Amelioration of Collagen-Induced Arthritis by Blockade of Inducible Costimulator-B7 Homologous Protein Costimulation

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Inducible Costimulator-B7 Homologous Protein Costimulation 1

Hideyuki Iwai,*† Yuko Kozono,* Sachiko Hirose,‡ Hisaya Akiba,§ Hideo Yagita,§ Ko Okumura,§ Hitoshi Kohsaka, † Nobuyuki Miyasaka, † and Miyuki Azuma2*§

B7 homologous protein (B7h)/B7-related protein 1 (B7RP-1) is a new member of the B7 family of costimulatory molecules that specifically interacts with inducible costimulator (ICOS) expressed on activated T cells. Collagen type II (CII)-induced arthritis (CIA) is an experimental model of arthritis that has been used to dissect the pathogenesis of human rheumatoid arthritis. In this study, we have investigated the effect of neutralizing anti-B7h mAb on the development and disease progression of CIA. Administration of anti-B7h mAb significantly ameliorated the disease as assessed by clinical arthritis score and histology in the joints, and a beneficial effect was also obtained by a delayed treatment after the onset of disease. Expression of ICOS and B7h was observed in the inflamed synovial tissue as well as in the draining lymph nodes (LNs) and expansion of ICOS+ T cells in the LN was reduced by the anti-B7h mAb treatment. Expression of mRNA for proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in the joints was inhibited by the treatment. Proliferative responses and production of IFN-γ and IL-10 upon restimulation with CII in vitro were significantly inhibited in LN cells from the anti-B7h mAb-treated mice. Serum anti-CII IgG1, IgG2a, and IgG2b levels were also reduced. Our present results showed a beneficial effect of the B7h blockade on CIA through anti-inflammatory actions and inhibition of both Th1- and Th2-mediated immune responses, suggesting that the ICOS-B7h interaction plays an important role in the pathogenesis of CIA and thus the blockade of this pathway may be beneficial for the treatment of human rheumatoid arthritis. The Journal of Immunology, 2002, 169: 4332–4339.

SUCCESSFUL T cell activation requires the engagement of the TCR with Ag/MHC as well as the engagement of costimulatory molecules provided by the cognate interactions of T cells with APCs (1, 2). CD28 is a most extensively characterized costimulatory molecule on T cells and interacts with CD80 and CD86 on APCs. CD28 is expressed on most naive T cells and the CD28 costimulatory pathway plays a particularly critical role in the initial activation of naive T cells. Blockade of CD28 pathway has been known to ameliorate experimental autoimmune diseases such as lupus, experimental autoimmune encephalomyelitis (EAE),3 diabetes, and arthritis (3–6).

Recently, new members of the CD28-B7 family have been identified. The inducible costimulator (ICOS) is one of such molecules expressed on activated T cells (7–10). The ICOS ligand, B7 homologous protein (B7h) (11)/B7-related protein 1 (B7RP-1) (12)/GL50 (13)/ligand of ICOS (14), hereafter designated B7h, is constitutively expressed on B cells and is inducible on monocytes and dendritic cells at low levels (15, 16). B7h expression in these APCs and fibroblasts could be induced by proinflammatory cytokines such as IFN-γ and TNF-α (11). Ligation of ICOS on activated T cells by mAb or B7h fusion proteins strongly enhanced the production of multiple cytokines including IL-4, IL-5, IFN-γ, and IL-10, whereas IL-2 production was not clearly enhanced (7, 15, 17). These results suggested a unique property of the ICOS-mediated costimulation distinct from the CD28-mediated costimulation. Earlier studies of the ICOS blockade (10, 18) and ICOS-deficient mice (19–21) suggested a predominant role of the ICOS costimulation in Th2-mediated humoral immune responses. However, recent studies also demonstrated the involvement of ICOS in Th1- and CD8+ T cell-mediated cellular immune responses (22–25). Another intriguing feature of the ICOS blockade distinct from the CD28 blockade is the fact that the ICOS blockade was effective at the effector phase, but not at the induction phase of both Th1- and Th2-mediated responses (26, 27). These results suggest that ICOS may be a potent costimulator for effector T cells.

The collagen-induced arthritis (CIA) model has been extensively used to elucidate pathogenic mechanisms relevant to human rheumatoid arthritis and to identify potential targets for therapeutic intervention (28). A murine model of CIA can be induced in genetically susceptible mice such as DBA/1 by intradermal injection of type II collagen (CII) in adjuvant. The development of CIA is known to be dependent on CD4+ T cell activation and Ab production against CII (28). In addition to these Ag-specific immune responses, local production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, is involved in the pathogenesis of CIA (29). In this study, we have examined the effects of the administration of mAb against B7h in the murine CIA model and investigated the involvement of the ICOS-B7h costimulatory pathway in the development and disease progression of CIA.
Materials and Methods

Mice

Specific pathogen-free 6- to 8-week-old male DBA/1J mice were purchased from the Japan Charles River Breeding Laboratories (Kanagawa, Japan) and maintained in the animal facility at the Tokyo Medical and Dental University (Tokyo, Japan). All mouse procedures were reviewed and approved by the Animal Care and Use Committee in the Tokyo Medical and Dental University.

Ig fusion protein, transfected mAbs, and immunofluorescence

The Ig fusion protein consisting of the extracellular portion of mouse ICOS (aa 1–147) (30) linked to the Fc portion of human IgG1 (ICOS-Ig) and OX40-Ig were prepared as described previously (31). Mouse B7h cDNA was generated by RT-PCR from peripheral macrophages of BALB/c mice and subsequently subcloned into a pMKITneo vector (kindly provided by Dr. K. Maruyama, Tokyo Medical and Dental University). Primers used to generate a full-length B7h cDNA were: sense, 5′-GACGACCTATCGAGCTAAAGTGCTCCTGG-3′ including an EcoRI cloning site and antisense, 5′-GAGCTCAGGAGGCGTTGATGTAATG-3′ including a XhoI cloning site. NRK-52E (NRK) and L cells were transfected with a B7h/pMKITneo vector by electroporation, drug selected, and cells expressing B7h were identified by staining with ICOS-ant. The anti-mouse B7h mAb (HK5.3, rat IgG2a) was generated by immunizing SD rats with mouse B7h-transfected L cells and fusing immune spleocytes with P1U1 myeloma cells and screened for binding to mouse B7h-transfected NRK cells.

Induction of CIA, Ab treatment, and clinical assessment of arthritis

CIA was induced as previously described with minor modifications (36). Briefly, male DBA/1J mice (7–10 wk old) were injected intradermally at the base of the tail with 200 μg of bovine CII (Collagen Research Center, Tokyo, Japan) in 0.05 M acetic acid, emulsified in CFA (Difco, Detroit, Tokyo, Japan) in 0.05 M acetic acid, emulsiﬁed with 7.5% (v/v) Freund’s complete adjuvant and was subsequently subcloned into a pMKITneo vector (kindly provided by Dr. K. Maruyama, Tokyo Medical and Dental University). Primers used to generate a full-length B7h cDNA were: sense, 5′-GACGACCTATCGAGCTAAAGTGCTCCTGG-3′ including an EcoRI cloning site and antisense, 5′-GAGCTCAGGAGGCGTTGATGTAATG-3′ including a XhoI cloning site. NRK-52E (NRK) and L cells were transfected with a B7h/pMKITneo vector by electroporation, drug selected, and cells expressing B7h were identified by staining with ICOS-ant. The anti-mouse B7h mAb (HK5.3, rat IgG2a) was generated by immunizing SD rats with mouse B7h-transfected L cells and fusing immune spleocytes with P1U1 myeloma cells and screened for binding to mouse B7h-transfected NRK cells.

For in vitro blocking experiments, anti-mouse CD80 (RM80, rat IgG2a) (32), CD86 (PO3, rat IgG2b) (32), CD154 (MR1, hamster IgG) (33), and CD134L (RM134L, rat IgG2b) (34) mAbs were used. All mAbs were purified from ascites as described previously (3, 32). Rat IgG (Sigma-Aldrich, St. Louis, MO) was used as a control reagent. For immunofluorescence analysis, PerCP-conjugated anti-CD3 mAb (145-25C11, hamster IgG) and FITC-conjugated anti-ICOS mAb (B10.5, rat IgG2a, kindly provided by JT Central Pharmaceutical Research Institute, Osaka, Japan) (35), or appropriate fluorochrome-conjugated control Ig were used. All fluorochrome-conjugated mAbs and control Ig were obtained from BD PharMingen (San Diego, CA), unless otherwise noted. For indirect staining, PE-conjugated anti-human IgG (Caltag Laboratories, Burlingame, CA) and PE-conjugated anti-rat IgG (Caltag Laboratories) were used for the second-step Abs. Immunofluorescence, flow cytometry, and data analysis were performed using FACS Calibur and CellQuest software (BD Biosciences, San Jose, CA).

CII-specific T cell proliferation and cytokine production

Five days or 10 wk after the second immunization, draining lymph nodes (LNs) and the spleen were removed. Single-cell suspensions of LN cells and erythrocyte-depleted splenocytes were prepared in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 5 × 10−3 M 2-ME, and antibiotics. For purification of CD4+ T cells, cells were treated with anti-I-A, anti-CD24, anti-CD45R, and anti-CD8 mAbs and rabbit complement and the purity of CD4+ T cells was confirmed to be >95% CD4+ cells by flow cytometry. Whole spleenocytes, LN cells, or CD4+ splenic T cells (2.5 × 105/well) were seeded in 96-well flat-bottom microtiter plates and cultured in the presence or absence of the indicated amounts of denatured (60°C, 30 min) bovine CII (dCII) for 72 h. In the culture with purified CD4+ T cells, spleenocytes from the mice at 5 days after the second immunization were treated with mitomycin C (50 μg/ml, 37°C, 30 min; Sigma-Aldrich) and the cells (2.5 × 105/well) were added as APCs. For in vitro mAb blocking experiments, LN cells from the mice 8 wk after the primary immunization were used and cultured as described above. The blocking mAb was added at the start of the assay. All cultures were pulsed with [3H]thymidine (0.5 μCi/well; DuPont/NEC, Boston, MA) for the last 16 h, harvested on a Micro 96 Harvester (Skatron, Lier, Norway), and the incorporated radioactivity was measured using a microplate beta counter (Micro Beta Plus; Wallac, Turku, Finland). Supernatants from similar cultures were collected after 96 h for measurement of cytokine production by ELISA. Aliquots of the supernatants were used for IL-2, IL-4, and IL-10 assays using ELISA kits (Ready-SET-Go!; eBioScience, San Diego, CA) according to the protocols recommended by the manufacturer.

ICOS, B7h, and proinflammatory cytokine expression in the synovium

In some experiments, the knee joints from CIA mice at day 5 were removed and subjected to immunohistological analysis. Cryostat sections were fixed in acetone and 1% paraformaldehyde. For detection of ICOS, the sections were stained with biotinylated anti-ICOS mAb (B10.5) followed by biotinylated rabbit anti-rat IgG Ab (DAKO-Japan, Kyoto, Japan), Alexa 488-conjugated streptavidin (Molecular Probes, Eugene, OR), normal rat serum for blocking, and then Alexa Fluor 594-conjugated rat anti-CD45R mAb (B220).

For detection of B7h, the sections were stained with biotinylated ICOS-Ig and then HRP-conjugated streptavidin followed by Alexa 488-tyramide (TSA kit 22; Molecular Probes). After blocking with normal rat serum, Alexa 594-conjugated rat anti-CD45R (B220) was further stained. Conjugation of Alexa 594 to anti-CD45R and CD4 mAbs was performed using a Alexa Fluor 594 protein labeling kit (Molecular Probes) according to the protocols recommended by the manufacturer. The stained sections were obtained with a fluorescence microscope (Olympus BX-50; Olympus, Tokyo, Japan) equipped with a charge-coupled device camera (PXL System; Photometrics, Tucson, AZ) and the image was analyzed by using IPLab Spectrum software (Signal Analytics, Vienna, VA).

For RT-PCR analysis, synovial tissues were isolated from the knee joints, and total RNA was extracted by using Concert cytoplasmic RNA reagent (Invitrogen, Tokyo, Japan). First-strand cDNA was synthesized using oligo(dT) primer and SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD). PCR was performed using the following primers: mouse TNF-α (sense, 5′-GGCACCACCGCTTCTGCT-3′ and antisense, 5′-ATGCGGTCTACAGGGG-3′), mouse IL-1β (sense, 5′-CTGAAATCCCTCCACTCTC-3′ and antisense, 5′-GGTCTGCTGATACCA GTTG-3′); mouse IL-6 (sense, 5′-TCTCTCCTGCAAGAGATTCT-3′ and antisense, 5′-TTCGCCAAGTCATCATCG-3′); mouse IL-12 (sense, 5′-GAATCTGGTCCATGTCTTG-3′ and antisense, 5′-TGGGTGCACGGC TGAAGT-3′); mouse B7h (sense, 5′-GGTCTCCGTGGTTTTGGGCTC-3′ and antisense, 5′-TGAAGTGTGCTGCAACGG-3′); and mouse GAPDH (sense, 5′-GCCCCACCGGCTTATACCTC-3′ and antisense, 5′-GACA CATTGGGGTAGGAAC-3′). For quantification of the PCR products, the amounts of cDNA were preliminarily normalized to produce the same amount of PCR products for GAPDH based on the intensity of the ethidium bromide-stained bands measured by the charge-coupled device imaging system (Densitograph AE-6920; Atto, Tokyo, Japan). The cDNA samples from four individual mice in each group were amplified on a DNA thermal cycler (PerkinElmer, Norwalk, CT) for 3 cycles except for 25 cycles of GAPDH. The PCR condition was as follows: 94°C for 1 min, followed by 58°C for 1 min, 72°C for 2 min with a 15-min extension at 72°C at the end. The PCR products were electrophoresed on agarose gel, stained with ethidium bromide, and the image was acquired using the Densitograph.

Histological and radiological assessments of arthritis

CIA mice were killed at day 35. Anteroposterior radiographs of the four limbs were obtained with a cabinet soft x-ray apparatus (CMB-2; Softex, Tokyo, Japan). Then, the hind paws were removed, fixed in Formalin, decalcified in 10% EDTA, embedded in paraffin, sectioned, and stained with H&E.
Liver samples were collected on days 7, 14, and 21, and the titers of anti-CII IgG Abs were measured by ELISA. Bovine CII (1 μg/ml) was coated onto microtiter plates (Maxisorb; Nunc, Roskilde, Denmark) overnight at 4°C. After blocking with 1% BSA in PBS, serially diluted serum samples were added and incubated for 1 h at room temperature. After washing, HRP-conjugated rabbit anti-mouse IgG1, IgG2a, or IgG2b Ab (Zymed Laboratories, San Francisco, CA) was added and incubated for 2 h at 37°C. After washing, Ab binding was visualized using o-phenylenediamine (Sigma-Aldrich). A standard serum composed of a mixture of sera from arthritic mice was added to each plate in serial dilutions and a standard curve was constructed. The standard serum was defined as 1 U and the Ab titers of serum samples were determined by the standard curve.

Statistical analysis
Significant differences between experimental groups were analyzed by the Mann-Whitney U test. Values of p < 0.05 were considered to be significant.

Results
Inhibitory effect of anti-B7h mAb on CII-specific T cell proliferative responses in vitro
We generated a mAb (HK5.3) against mouse B7h by immunizing SD rats with B7h transfectants. Similar to the staining with ICOS-Ig, HK5.3 specifically bound to B7h-transfected NRK (B7h/NRK) cells, but not to parental NRK cells (Fig. 1A). Preincubation with HK5.3, but not with control rat IgG (data not shown) efficiently blocked the ICOS-Ig binding to B7h/NRK cells, indicating the specific binding of HK5.3 to the ICOS ligand B7h. To evaluate the functional inhibitory effects of this anti-B7h mAb, we examined the effect of this mAb on CII-specific proliferative responses in vitro. LN cells from CII-immunized mice were stimulated with 30 μg/ml dCII and a panel of mAbs against costimulatory molecules (Fig. 1B) and the titrated amount of anti-B7h mAb (Fig. 1C) was added to the culture. The addition of dCII dramatically enhanced proliferative responses and this enhanced proliferation was clearly inhibited by anti-B7h mAb as well as by anti-CD80 and CD86 mAbs, anti-CD134L mAb, or anti-CD154 mAb. The inhibitory effect of anti-B7h mAb occurred in a dose-dependent manner. These results indicate an inhibitory activity of anti-B7h mAb and a substantial involvement of B7h in the CII-specific T cell responses in vitro.

Preventive and therapeutic effects of anti-B7h mAb on CIA
To investigate the role of ICOS-B7h-mediated T cell costimulation in the development of autoimmune arthritis, we examined the effects of anti-B7h mAb on the development of CIA. DBA/1J mice were immunized twice with bovine CII in CFA to elicit CIA. Four groups of mice were i.p. administered either 50, 100, and 300 μg of anti-B7h mAb or 100 μg of control IgG every other day from day −1 for four times. As shown in Fig. 2A, the mice treated with control IgG developed severe arthritis. In contrast, the administration of anti-B7h mAb significantly ameliorated the clinical manifestations of CIA in a dose-dependent manner. As summarized in Table I, the treatment with 100 μg of anti-B7h mAb significantly delayed the day of onset and decreased the mean arthritis score and the mean number of arthritic paws, although the incidence of disease was not affected. The amelioration of clinical arthritis by the anti-B7h mAb treatment was confirmed by histopathological examination of the joints. The hind paw sections from the control IgG-treated mice at day 35 showed infiltration of mononuclear cells, synovial hyperplasia, pannus formation, cartilage destruction, and bone erosion that are characteristic features of arthritis (Fig. 3c). These features were clearly ameliorated by the anti-B7h mAb treatment (Fig. 3d). Radiological examination also showed the prevention of bone erosion by the anti-B7h mAb treatment (Figs. 3, a and b).

To investigate whether the treatment with anti-B7h mAb is still effective after the onset of disease, we have performed a delayed treatment. Since day 5 was the mean day of onset in the control IgG-treated mice as shown in Table I, we started a similar treatment from day 5 and examined the clinical scores. The delayed treatment was also effective in reducing the clinical arthritis scores (Fig. 2B). These results demonstrated that the treatment with anti-B7h mAb either before or after the onset of disease could inhibit...
then weakly on CD11c/H11001/H9262 in injected i.p. with 100 p.

5, 7, 9, and 11. Data are expressed as the mean of seven mice in each group.

To determine the expression of ICOS and B7h in the in

Expression of ICOS and B7h in the joints and the draining LNs

were clearly enhanced as compared with that in the naive mice and this enhancement was reduced in the anti-B7h mAb-treated mice (1.6 ± 0.3 × 10^7 cells). However, the ratio of T (CD3^+), B (CD45R^-CD3^+), or non-T/B (CD3^-CD45R^-) LN cells was not affected by the anti-B7h mAb treatment (data not shown). ICOS^+ T cells were clearly increased in the control IgG-treated CIA mice (8.2 ± 1.0%) as compared with naive mice (4.9 ± 0.3%; data not shown), but this increase was significantly inhibited in the anti-B7h mAb-treated mice (5.3 ± 0.3%; Fig. 4C). Consistent with a previous report (15), a constitutive expression of B7h was observed on B cells in naive mice and this expression on LN-B cells was not clearly affected by the CII immunization and the anti-B7h mAb treatment (data not shown). These results demonstrated a substantial expression of ICOS and B7h in the local joints as well as in the draining LNs in CIA mice.

Down-regulation of proinflammatory cytokines in the joints by anti-B7h mAb treatment

Since the inflammatory process in the synovium plays a major role in the development of arthritis (29), we next examined the change of proinflammatory cytokine expression in the synovium by the treatment. Consistent with previous reports (29, 37), high levels of TNF-α, IL-1β, and IL-6 mRNA were observed in the synovial tissues from the control IgG-treated mice, but the treatment with anti-B7h mAb significantly inhibited the expression of these proinflammatory cytokines (Fig. 5). These results suggested that the disease severity of CIA, suggesting the involvement of the ICOS-B7h pathway in the pathogenesis.

Expression of ICOS and B7h in the joints and the draining LNs

To determine the expression of ICOS and B7h in the inflamed joints and the draining LN cells, immunohistological staining and flow cytometry were performed using a specific mAb against ICOS and B7h or ICOS-Ig. ICOS expression was observed on both CD4^+ and CD4^- (presumably CD8^- T) cells in the synovium of the inflamed joints from the control IgG-treated mice on day 5 (Fig. 4Ab). We first performed immunohistological staining using anti-B7h mAb; however, the positive staining was not observed even in the spleen and LN as well as in the synovial tissue, suggesting that this anti-B7h mAb (HK5.3) is not applicable to immunohistological staining. We therefore used ICOS-Ig for detection of B7h expression. Positive cells were observed within some of the CD45R^-B cells (Fig. 4Ac) and CD11b^- macrophages, and then weakly on CD11c^- dendritic cells (data not shown) in the inflamed joints. To further assess the change of ICOS and B7h expression in the inflamed joints, the mRNA levels for ICOS and B7h were compared between naive and the control IgG-treated CIA mice. The mRNA expression for ICOS and B7h in the joints was significantly enhanced in the CIA mice (Fig. 4B). We next investigated draining LN cells. The mean total number of LN cells in the control IgG-treated CIA mice (2.5 ± 0.1 × 10^7 cells) was clearly enhanced as compared with that in the naive mice and this enhancement was not clearly affected by the anti-B7h mAb treatment (data not shown). ICOS^+ T cells were clearly increased in the control IgG-treated CIA mice (8.2 ± 1.0%) as compared with naive mice (4.9 ± 0.3%; data not shown), but this increase was significantly inhibited in the anti-B7h mAb-treated mice (5.3 ± 0.3%; Fig. 4C). Consistent with a previous report (15), a constitutive expression of B7h was observed on B cells in naive mice and this expression on LN-B cells was not clearly affected by the CII immunization and the anti-B7h mAb treatment (data not shown). These results demonstrated a substantial expression of ICOS and B7h in the local joints as well as in the draining LNs in CIA mice.

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* CIA was induced as described in Fig. 2 and treated with either 100 µg of control IgG or anti-B7h mAb on days −1, 1, 3, and 5. Values represent the mean ± SD from each group of 26 mice.

* Statistically different from the control IgG-treated group (p < 0.05).

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anti-B7h mAb treatment reduced the arthritic manifestations by down-regulating the expression of proinflammatory cytokines in the joints.

Inhibition of CII-specific T cell proliferation and cytokine production by anti-B7h mAb treatment

The intervention of the ICOS-B7h costimulatory pathway by anti-B7h mAb might modulate CII-specific T cell responses and might affect the Th1/Th2 balance. To address these possibilities, splenocytes and LN cells at day 5 were isolated from the CIA mice, and proliferative responses and cytokine production against CII were examined. Splenocytes from the control IgG-treated mice proliferated well in response to dCII, whereas splenocytes from naive mice did not proliferate even at a high concentration of dCII (Fig. 6Aa). Splenocytes from the anti-B7h mAb-treated mice showed significantly reduced proliferative responses to dCII as compared with those from the control IgG-treated mice. A similar inhibitory effect of the anti-B7h mAb treatment was observed when CD4⁺ splenic T cells (Fig. 6Ab) or LN cells (Fig. 6Ac) were used as responder cells. These results suggested that the anti-B7h mAb treatment at the second immunization inhibited the expansion of CII-specific T cells in the LN and spleen. We next examined the Th1 and Th2 cytokine production. LN cells from the control IgG- or anti-B7h mAb-treated mice were stimulated with 30 μg/ml dCII for 96 h, and IFN-γ, IL-4, and IL-10 in the supernatants were measured by ELISA. LN cells from the control IgG-treated mice produced high levels of IFN-γ and IL-10 in response to CII, but the production of these cytokines by LN cells was greatly reduced in the anti-B7h mAb-treated mice (Fig. 6B). IL-4 production was undetectable in both groups of mice in this culture condition (data not shown). Inhibitory effect by the anti-B7h mAb treatment on IFN-γ production was persistently observed even at 10 wk after the second immunization (Fig. 6C). These results suggested that the anti-B7h mAb treatment prevented the differentiation and/or expansion of CII-specific Th1 and Th2 cells and prolonged the inhibitory effect on Th1-mediated responses.

Inhibition of anti-CII IgG1, IgG2a, and IgG2b Ab production by anti-B7h mAb treatment

It is well known that IgG2a production is mainly induced by the Th1 cytokine IFN-γ and other IgG isotypes are regulated by Th2 cytokines such as IL-4, IL-5, IL-6, and IL-10 (38, 39). We thus investigated the anti-CII IgG1, IgG2a, and IgG2b Ab levels in the sera from the control IgG- or anti-B7h mAb-treated mice at 7, 14, and 21 days after the second immunization. The serum levels of anti-CII IgG1, IgG2a, and IgG2b were dramatically increased in response to the second immunization, but all of these responses were significantly suppressed by the anti-B7h mAb treatment (Fig. 7). These results indicated that the anti-B7h mAb treatment inhibited the production of anti-CII Abs that is dependent on either Th1
FIGURE 5. Expression of proinflammatory cytokines in the joints. A, The joints of hind paws from control IgG- or anti-B7h mAb-treated mice at day 5 were removed and mRNA expression for TNF-α, IL-1β, and IL-6 was assessed by RT-PCR. The expected product sizes for TNF-α, IL-1β, and IL-6 are 452, 297, and 248 bp, respectively. Data represent four individual mice from each group. B, The unit values were measured as described in Fig. 4 and the mean ± SEM of unit values from four control IgG (□) and anti-B7h mAb (■)-treated mice are shown. Similar results were obtained in three independent experiments. *, Statistically different from the control IgG-treated group (p < 0.05).

or Th2 cells. Since the anti-CII Abs have been implicated in the pathogenesis of CIA (28), the reduced production of anti-CII Abs might also be responsible for the ameliorating effect of the anti-B7h mAb treatment.

Discussion

In this study, we demonstrated the involvement of the ICOS-B7h costimulatory pathway in the pathogenesis of an experimental murine autoimmune arthritis. The administration of anti-B7h mAb ameliorated the clinical and histological manifestations of CIA. A similar beneficial effect was also obtained by a delayed treatment after the onset of arthritis. Expression of proinflammatory cytokines in the joints and both cellular and humoral responses to CII were significantly inhibited by the anti-B7h mAb treatment, which appeared to result in a beneficial effect.

Various costimulatory molecules, including CD28-CD80/CD86 (6), CD40-CD40 ligand (L) (40), and CD134-CD134L (41), have been implicated in the pathogenesis of CIA. Blockade of the CD28 costimulatory pathway inhibited the development of CIA in mice and rats (6, 42) and CD28-deficient mice were highly resistant to CIA (43), suggesting a critical involvement of CD28 in the induction of CIA. Unlike the constitutive expression of CD28 on naive T cells, ICOS is not expressed on naive T cells but induced after activation (7, 18, 27). In contrast, a considerable level of B7h is expressed on B cells without stimulation (12, 13, 15), whereas CD80 and CD86 are induced by stimulation. Consistent with these reports, we observed a constitutive expression of B7h on B cells in the LNs and the increased number of ICOS+ T cells in the LNs after immunization. In addition to these observations, we first demonstrated the expression of ICOS on T cells and B7h on B cells, macrophages, and dendritic cells in the inflamed joints at protein levels and the increased mRNA expression in the CIA mice. Although further studies are required for the detection of B7h expression on other types of cells in the synovium, our results suggest the possible involvement of ICOS-B7h interactions at the site of joints as well as in the draining LNs.

The actual involvement of the ICOS-B7h interaction in the pathogenesis of CIA was verified by the administration of neutralizing anti-B7h mAb. Blockade of the ICOS costimulatory pathway by anti-B7h mAb resulted in amelioration of inflammatory arthritis as assessed by clinical scoring and histological and radiological examinations. However, this treatment failed to decrease the incidence of disease, while similar treatment with CTLA-4Ig (6), anti-CD154 mAb (40), or anti-CD134L mAb (41) substantially decreased the incidence. Nevertheless, it should be noted that the delayed short-term treatment with anti-B7h mAb was also effective for preventing the progression of disease. In contrast, the treatment with anti-CD4 mAb (44), anti-CD134L mAb (41), or anti-CD80 and CD86 mAbs (6) was not or was less effective after the arthritis had been initiated. These results are consistent with the recent observations in EAE and allergic airway inflammation models, where the blockade of ICOS at the peak of disease, but not during Ag priming, dramatically ameliorated ongoing inflammatory responses (26, 27). Since ICOS is not expressed on naive T cells, it

FIGURE 6. Effects of anti-B7h mAb treatment on CII-specific T cell proliferation and cytokine production. A, Whole splenocytes (a), CD4+ splenic T cells (b), and LN cells (c) from naive mice (□) and the CIA mice treated with control IgG (○) and (■) or anti-B7h mAb (●) and (■) at day 5 were cultured in the presence or absence of the indicated amounts of dCII for 72 h. In the culture of CD4+ T cells, the mitomycin C-treated immunized splenocytes were added as APCs. Cultures were pulsed with [3H]thymidine and the incorporated radioactivity was measured. B, The supernatants of LN cells after a 96-h culture were assessed for IFN-γ and IL-10 production by ELISA. C, LN cells from the CIA mice treated with control IgG (□) or anti-B7h mAb (■) at 10 wk after the secondary immunization were cultured, and proliferation and cytokine production were measured as described above. IL-4 and IL-10 production were undetectable in this culture condition (data not shown). All data are shown as the mean ± SEM of four mice in each group. Data are representative of three independent experiments with similar results. *, Statistically different (p < 0.05).
is reasonable that the blockade of ICOS was less effective for preventing the priming of T cells. In contrast, ICOS is expressed on activated T cells and thus the ICOS-B7h interaction may play its primary role in costimulation of already Ag-primed T cells. Consistent with this notion, the expansion of ICOS+ T cells in the draining LN upon second immunization was markedly inhibited by the anti-B7h mAb treatment. In addition, ICOS may also play a costimulatory role in the activation of ICOS+ effector T cells infiltrating in the target tissues as discussed above.

The amelioration of CIA by the anti-B7h mAb treatment appeared to be correlated with the reduction of CIIC-specific T and B cell responses. T cell proliferative responses to CII and both IFN-γ and IL-10 production were significantly inhibited by the treatment. In addition, a marked reduction in all IgG1, IgG2a, and IgG2b subclasses of serum anti-CII Abs was observed. These results suggested that both Th1- and Th2-mediated immune responses against CII were comparably inhibited by the anti-B7h mAb treatment.

It has been reported that the blockade of the ICOS pathway by ICOS-Ig fusion protein or a neutralizing anti-ICOS mAb exhibited prominent inhibitory effects in the effector phase of the Th2-mediated immune responses (10, 27, 45). ICOS is preferentially expressed on Th2 cells at higher levels than on Th1 cells (10, 18, 27) and the blockade of ICOS in vitro preferentially reduced the production of Th2 cytokines such as IL-4 and IL-10 (18). Several reports demonstrated the failure to inhibit Th1-mediated immune responses by the ICOS blockade (10, 45, 46). On the other hand, a successful inhibition of Th1-mediated immune responses such as acute allograft rejection (22) and EAE (23, 26) has been also demonstrated. Our present results add a new example in which the blockade of the ICOS-B7h interaction resulted in inhibition of both Th1- and Th2-mediated immune responses.

In addition to the CIIC-specific immune responses mediated by T and B cells, locally produced proinflammatory cytokines also play a critical role in the development of arthritis. Among such cytokines, TNF-α, IL-1β, and IL-6 are crucial and the intervention of their actions could be a potential strategy for the treatment of arthritis (29, 47–50). We here showed that the treatment with anti-B7h mAb efficiently reduced the expression of these proinflammatory cytokines in the inflamed joints. It has been reported that TNF-α induced B7 expression on fibroblasts and nonlymphoid cells (11, 12) and that ICOS costimulation strongly enhanced TNF-α production by T cells (51). Therefore, the reciprocal induction of TNF-α and B7 through the ICOS pathway may amplify and perpetuate the local inflammation. In the acute allograft rejection model, the ICOS blockade suppressed the production of chemokines as well as IFN-γ and IL-10 (22). Thus, the ICOS-B7h interaction may be involved in the regulation of multiple cytokines and chemokines that control local inflammatory responses.

In conclusion, the blockade of ICOS costimulation by anti-B7h mAb ameliorated CIA through anti-inflammatory actions and suppression of both Th1- and Th2-mediated responses. Intervention of the ICOS costimulatory pathway may be a novel strategy for the treatment of human rheumatoid arthritis and possibly other chronic inflammatory diseases.

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