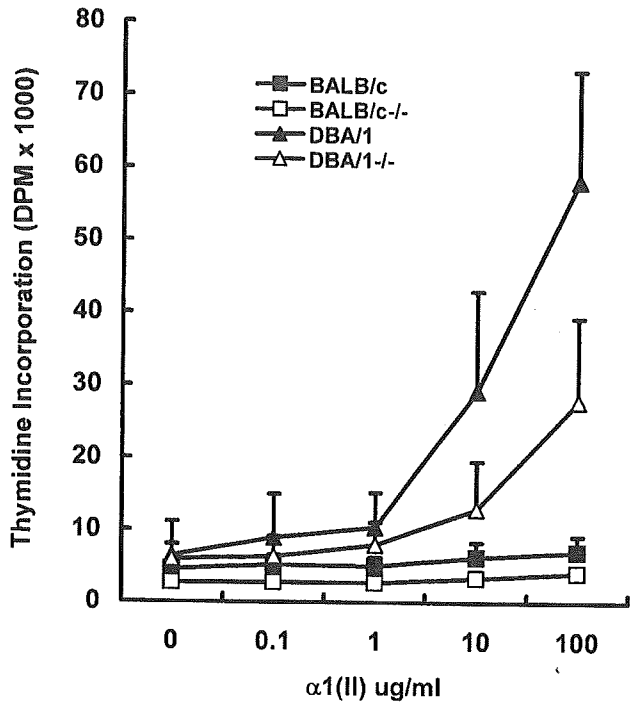

Figure 6. T cell proliferative response to type II collagen (CII). Mice were immunized with CII, as described in Materials and Methods. Popliteal lymph node cells were harvested and cultured with α1(II) at 0.1, 1, 10, and 100 μg/ml for 72 hours, and the responses were measured by incorporation of tritium-labeled thymidine. In each experiment, cells from 2 BALB/c, 2 BALB/c$^{-/-}$, 2 DBA/1, and 2 DBA/1$^{-/-}$ mice were tested. The proliferative response to α1(II) at 100 μg/ml was stronger in DBA/1 mice than in BALB/c mice ($P < 0.05$). Among DBA/1 mice, T cell responses to α1(II) at 10 μg/ml and 100 μg/ml were stronger in mice not deficient in interleukin-1 receptor antagonist (IL-1Ra) than in IL-1Ra$^{-/-}$ mice ($P < 0.01$) ($^{-/-}$ = IL-1Ra$^{-/-}$). Among BALB/c mice, there was no significant difference between those with and those without IL-1Ra, with α1(II) at any dose. Data were obtained from 2 independent experiments. Values are the mean and SD of each group. Statistical analysis was performed by analysis of variance, univariate analysis of variance, and Bonferroni adjustment for multiple comparisons.

against CII, immunoglobulin, and double-stranded DNA (15). However, the data presented here do not support the notion of a major role for autoimmunity to CII in the pathogenesis of spontaneous arthritis. The anti-CII levels in arthritic BALB/c$^{-/-}$ mice do not approach those seen in CIA. Even after immunization with CII, specific antibody levels in BALB/c mice remained at low levels. There was also little T cell reactivity with CII, as measured by the proliferative response of lymph node cells. Culture of lymph node cells in α1(II) at 4 different doses for 72 hours showed a proliferative response in DBA/1 mice that was at least 10 times stronger than in BALB/c mice, and there was a clear dose-response in DBA/1 mice but not in BALB/c mice. Furthermore, immunizing arthritic BALB/c mice with CII did not result in the exacerbation of arthritis.

These data are consistent with previous observations that CIA is linked to MHC genes and that only H-2^q and H-2^r are high responders. This is due to binding of CII-immunodominant epitopes by MHC-encoded, antigen-presenting molecules. BALB/c mice are H-2^d low responders to CII and resistant to CIA (4,21). It is not surprising that IL-1Ra deficiency does not alter the CII responsiveness of this strain, since it would not affect the lack of binding of CII epitopes by H-2^d. It has been estimated that the MHC haplotype contributes ~50% of the susceptibility to CIA (22). The data presented here and previous data (15,23) do not show a major contribution by MHC to IL-1Ra$^{-/-}$ spontaneous arthritis, although additional studies would be required to establish that fact.

These studies do not identify the cause of spontaneous arthritis in BALB/c mice. While this strain is resistant to CIA, it is susceptible to arthritis after immunization with other cartilage components, including proteoglycan (aggrecan), link protein, and versican (24–26). It is possible that one of these other components is the major contributing factor or that even low levels of autoimmunity to multiple cartilage components may have an additive or multiplicative effect in inducing arthritis.

It was postulated by Adarichev and coworkers (21) that CIA exhibits characteristics more consistent with a major influence of a single gene or a small group of genes, whereas proteoglycan-induced arthritis on the BALB/c background is dependent on multiple genes. Consistent with a multigenic effect in BALB/c, we found that for 7 of the 10 cytokines tested, BALB/c$^{-/-}$ mice were hyperresponsive relative to DBA/1 or B10.DR1 mice. If susceptibility is related to cytokine production, they may be closer to the threshold for development of arthritis. It has been shown previously that IL-1, IL-17, and TNFα synergize in the up-regulation of inflammatory mediators and in the release of collagen and proteoglycan from cartilage (27,28). It is interesting that IL-1 and TNFα are monocyte derived, whereas IL-17 is produced almost exclusively by T cells (29).

It has previously been shown that arthritis in both CIA-susceptible and IL-1Ra$^{-/-}$ mice is dependent on IL-17 and TNFα (16,23). We found that spleen cells from DBA/1$^{-/-}$ mice stimulated with anti-CD3 express approximately the same levels of TNFα as BALB/c$^{-/-}$ mice. Thus, TNFα, while necessary, is not sufficient for

the induction of arthritis. On the other hand, BALB/c$^{-/-}$ mice produced much higher levels of IL-17 than either DBA/1$^{-/-}$ or B10.DR1$^{-/-}$ mice. There was a particularly striking increase in IL-17 in BALB/c$^{-/-}$ mice relative to the parental strain, in comparison with the other strains. This cytokine showed a 12-fold increase in BALB/c mice that was greater than that of any other cytokine tested. These data suggest that IL-17 may be a driving force in the development of the spontaneous arthritis and that T cells make a major contribution.

Deficiency in the IL-1Ra gene had a profound effect on the T cell response in all of the strains tested. It led to increased numbers of cells in the draining lymph nodes. In particular, the lymph node cell numbers in BALB/c$^{-/-}$ mice were higher than in wild-type parental mice. In spite of increased numbers of cells, in the CIA-susceptible strains, IL-1Ra$^{-/-}$ mice had a weaker proliferative response to CII than the respective parental strains. This was an unexpected finding and the basis is not clear, since the production of cytokines by spleen cells was increased. This means that the amount of cytokine per cell was disproportionately increased.

In B10.DR1$^{-/-}$ and DBA/1$^{-/-}$ mice, arthritis after immunization with CII appeared earlier and was more severe compared with the parental strains. In many of our experiments using DBA/1$^{-/-}$ mice, the arthritis was so severe that it was necessary to terminate the observation period early. This may have been due in part to increased antibody production in IL-1Ra$^{-/-}$ mice. An important contributor to CIA is antibody to CII and, in particular, IgG2a and IgG2b antibodies (30). It has previously been shown that IL-1 enhances antibody production through induction of CD40 (31). In the CIA-susceptible strains, T cell cytokine production and, in particular, IL-17 levels were lower than in BALB/c mice.

In summary, IL-1Ra deficiency leads to spontaneous arthritis only in some strains of mice. This difference appears to be due to non-MHC genes. The CIA-susceptible strains tested in the experiments reported here are resistant to spontaneous arthritis. T cells from the BALB/c$^{-/-}$ spontaneous arthritis–susceptible strain exhibited higher levels of multiple cytokines, although the relative increases over the parental wild-type strain were modest, with the exception of IL-17. These data suggest that CIA and the spontaneous arthritis of IL-1Ra deficiency may be dependent on different pathogenic mechanisms. In BALB/c$^{-/-}$ mice, there appears to be an increase in multiple cytokines but with a substantial contribution by IL-17 that is largely, if not exclusively, produced by T cells. Arthritis in DBA/1 and B10.DR1 mice depends more on antibody to CII. In these strains, IL-1Ra deficiency fails to induce spontaneous arthritis but drives an enhanced antibody response. Thus, a variety of pathways are affected by IL-1, and several may contribute to increased severity of arthritis.

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CD28-Dependent Differentiation Into the Effector/Memory Phenotype Is Essential for Induction of Arthritis in Interleukin-1 Receptor Antagonist–Deficient Mice

Motoko Kotani,[1] Kazuya Hirata,[1] Shuhei Ogawa,[1] Katsuyoshi Habiro,[1] Yasuo Ishida,[2] Seiichi Tanuma,[1] Reiko Horai,[3] Yoichiro Iwakura,[3] Hidehiro Kishimoto,[1] and Ryo Abe[1]

Objective. **Interleukin-1 receptor antagonist (IL-1Ra)–deficient mice on a BALB/c background spontaneously develop a chronic inflammatory polyarthropathy closely resembling that of rheumatoid arthritis in humans. To elucidate the role of CD28 costimulatory signals in the development of this disease, we studied IL-1Ra/CD28–double-deficient mice.**

Methods. **We crossed IL-1Ra–deficient mice with CD28–deficient mice and observed the incidence and severity of arthritis. To investigate functions of IL-1Ra/CD28–double-deficient T cells, cells were stimulated with CD3 monoclonal antibody or allogeneic antigen-presenting cells (APCs) and their proliferative responses and levels of cytokine production were measured.**

Results. **Disease severity was lower in IL-1Ra/CD28–double-deficient mice than in mice that were deficient only in IL-1Ra, although incidence of arthritis was not affected by the presence or absence of CD28. When pathogenic IL-1Ra–KO T cells were transferred into nude mice, severe arthritis developed. Even though T cells from double-deficient mice showed the same diminished proliferative capacity as was seen in T cells from CD28–single-deficient animals, nude mice into which double-deficient T cells were transferred never developed arthritis.**

Conclusion. **These findings indicate that IL-1Ra/CD28–double-deficient T cells can be activated by IL-1Ra–deficient activated APCs, resulting in induction of arthritis; however, these T cells did not induce the disease under normal conditions, because they did not differentiate into effector/memory phenotype.**

Interleukin-1 (IL-1) is a proinflammatory cytokine that has important roles in inflammation, host defense, and the neuro–immuno–endocrine network (1). The 3 gene products, IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1Ra), bind to IL-1 receptors. IL-1α and IL-1β exert similar biologic activities through IL-1 receptor type I (IL-1RI) (2), whereas IL-1Ra is a naturally occurring inhibitor of IL-1 that acts by competitively binding to the receptor (3,4). IL-1 signaling is also regulated by IL-1RII, a decoy receptor (5). IL-1Ra production is induced by a number of cytokines, viral products, and acute-phase proteins and is augmented in patients with autoimmune and inflammatory diseases, suggesting that this cytokine may play a regulatory role in these diseases (6).

IL-1Ra–deficient (IL-1Ra–knockout [IL-1Ra–KO]) mice on a BALB/c background have been shown to spontaneously develop a chronic inflammatory polyarthropathy that closely resembles rheumatoid arthritis (RA) in humans (7). Histopathologic analysis demonstrates marked synovial and periarticular inflammation, accompanied by articular erosion caused by invasion of granulation tissue. Overexpression of proinflammatory cytokine genes has been observed in the joints of these mice even before the onset of the disease, and elevations of serum immunoglobulin levels and autoantibody production have been observed, suggesting involvement of autoimmunity in the development of this disease.

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[1]Motoko Kotani, PhD, Kazuya Hirata, MPS, Shuhei Ogawa, PhD, Katsuyoshi Habiro, PhD, Seiichi Tanuma, PhD, Hidehiro Kishimoto, MD, PhD, Ryo Abe, MD, DMS: Tokyo University of Science, Chiba, Japan; [2]Yasuo Ishida, MD, PhD: Teikyo University School of Medicine, Ichihara Hospital, Chiba, Japan; [3]Reiko Horai, PhD (current address: NIH, Bethesda, Maryland), Yoichiro Iwakura, DS: University of Tokyo, Tokyo, Japan.

Address correspondence and reprint requests to Ryo Abe, MD, DMS, Division of Immunobiology, Research Institute for Biological Sciences, Tokyo University of Science, 2669 Yamazaki, Noda, Chiba 278-0022, Japan. E-mail: rabe@rs.noda.tus.ac.jp.

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It is widely accepted that T cells play important roles in the development of various autoimmune and inflammatory diseases. Studies involving autoreactive T cell transfer into mice and of transgenic mice carrying self-reactive T cell receptors (TCRs) have revealed that breaking of T cell tolerance could provoke autoimmune disease. Therapeutic interventions targeted toward T cells, such as T cell vaccination (8) or the use of a T cell–suppressive agent (9), have led to successful outcomes. In IL-1Ra–KO mice, T cells also play a very important role in the pathogenesis of arthritis, because T cells from arthritic mice could transfer the disease into naive nude mice (10).

Recent studies, however, show that non–T cell populations such as neutrophils (11) and mast cells (12) are also involved in the development of autoimmune disease. For example, Kontoyiannis et al reported that mice deficient in tumor necrosis factor (TNF) AU-rich elements, which regulate TNF biosynthesis, develop arthritis without mature T and B cells (13); those authors proposed that inflammatory cytokines could induce arthritis without T cells. Furthermore, Plows and colleagues have shown that even in collagen-induced arthritis (CIA), which is the best-characterized animal model for human RA and in which the involvement of class II major histocompatibility complex–restricted T cells and autoantibodies against type II collagen (CII) has been repeatedly shown, severe arthritis could be induced in the absence of mature T and B cells (14). These observations clearly reveal the complexity of the disease pathogenesis.

CD28 is the major costimulatory molecule for T cell proliferation and cytokine production, which are essential for the development of effector function of T cells or antibody production from B cells (15–17). In the absence of this signal, TCR signals could induce antigen-specific nonresponsiveness (anergy) of T cells (18). Costimulatory signals provided by CD28 are also known to be involved in autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) (19,20), CIA (21,22), and systemic autoimmunity induced by the chronic graft-versus-host reaction (23) or developed in MRL-*lpr/lpr* mice (24). To assess the role of T cells, particularly their costimulatory signals, in the development of RA, we generated IL-1Ra–KO mice on a CD28-KO background and investigated the role of CD28 costimulatory function in T cells in the pathogenesis of the disease.

MATERIALS AND METHODS

Mice. BALB/c mice were obtained from Sankyo (Tokyo, Japan). IL-1Ra–KO mice were produced as previously described (25) and were backcrossed to BALB/c mice for 12 generations. CD28-KO mice were generated as previously described (26) and were backcrossed to BALB/c mice for 10 generations. IL-1Ra/CD28–double-deficient (dKO) mice were bred in our facility. The experiments described herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Japan National Research Council.

Clinical evaluation of arthritis. The incidence of arthritis was judged macroscopically. Each joint was examined weekly, and the severity of arthritis was graded on a scale of 0–3 for each paw, based on the degree of redness and swelling (grade 0 = normal; grade 1 = mild swelling of the joint and/or redness of the footpad; grade 2 = obvious swelling of the joint; grade 3 = severe swelling and fixation of the joint). A severity score was calculated for the hind limbs (maximum score of 6).

Measurement of serum antibody levels. Serum IgG levels were assessed by enzyme-linked immunosorbent assay (ELISA) as previously described (27), using antibodies purchased from Southern Biotechnology (Birmingham, AL) (27). Briefly, ELISA plates (Nalge Nunc International, Roskilde, Denmark) were coated with goat anti-mouse IgG (heavy and light chain), and then with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) to prevent nonspecific protein binding. Serum samples, diluted 1:500, 1:1,000, and 1:2,000 in PBS, were added and incubated for 1 hour at room temperature or overnight at 4°C. Experiments were carried out in duplicate. The plates were washed with ELISA buffer (PBS supplemented with 0.05% Tween 20 [Wako, Osaka, Japan]) and incubated for 1 hour at room temperature with horseradish peroxidase (HRP)–conjugated goat antibodies specific for each subclass. After washing, ABTS (Sigma, St. Louis, MO) was added for detection of HRP activity (optical density at 450 nm). The plates were read with an automated ELISA reader (Microplate Reader model 3550; Bio-Rad, Hercules, CA). Antibody concentrations were calculated by using the linear ranges of the dilution, and standard curves were generated with purified mouse Ig (Zymed, San Francisco, CA).

Measurement of cytokine levels. Concentrations of IL-2, IL-4, and interferon-γ (IFN-γ) were measured by ELISA. Spleen cell suspensions from mice of each genotype were cultured in 96-well plates for 48 hours at a concentration of 5×10^6 cells/ml with anti-CD3 monoclonal antibody in the presence or absence of anti-CD28 monoclonal antibody. The supernatants were transferred to other 96-well plates coated with antibodies against each cytokine and incubated for 1 hour at room temperature. After washing, wells were incubated with biotinylated antibodies against each of the cytokines for 1 hour at 37°C and then with streptavidin-HRP for 1 hour at room temperature. Finally, ABTS was added for detection of HRP activity. The plates were read with an automated ELISA reader (Bio-Rad).

Mixed lymphocyte reaction. T cell depletion was carried out by treatment of mouse spleen cells with rabbit anti-mouse brain serum plus guinea pig complement (Gibco, Chagrin Falls, OH). To obtain a T cell–enriched population, spleen cells were applied to plates coated with rabbit antibodies specific for mouse IgM and incubated for 30 minutes at 37°C in a CO_2 incubator. Nonadherent cells were then collected. The T cell–enriched population (2×10^5) was cultured with allogeneic stimulator cells ($1–9 \times 10^5$) in 200 μl of complete medium (RPMI 1640 supplemented with 10% fetal

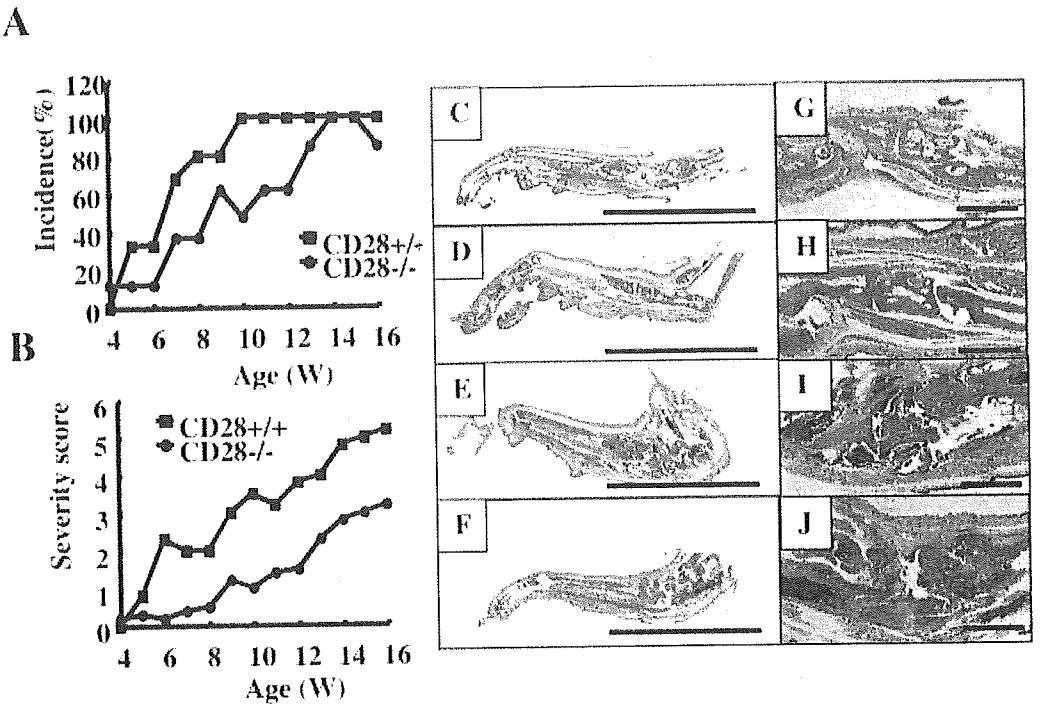

Figure 1. Involvement of CD28 in the progression of arthritis in interleukin-1 receptor antagonist–knockout (IL-1Ra–KO) mice, despite the lack of necessity of CD28 for development of the disease. **A**, Incidence rates and **B**, mean severity scores of arthritis at ages 4–16 weeks in mice deficient in IL-1Ra only ($CD28^{+/+}$) (n = 9) compared with mice deficient in IL-1Ra and CD28 (IL-1Ra/CD28–double-KO [dKO; $CD28^{-/-}$]) (n = 8). **C–J**, Paraffin-embedded tissue sections of ankle joints were stained with hematoxylin and eosin, and histologic sections from **C** and **G**, wild-type mice, **D** and **H**, CD28–single-KO (sKO) mice, **E** and **I**, IL-1Ra–sKO mice, and **F** and **J**, IL-1Ra/CD28–dKO mice were studied. Bars in **C–F** = 1 cm; bars in **G–J** = 1 mm.

calf serum, penicillin, streptomycin, 10 m*M* HEPES [pH 7.55], and 50 μM 2-mercaptoethanol) in 96-well flat-bottomed plates (BD Discovery Labware, Bedford, MA). The cultures were pulsed with 0.5 μCi of ^{3}H-thymidine for the final 8 hours of a 96-hour culture.

Flow cytometry. Spleen cell suspensions from individual mice were prepared in fluorescence-activated cell sorting medium (PBS plus 0.1% BSA [2153; Sigma] and 0.1% sodium azide). Cells (10^6/tube) were first incubated with unlabeled anti–Fc receptor (2.4G2) to block nonspecific binding and then stained with antibodies. Incubations were performed for 20 minutes. A FACSCalibur with CellQuest software (BD Biosciences, San Jose, CA) was used for flow cytometric analysis.

Adoptive T cell transfer. For T cell purification, spleen and axillary, inguinal, brachial, cervical, and popliteal lymph node cells were washed and plated onto anti-Ig antibody–coated plastic plates, and nonadherent cells were collected. T cells were further selected with biotinylated anti-mouse Thy1.2 and streptavidin magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) by passing through a MACS column (Miltenyi Biotec). Prepared cells (1.5×10^7; >99% CD3+) were resuspended in 0.4 ml PBS and injected intravenously into BALB/c-*nu/nu* mice. Incidence of arthritis was judged macroscopically.

RESULTS

Involvement of CD28 in the progression of arthropathy in IL-1Ra–KO mice. To investigate the role of CD28-mediated costimulatory signals in the progression of arthritis in mice deficient for IL-1Ra, IL-1Ra–KO mice were crossed with CD28-KO mice, and mice deficient for both IL-1Ra and CD28 were generated. Disease incidence and severity scores were monitored weekly from age 4 weeks to age 16 weeks. At age 4 weeks, arthropathy developed in some of the IL-1Ra–single-KO (sKO) mice, and the incidence approached 100% by approximately age 10 weeks (Figure 1A). Although the onset of the disease was delayed for ~3 weeks in dKO mice compared with IL-1Ra–single-KO mice, arthritis had developed in almost all of the dKO mice by age 14 weeks. Arthritis severity was mild in dKO mice, indicating the importance of CD28 costimulatory signals in the progression of the disease (Figure 1B).

Consistent with the low degree of clinical sever-

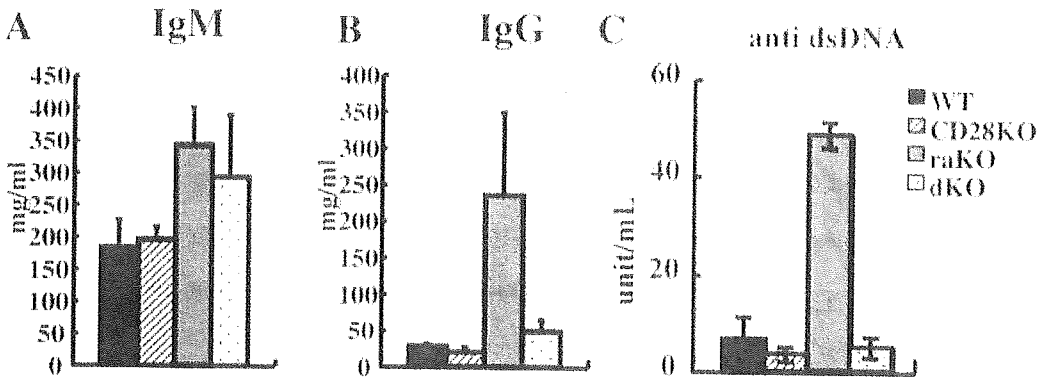

Figure 2. Serum Ig and anti–double-stranded DNA (anti-dsDNA) levels in interleukin-1 receptor antagonist (IL-1Ra)/CD28–double-knockout (dKO) mice compared with wild-type (WT) mice and mice deficient in only IL-1Ra or only CD28. Serum Ig levels were measured by sandwich enzyme-linked immunosorbent assay. Values are the mean ± SD.

ity, histologic analysis clearly showed that paw thickness and synovial hyperplasia were reduced in the ankle joints of dKO mice compared with IL-1Ra–sKO mice (Figures 1E and F). Interestingly, despite the substantial reduction in clinical symptoms, the main histologic characteristics of the arthritis, i.e., hyperplasia of the synovial lying cells, infiltration of neutrophils, and formation of pannus, although somewhat diminished, were still clearly observed in dKO mice (Figure 1J). These results indicate the existence of a CD28-independent pathway for induction of arthropathy.

Reduction in serum IgG levels in IL-1Ra/CD28–dKO mice. Autoantibodies play an important role in the development of arthritis, and the elevation of serum Ig levels is a characteristic feature of arthritis in IL-1Ra–KO mice (7). We therefore measured serum Ig levels in each mouse at age 16 weeks, by ELISA. Concentrations of IgM in dKO mice were as high as those in IL-1Ra–sKO mice (Figure 2A). In contrast, IgG levels in dKO mice were comparable with those in normal mice even at age 16 weeks (Figure 2B), an age at which all dKO mice had developed arthritis with severity scores of 2–3. Serum levels of IgG-class anti–double-stranded DNA in dKO mice were also reduced compared with those in IL-1Ra–sKO mice (Figure 2C). Since disease severity in IL-1Ra/CD28–dKO mice was significantly lower than that in IL-1Ra–sKO mice, lower production of IgG antibodies may correlate with severity of the arthritis.

Phenotype and function of T cells. We examined the phenotype of CD4+ T cells from arthritis-prone IL-1 Ra–sKO and dKO mice (Figure 3A). As expected, the population of memory phenotype $CD44^{high}$,CD4+ T cells was expanded (29%) in IL-Ra–sKO mice compared with wild-type (WT) control mice (19%). The $CD62L^{low}$ and $CD45RB^{low}$ populations, other markers for the T cell memory phenotype, were also expanded in IL-Ra–sKO mouse CD4+ T cells. The memory phenotype of CD4+ T cells, however, was greatly reduced in the dKO mice; these levels were comparable with those in the CD28-sKO mice, suggesting that dKO mouse T cells could not survive after activation or may not differentiate into memory T cells.

To determine whether dKO mouse T cells can be fully activated by stimulation via TCRs, surface expression of activation markers or other costimulation molecules on CD4+ T cells was investigated by flow cytometry following stimulation with soluble 2C11 and antigen-presenting cells (APCs) (Figure 3B). CD25 up-regulation upon CD3 stimulation was impaired in CD4+ T cells from dKO mice, suggesting that these cells have a low capacity for activation. Up-regulation of OX40 and inducible costimulator (ICOS) was also significantly lower in dKO and CD28-sKO mice than in WT or IL-1Ra–sKO mice. Since OX40 (CD134) and ICOS are thought to play key roles in the induction of arthritis by promoting production of Th1 cytokines, dKO mouse T cells were not capable of triggering the pathogenesis leading to arthritis development. In contrast, expression of CD40 ligand (CD40L) was not affected by CD28 disruption (Figure 3B).

To assess the differences between T cell activation states in vivo in each mouse genotype, cytokine production in response to stimulation with plate-bound anti-CD3 was measured. T cell–enriched splenocytes from WT, CD28-sKO, IL-1Ra–sKO, and dKO mice were stimulated, and IL-2, IL-4, and IFNγ concentrations in the supernatants were measured by ELISA after

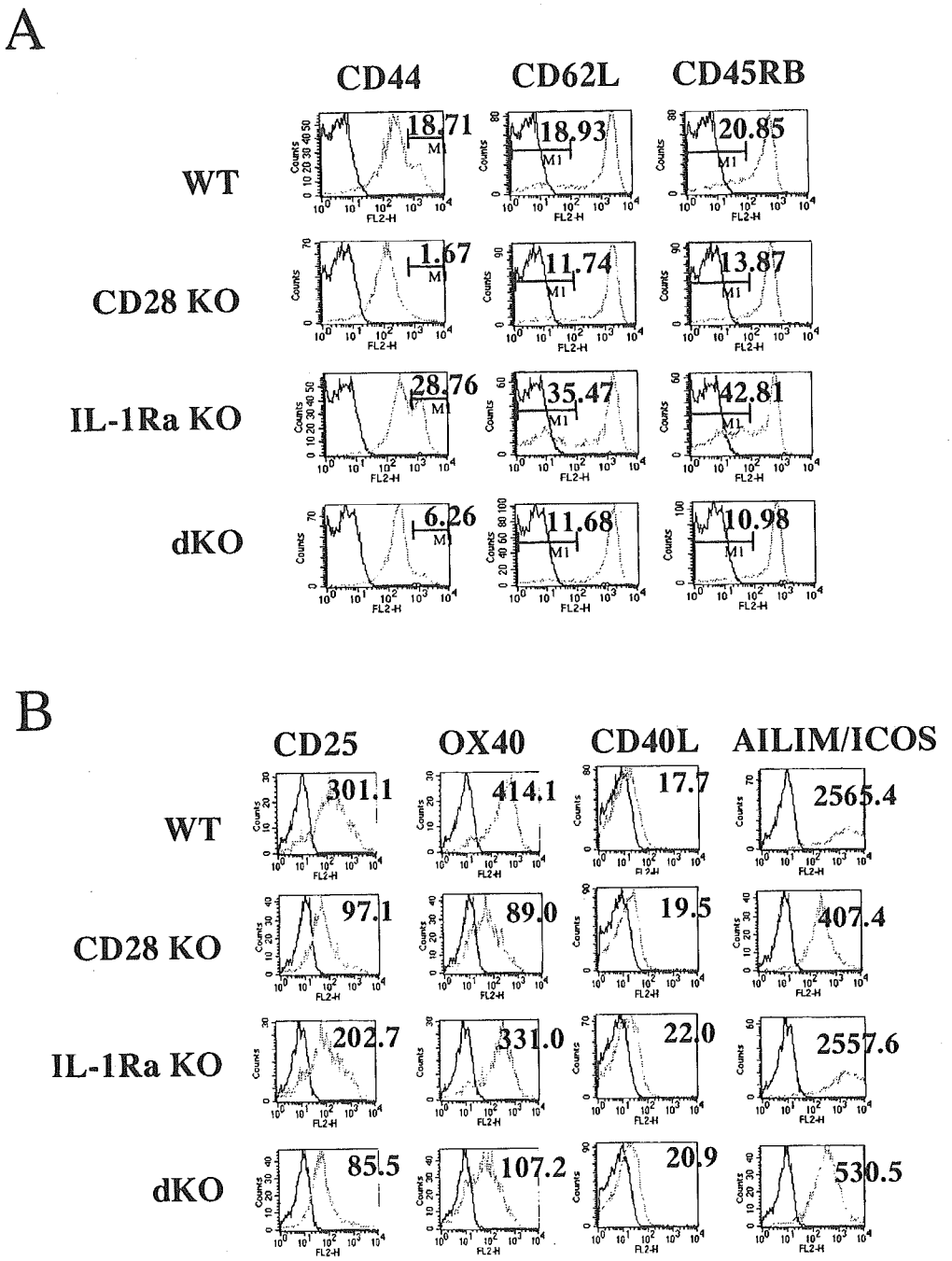

Figure 3. Expression of surface activation markers on CD4+ T cells. **A**, Splenocytes from mice of each genotype were treated with anti-CD44, anti-CD62 ligand (anti-CD62L), and anti-CD45RB monoclonal antibodies (mAb). **B**, Splenocytes from mice of each genotype were stimulated with soluble anti-CD3 mAb (2C11) for 12 hours (3 hours for CD40L) and the surface expression of CD25, OX40, and CD40L was analyzed by flow cytometry. Shaded lines represent stained cells; solid lines represent unstained cells. OX40 = CD134; AILIM = activation-inducible lymphocyte immunomodulatory molecule; ICOS = inducible costimulator (see Figure 2 for other definitions).

48 hours and 72 hours of culture. Interestingly, T cells from IL-1Ra–sKO mice were able to produce IL-4 and IFNγ whereas T cells from WT, CD28-sKO, and dKO mice were not (Figure 4), suggesting that IL-1Ra–sKO mouse T cells were already activated in vivo and had differentiated into effector cells. As expected, cytokine secretion levels in dKO mouse T cells were the same as in cells from CD28-sKO mice even though the dKO

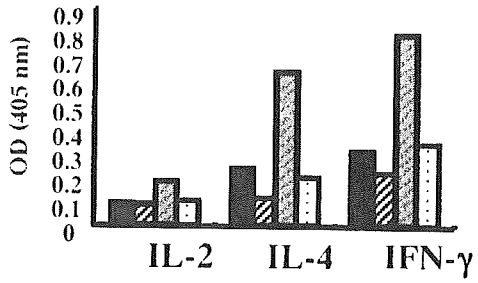

Figure 4. Cytokine expression by splenocytes from WT mice (solid bars), CD28-sKO mice (hatched bars), IL-1Ra–sKO mice (shaded bars), and IL-1Ra/CD28–dKO mice (dotted bars). Splenocytes were stimulated with plate-coated antibody against CD3 (2C11). After 48 hours and 72 hours, culture supernatant was collected and concentrations of interleukin-2 (IL-2), IL-4, and interferon-γ (IFNγ) in the culture supernatants were measured by sandwich enzyme-linked immunosorbent assay. For measurement of IL-2 concentrations, supernatants collected after 48 hours were used; for IL-4 and IFNγ, supernatants collected after 72 hours were used. Values are the mean. OD = optical density (see Figure 2 for other definitions).

mouse T cells were from animals with arthritis, indicating that CD28 signals were essential for differentiation of autoreactive T cells into effector T cells. Thus, the lack of memory phenotype T cells in dKO mice (Figure 3A) indicated that dKO T cells fail to be activated and to differentiate to effector/memory T cells.

Inability of IL-1Ra/CD28–dKO mouse T cells to transfer arthritis to nude mice. To test the possibility that dKO mouse T cells were not pathogenic and to evaluate the role of T cells in the development of arthritis in this model, we transferred dKO mouse T cells into nude mice and reconstructed the environment in which only the T cells were IL-1Ra deficient. Purified T cells (1.5×10^7/mouse) from arthritic IL-1Ra–sKO or dKO mice (16 weeks old) were transferred into nude mice, and disease incidence and severity scores were recorded 0–10 weeks after cell transfer. Interestingly, dKO T cells, even though they were collected from arthritic mice, did not induce arthritis in nude mice, whereas IL-1Ra–sKO T cells caused arthritis in 5 of 7 nude mice (Figure 5). Histologic analysis also revealed no sign of inflammation in the joints of dKO T cell–transferred nude mice (results not shown). These results indicate the absolute requirement for CD28-mediated costimulation in order for IL-1Ra T cells to obtain and exhibit the ability to induce arthropathy.

Antigen-presenting capacity of splenocytes from IL-1Ra/CD28–dKO mice. As shown in Figure 1, dKO mice developed mild but definite signs of arthritis. Combined with the other results described above, this should suggest that non–T cell populations would be involved in the development of arthritis in these mice. In fact, APCs from IL-1Ra–KO mice are known to have hyperfunction in T cell priming by inducing the expression of CD40L and OX40 on the T cell surface (28). It is therefore conceivable that the delay in initial development of arthritis was caused by insufficient T cell function with the lack of CD28-costimulatory signals, whereas later progression of arthritis was due to hyperactivation of APC capacity.

To investigate the ability of APCs to prime dKO T cells, T cell–depleted splenocytes from mice of each genotype were cocultured with naive T cells from B10BR mice, and alloreactivity of B10BR T cells was

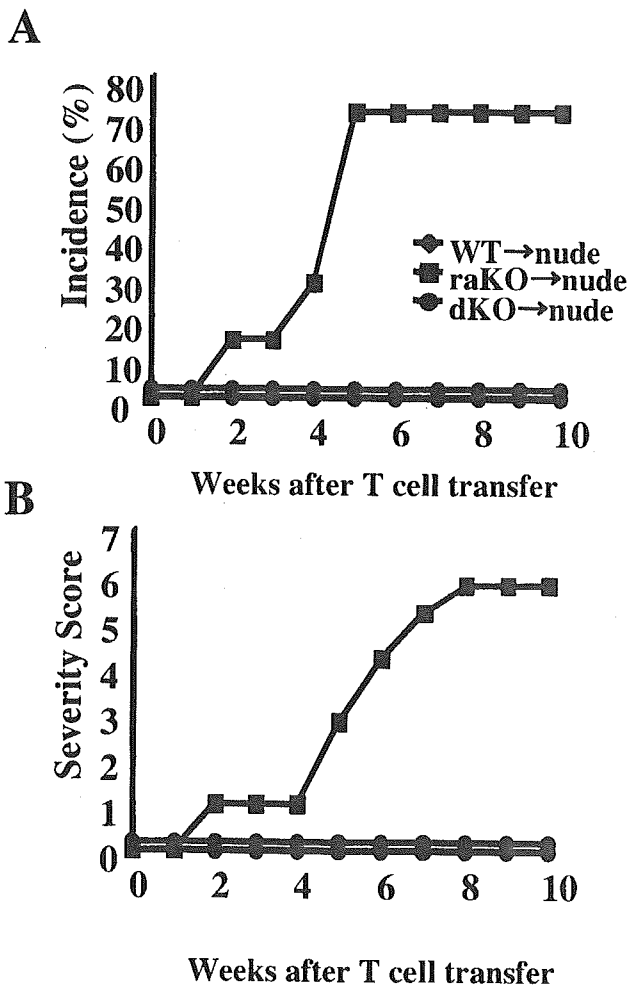

Figure 5. Inability of T cells from IL-1Ra–KO mice to transfer arthritis to nude mice. Cells were harvested from the spleen and lymph nodes of each mouse, and T cells were purified with biotinylated anti-Thy1.2 monoclonal antibody and streptavidin magnetic beads, using MACS. T cells (1.5×10^7/mouse) were transferred into nude mice via the tail vein. The incidence rates (**A**) and mean severity scores (**B**) were recorded weekly for 10 weeks after cell transfer. See Figure 2 for definitions.

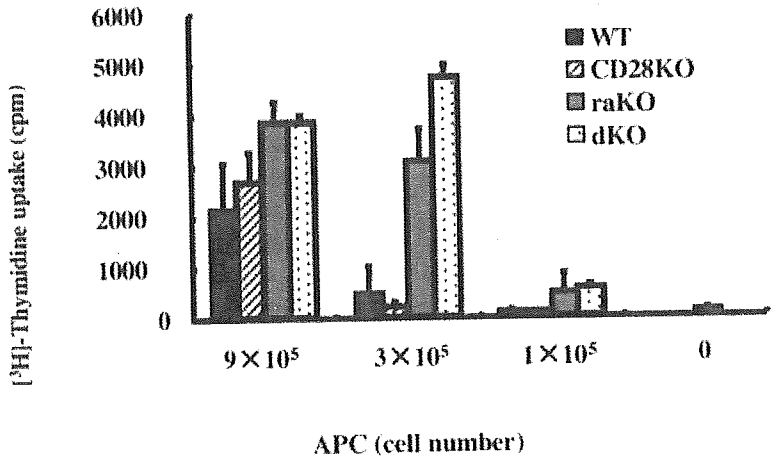

Figure 6. Antigen-presenting capacity of splenocytes from IL-1Ra/CD28–dKO mice. T cell–depleted splenocytes from dKO mice were irradiated and cocultured with T cells from B10BR mice. After 4 days of culture, cells were pulsed with ^{3}H-thymidine. Values are the mean and SD. Similar results were obtained in 3 independent experiments. APC = antigen-presenting cells (see Figure 2 for other definitions).

evaluated by measurement of ^{3}H-thymidine incorporation. APCs from dKO mice and from IL-1Ra–sKO mice exhibited a strong ability to stimulate allospecific T cells compared with APCs from WT or CD28-sKO mice (Figure 6). This implies the existence of CD28-independent activation pathways of T cells due to excess IL-1 signaling. These results suggest that such increases in the capacity of APCs may contribute to the development of arthritis in dKO mice, via a CD28-independent pathway.

DISCUSSION

In the absence of IL-1Ra, a competitive inhibitor of IL-1, inflammation has been shown to be enhanced and prolonged, and mice lacking IL-1Ra spontaneously develop a severe inflammatory polyarthropathy (25). Previous reports have suggested that T cells play a critical role in the induction of arthritis because arthritis can be transferred into nude mice by adoptive transfer of pathogenic IL-1Ra–KO T cells (10) (Figure 5). Although the role of T cells in the development of arthritis has been well described, little is known of the role of costimulatory molecules, especially CD28, on T cells in the pathogenesis of autoimmune arthritis. In this study, we established CD28-deficient mice on the IL-1Ra–KO BALB/c background to examine this question. We found that the severity of arthritis was reduced in the absence of CD28 in the IL-1Ra–KO mice, although these mice still developed arthritis. We also observed lower titers of IgG in the serum of these mice, indicating that there was little T helper cell function. Even though the expression of other activation markers, e.g., CD25 (Figure 3B) and CD69 (data not shown), was reduced, CD40L was up-regulated on IL-1Ra/CD28–dKO mouse T cells. These results suggest that with persistent signaling from the IL-1R, T cells could be activated transiently without CD28-mediated signals and could express CD40L, thus providing a costimulatory signal to APCs, resulting in their activation and the activation of synovial lining cells.

Costimulatory signals provided by CD28 are known to be involved in autoimmune diseases, including EAE (19,20). Treatment with large doses of immunized antigen, however, can lead to the development of EAE in CD28-KO mice. In this neuropathy in CD28-KO animals, CD40 (29) and OX40 (30) pathways have been shown to be involved in the development of the disease. Nakae et al previously showed that splenic adherent cells from IL-1Ra–KO mice could prime antigen-specific T cells more efficiently than WT cells, and that this predominant antigen-presenting capacity in IL-1Ra–KO mice correlated with the surface expression of CD40 and OX40 (28). Based on these findings, they suggested that excess IL-1 signaling could activate arthritogenic T cells via CD40- and/or OX40-mediated pathways, in a CD28 costimulation–independent manner. We have shown that administration of anti-CD40L or anti-OX40L could ameliorate the arthropathy (10). Thus, CD40 and OX40

signals also play important roles in the developmental mechanism of arthropathy, via a CD28-independent pathway, in IL-1Ra–KO arthropathy. The critical involvement of TNFα and IL-17 in IL-1Ra–KO mouse arthritis has been recently reported (10,31), and this involvement may also play a role in the development of arthritis in dKO mice.

The observation that the IL-1Ra/CD28–dKO mice still developed arthritis (Figure 2) suggests that the role of T cells in the development of arthritis in IL-1Ra–KO mice may be limited. CIA can be induced by 2 different procedures, immunization with CII protein or injection of anti-CII antibodies plus lipopolysaccharide. With the first method, T cells are required for disease development, but with the latter, arthritis is induced via a T cell–independent pathway. In K/BxN mice, which are transgenic for glucose-6-phosphate isomerase (GPI)–specific TCRs, anti-GPI autoantibody deposition at the surface of cartilage triggers infiltration of neutrophils into the joints, in a complement-dependent manner (32). Both in mice with CIA and in K/BxN mice, activation of autoreactive T cells is the initial event in the disease, although once autoantibodies are produced, T cells are no longer required for disease progression. In support of this idea, the APC capacities of IL-1Ra–KO and IL-1Ra/CD28–dKO mice were highly augmented as compared with those of WT or CD28-KO mice (Figure 6). It is possible that APCs and other types of inflammatory cells are constitutively activated in the absence of IL-1Ra. Thus, both IL-1Ra–KO and IL-1Ra/CD28–dKO mice are susceptible to severe inflammation as seen in the joints of IL-1Ra–deficient arthritic animals, in which massive infiltration of neutrophils and up-regulation of the expression of genes associated with the classical complement cascade are observed (Fujikado N, et al: unpublished observations).

In CD28-deficient mice, we also observed a lack of CD4+,CD25+ T regulatory cells, which are known to play a key role in maintaining peripheral self-tolerance (33). In the absence of CD28 molecules, T cells acquire little or no effector function. Thus, autoimmune disease usually does not develop in CD28-deficient mice on the normal background. In NOD mice, however, a lack of CD28 molecules on T cells greatly enhances the development and severity of diabetes, even though CD28-KO NOD mouse T cells have little helper ability (34). In IL-1Ra/CD28–dKO mice, the important T regulatory cell population is missing, so other types of activated inflammatory cells, e.g., natural killer cells and macrophages, could still cause inflammation in the joints.

T cells from dKO mice did not induce arthritis when transferred into nude mice (Figure 5), although continuous IL-1 signals in IL-1Ra–sKO mice could induce arthritis in a CD28-independent manner. These results indicate that, in nude mice, complete activation of T cells by costimulatory molecules including CD28 is absolutely required in order to cause disease, unless APC capacity is dysregulated. In addition, OX40 may contribute to this inability of dKO T cells to induce arthritis since, as shown in Figure 3, surface expression of OX40 on dKO mouse T cells was greatly reduced. In fact, administration of anti-OX40L monoclonal antibody has been shown to ameliorate the disease in IL-1Ra–sKO mice (10). Villegas and colleagues showed that CD28-KO mice chronically infected with *Toxoplasma gondii* were susceptible to rechallenge with the same strain (35). Memory phenotype T cells ($CD44^{high}$,$CD62L^{low}$) were only scarcely found in dKO mice (Figure 3A), suggesting that activated T cells from dKO mice were not able to survive and become effector/memory T cells. Taken together, these findings indicate that CD28-deficient T cells lack the ability to differentiate into effector/memory cells. In our preliminary experiments, the ability of thymocytes from IL-Ra–sKO mice to induce arthritis in nude mice was significantly lower than that of peripheral T cells, suggesting that T cells must differentiate into effector/memory cells in order to gain the ability to induce arthritis in normal mice.

The results described herein allow us to propose a mechanism of CD28-dependent and/or -independent arthropathy development in this model of arthritis. Specifically, we suggest that, in IL-1Ra–KO mice, excess IL-1 signal acts on both T cells and APCs, T cell tolerance is broken by this signal, and antibody production and expansion of synovial lining cells is induced. T cells may contribute to the initiation of the disease by expressing CD40L, which stimulates APCs. Activated APCs produce more inflammatory cytokines such as IL-1, IL-12, and TNFα which induce T cell surface expression of OX40. OX40 signals enhance expression of IL-17, which is known to be produced by activated/memory T cells and has a crucial role in the development of arthritis in this model system (31). In the absence of CD28, although CD40L expression is still induced, low expression levels of OX40 and the scarceness of effector/memory cells may interfere with progression of the disease.

Various factors are known to be involved in the development of autoimmune diseases. Blockade of CD28 costimulatory signals is effective in the treatment of T cell–mediated autoimmune disease. However, because of the complexities of disease development, con-

sideration of T cell–independent mechanisms is also important in the search for effective therapies for RA.

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Mutual augmentation of the induction of the histamine-forming enzyme, histidine decarboxylase, between alendronate and immuno-stimulants (IL-1, TNF, and LPS), and its prevention by clodronate

Xue Deng [a,b], Zhiqian Yu [a,b], Hiromi Funayama [a], Noriaki Shoji [b], Takashi Sasano [b], Yoichiro Iwakura [c], Shunji Sugawara [a], Yasuo Endo [a,*]

[a] *Department of Molecular Regulation, Graduate School of Dentistry, Tohoku University, Seiryo-machi, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan*
[b] *Department of Oral Diagnosis, Graduate School of Dentistry, Tohoku University, Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan*
[c] *Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan*

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Abstract

Nitrogen-containing bisphosphonates (N-BPs), powerful anti-bone-resorptive drugs, have inflammatory side effects, while histamine is not only an inflammatory mediator, but also an immuno-modifier. In murine models, a single intraperitoneal injection of an N-BP induces various inflammatory reactions, including the induction of the histamine-forming enzyme histidine decarboxylase (HDC) in tissues important in immune responses (such as liver, lungs, spleen, and bone marrow). Lipopolysaccharide (LPS) and the proinflammatory cytokines IL-1 and TNF are also capable of inducing HDC. We reported previously that in mice, (i) the inflammatory actions of N-BPs depend on IL-1, (ii) N-BP pretreatment augments both LPS-stimulated IL-1 production and HDC induction, and (iii) the co-administration of clodronate (a non-N-BP) with an N-BP inhibits the latter's inflammatory actions (including HDC induction). Here, we add the new findings that (a) pretreatment with alendronate (a typical N-BP) augments both IL-1- and TNF-induced HDC elevations, (b) LPS pretreatment augments the alendronate-induced HDC elevation, (c) co-administration of clodronate with alendronate abolishes these augmentations, (d) alendronate does not induce HDC in IL-1-deficient mice even if they are pretreated with LPS, and (e) alendronate increases IL-1β in all tissues tested, but not in the serum. These results suggest that (1) there are mutual augmentations between alendronate and immuno-stimulants (IL-1, TNF, and LPS) in HDC induction, (2) tissue IL-1β is important in alendronate-stimulated HDC induction, and (3) combination use of clodronate may have the potential to reduce the inflammatory effects of alendronate (we previously found that clodronate, conveniently, does not inhibit the anti-bone-resorptive activity of alendronate).

Keywords: Bisphosphonates; Histidine decarboxylase; Lipopolysaccharide (LPS); Interleukin-1 (IL-1); Clodronate; Alendronate

Introduction

Among the bisphosphonates (BPs), many nitrogen-containing bisphosphonates (N-BPs, including the aminobisphosphonates) have bone resorption inhibitory activities that are much stronger than those of non-N-BPs (Geddes et al., 1994; Rodan and Fleisch, 1996; Rogers et al., 2000). These drugs are important against diseases involving an enhanced bone resorption (osteoporosis, tumoral osteolysis, tumoral hypercalcemia, osteogenesis imperfecta, Paget disease, and rheumatoid arthritis). The anti-tumor activity of N-BPs may also be clinically useful against bone-metastatic tumors (Lipton, 2004). However, most N-BPs have undesirable inflammatory side effects, such as fever, increase in acute-phase proteins, gastrointestinal disturbance, and ophthalmic inflammation (Adami et al., 1987; Siris, 1993; Macarol and Frauenfelder, 1994; Sauty et al., 1996; Fleisch, 1997; Thiébaud et al., 1997). Although non-steroidal anti-inflammatory drugs (NSAIDs) are currently used for treating the fever (Rauch and Glorieux, 2004; Robinson et al., 2004), NSAIDs themselves have ulcerogenic side effects. Moreover, N-BP treatment can lead

Abbreviations: BP, bisphosphonates; HDC, histidine decarboxylase; IL-1, interleukin 1; KO, knockout; LPS, lipopolysaccharide; N-BP, nitrogen-containing bisphosphonate; NSAID, non-steroidal anti-inflammatory drug; TNF, tumor necrosis factor.

* Corresponding author. Fax: +81 22 717 8322.
E-mail address: endo@pharmac.dent.tohoku.ac.jp (Y. Endo).

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to jaw osteonecrosis (Ruggiero et al., 2004; Bagan et al., 2005) and, in children with osteogenesis imperfecta, to a potentially serious influenza-like reaction (Munns et al., 2004). Thus, development of a safe method to prevent their inflammatory side effects might increase the scope for applying N-BPs.

In murine-model experiments, a single intraperitoneal injection of an N-BP induces a variety of inflammatory reactions [including a prolonged induction of the histamine-forming enzyme, histidine decarboxylase (HDC)] together with changes in hematopoiesis (Endo et al., 1993; Nakamura et al., 1999). In such experiments, N-BPs induce a dose-dependent elevation of HDC activity at 5–40 μmol/kg (1.5–12 mg/kg) (Endo et al., 1993). These doses are larger than those used in clinical trials in terms of mg/kg. However, as described above, inflammatory reactions occur in many patients treated with intravenous N-BPs and also in significant numbers of patients treated with lower doses of oral N-BPs (Harinck et al., 1987; Schweitzer et al., 1995; Fleisch, 1997; Ruggiero et al., 2004). This suggests that human patients may be very sensitive to the inflammatory actions of N-BPs, and/or that the inflammatory actions of N-BPs might be augmented under certain conditions. Most patients with bone disorders require prolonged treatment with N-BPs, and such patients are often suffering from, or may catch, infectious diseases during the course of the treatment. Since pretreatment with an N-BP augments HDC induction by lipopolysaccharide (LPS, a cell-surface constituent of gram negative bacteria) (Sugawara et al., 1998; Funayama et al., 2000; Yamaguchi et al., 2000), we supposed that (i) N-BP treatment might augment the inflammatory reactions that occur when a patient catches an infectious disease and (ii) an infection might augment the inflammatory actions of N-BPs.

IL-1 and TNF (endogenous pyrogens) are implicated in various diseases, including those of the central nervous system (Dinarello, 1996; MacEwan, 2002; Anisman et al., 2003; Chesnokova and Melmed, 2002; Leon, 2002; Wang and Shuaib, 2002). LPS is a potent stimulator of the production of these cytokines, and indeed, they largely or partly mediate the actions of LPS (Dinarello, 1984, 1996; Beutler and Cerami, 1989). In addition to N-BPs and LPS, IL-1 and TNF also induce HDC in mice (Endo, 1989; Endo et al., 1986, 1992). Although LPS-stimulated HDC induction is not reduced in the IL-1-deficient mouse, N-BPs induce neither HDC elevation nor other inflammatory reactions in this artificial mutant mouse (Yamaguchi et al., 2000), indicating that the inflammatory actions of N-BPs may depend entirely on IL-1. We previously found that pretreatment of mice with alendronate (a typical N-BP or aminobisphosphonate) markedly augments both the IL-1 production and HDC induction stimulated by LPS (strangely, however, alendronate itself did not increase IL-1 in the blood) (Sugawara et al., 1998). These findings led us to hypothesize that N-BPs might also augment the inflammatory actions of IL-1 and TNF, the production of which may be enhanced in various inflammatory or infectious diseases.

Combined administration of an N-BP and clodronate (a non-N-BP) to mice largely abrogates the inflammatory actions of all N-BPs tested (Endo et al., 1999). We recently found that this combination retains the strong anti-bone-resorptive activity of alendronate (Monma et al., 2004), and in support of this in vivo observation, clodronate does not inhibit (rather, it enhances) the anti-bone-resorptive activity exhibited by low concentrations of alendronate in vitro (Frith and Rogers, 2003). We hypothesized that co-administration of clodronate and an N-BP might thus represent a strategy for preventing or reducing the latter's inflammatory actions while preserving its powerful anti-bone-resorptive activity.

As described above, HDC is commonly induced by N-BPs, LPS, IL-1, and TNF, and histamine is recognized not only as an inflammatory mediator but also as a regulator of immune responses, including the Th1/Th2 balance and hematopoiesis (Schneider et al., 2002). Here, to help test the basis of the hypotheses described above, we examined (i) HDC induction by alendronate in LPS-pretreated mice, (ii) HDC induction by IL-1 or TNF in alendronate-pretreated mice, (iii) changes in the levels of IL-1α, IL-1β, and TNFα in the serum and tissues of mice given alendronate, and (iv) the effects of co-administration of alendronate and clodronate in these experiments.

Materials and methods

Mice. BALB/c female mice (6–7 weeks of age) were obtained from the facility for experimental animals in Tohoku University. Homozygous BALB/c mice deficient in both IL-1α and IL-1β (IL-1KO mice) were provided by Dr. Iwakura (Tokyo University). IL-1KO mice were established by back-crossing to BALB/c mice from original IL-1KO mice (Horai et al., 1998). These mice were bred in our laboratory. All experiments complied with the Guidelines for Care and Use of Laboratory Animals in Tohoku University.

Reagents. Alendronate, clodronate (both synthesized by ourselves; Endo et al., 1999), or a mixture of the two was dissolved in sterile saline, the pH of the solution being adjusted to 7 with NaOH. Human recombinant IL-1β (Ohtsuka Pharmaceutical Co., Tokushima, Japan) and TNFα (Dainippon Pharmaceutical Co., Osaka, Japan) were each dissolved in sterile saline. A lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 prepared by Westphal's method was obtained from Difco Laboratories (Detroit, MI, USA) and dissolved in sterile saline. These reagents were injected intraperitoneally (i.p.) (0.1 ml/10 g body weight), except in one experiment in which LPS was injected intravenously (i.v.). RPMI 1640 solution, Triton X-100, 1 M HEPES solution, bovine serum albumin, and gentamicin sulfate solution were obtained from Wako (Osaka, Japan). Proteinase inhibitor cocktail was from Sigma (St. Louis, MO, USA). Experimental protocols are described in the text or in the legend to the figure relating to each experiment. Previous studies have shown that (i) the inflammatory effects of N-BPs and the suppressing effect of clodronate are dose-dependent, and (ii) alendronate induces nearly a maximum HDC induction at 40 μmol/kg, an effect that is largely abolished by co-administration of the same dose of clodronate (Endo et al., 1993, 1999). Consequently, we chose that dose for both alendronate and clodronate in the present study.

Assay of HDC activity. HDC activity was assayed as described previously (Endo et al., 1993, 1998). Briefly, mice were decapitated and the tissues rapidly removed and stored in a jar with dry ice. Each tissue sample (less than 250 mg), having been put into a cooled Teflon tube with phosphorylated cellulose and 2.5 ml of ice-cold 0.02 M phosphate buffer (pH 6.2) containing pyridoxal 5′-phosphate and dithiothreitol, was then homogenized. The supernatant obtained after centrifugation of the homogenate was used as the enzyme solution. The histamine in the tissues was bound to the phosphorylated cellulose and was removed almost completely from the enzyme solution by the centrifugation. Reaction mixture (1 ml) containing the enzyme solution was incubated at 37 °C for 3 h with histidine. After the enzyme reaction had been terminated by adding $HClO_4$, the histamine formed during the incubation was separated by chromatography on a small phosphorylated cellulose column, then quantified

fluorometrically. HDC activity was expressed as nmol of histamine formed during a 1-h period of incubation by the enzyme contained in 1 g (wet weight) of each tissue (nmol/h/g).

Determination of cytokines in serum. Blood was collected directly into test tubes following decapitation. Serum was recovered by centrifugation at 2000 *g* at 4 °C, then stored at −80 °C until used. The IL-1α and IL-1β in the serum were assayed using ELISA kits (Endogen, Cambridge, MA, USA), while TNFα was measured using an ELISA kit from Biosource (Camarillo, CA, USA). The assay procedures were performed exactly as described by the manufacturer.

Determination of cytokines in tissues. Frozen tissues (liver, lungs, and spleen) were homogenized in RPMI 1640 solution containing Triton X-100 (5 μl/ml), HEPES (10 μmol/ml), bovine serum albumin (100 μg/ml), gentamicin sulfate (50 μg/ml), and proteinase inhibitor cocktail (10 μl/ml) (Sasaki et al., 2000). The protease inhibitor cocktail contains 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A, and E-64. The supernatant obtained by centrifugation (10,000 *g* for 10 min at 4 °C) of the homogenate was then assayed for each cytokine as follows. IL-1α and IL-1β were measured using ELISA kits from Endogen (Cambridge, MA, USA), while TNFα was measured using an ELISA kit from Biosource (Camarillo, CA, USA). The assay procedures were performed exactly as described by the manufacturer. The amount of each cytokine is expressed as nanogram per gram wet tissue.

Data analysis. Experimental values are given as mean ± standard deviation (SD). The statistical significance of the difference between two means was evaluated using a Dunnett's multiple comparison test. *P* values less than 0.05 were considered to be significant.

Results

HDC induction and IL-1β production by LPS in mice previously given alendronate

We used male mice in our previous studies. In those studies, (a) the maximum HDC elevation occurred 3–4 days after an i.p. injection of alendronate (Endo et al., 1993), but the induction of HDC by LPS was more transient, reaching maximum 2–4 h after an i.p. injection of LPS (Endo, 1982, 1989), and (b) the HDC induction and IL-1β production stimulated by LPS were much greater in alendronate-treated mice than in saline-treated mice (Sugawara et al., 1998). In the present study, we decided to use female mice for all experiments because N-BPs are widely used in female patients with osteoporosis, and there are gender differences in some effects of BPs (Bonabello et al., 2003; Journe et al., 2004). We first examined whether an alendronate-induced augmentation, as previously observed in male mice, occurs in female mice, too. LPS was injected at 3 days after an injection of alendronate or saline, and the results showed that both the HDC induction and IL-1β production stimulated by LPS were markedly augmented in alendronate-pretreated female mice (Fig. 1). In contrast, as we previously found in male mice (Sugawara et al., 1998), the LPS-induced TNFα production was reduced in alendronate-pretreated female mice (Fig. 1).

IL-1- or TNF-induced elevation of HDC activity in alendronate-pretreated mice, and inhibitory effects of clodronate

IL-1 or TNF, like LPS, induces a maximal HDC elevation at 2–4 h after its injection (Endo, 1989), while (as described above) alendronate induces a maximal HDC elevation at 3–4 days after its injection. We therefore examined the effects of IL-1 and TNF on HDC activity in female mice given alendronate 3 days before an injection of IL-1 or TNF (Fig. 2). In the liver and lungs, the HDC elevations in mice given alendronate before IL-1 were higher than those in mice given alendronate alone or IL-1 alone, the effects being synergistic in

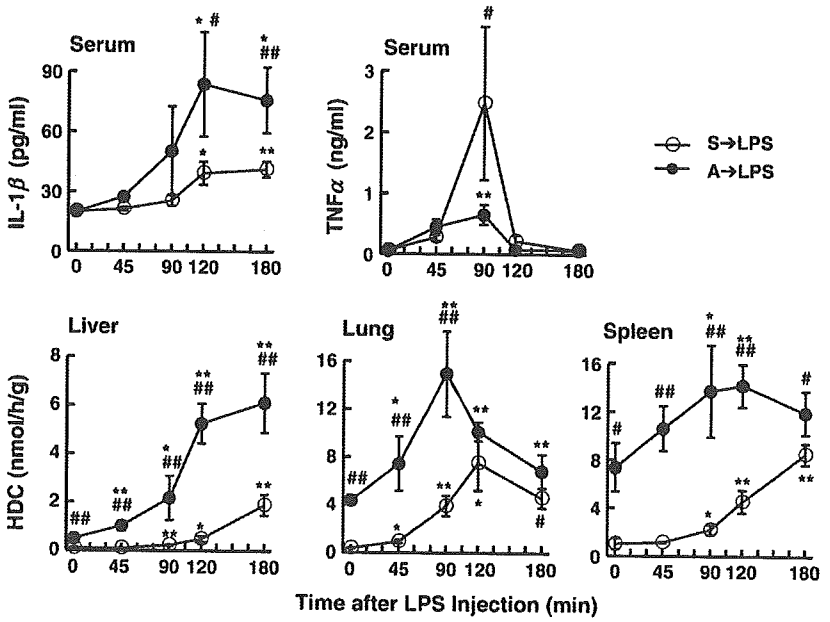

Fig. 1. Augmentation of LPS-induced HDC elevation and IL-1 production in mice given alendronate (A). Saline (S) or A (40 μmol/kg) was injected (i.p.) into female mice. Three days later, the mice were injected with LPS (0.1 mg/kg, i.p.). Blood was collected by decapitation at the indicated times and assayed for IL-1β and TNFα. Each value is the mean ± SD from four mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. time 0. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. S → LPS at the corresponding time. Note that the scales for IL-1β and TNFα are picograms per milliliter and nanograms per milliliter, respectively.