Effective Treatment of Adjuvant Arthritis with a Stimulatory CD28-specific Monoclonal Antibody

MARTA RODRÍGUEZ-PALMERO, ÀNGELS FRANCH, MARGARIDA CASTELL, CARME PELEGRÍ, FRANCISCO J. PÉREZ-CANO, CHRISTOPH KLEINSCHNITZ, GUIDO STOLL, THOMAS HÜNIG, and CRISTINA CASTELLOTE

ABSTRACT. ***Objective.*** To determine the immunomodulatory effects of the anti-rat CD28 monoclonal antibody (Mab) JJ316 on the onset of rat adjuvant arthritis (AA). JJ316 is a superagonistic Mab that induces polyclonal T cell proliferation in the absence of T cell receptor (TCR) ligation and promotes the expansion of regulatory T cells.

Methods. Female Wistar rats in which AA was induced were treated with JJ316 on Day 0 and Day 9 postinduction. A parallel treatment with JJ319, a "conventional" CD28-specific Mab that costimulates anti-TCR triggered proliferation, was performed. Severity of arthritis was monitored by means of an arthritic score, and by recording hindpaw volume and body weight increases. Serum antibodies against the AA-inducing mycobacteria were also determined by ELISA. To ascertain the effect of JJ316 on T lymphocytes *in vivo*, blood CD4+CD45RC^{high} (Th1-like) and CD4+CD45RC^{low} (Th2-like) cells were analyzed by flow cytometry, and the relative levels of interleukin 2 (IL-2), IL-10, and interferon-γ (IFN–γ) mRNA in synovial tissue were measured by real-time reverse transcription-polymerase chain reaction.

Results. JJ316 efficiently prevented the inflammatory process of AA. This effect was associated with a specific decrease in the blood CD4+CD45RC^{high}/CD4+CD45RC^{low} T cell ratio and high IL-10 mRNA expression in the synovia. In addition, anti-mycobacteria antibody levels decreased in JJ316 treated animals. In contrast, administration of the conventional anti-CD28 Mab JJ319 did not improve inflammation.

Conclusion. JJ316, a stimulatory CD28-specific Mab known to promote Th2 function and the expansion of regulatory T cells, provides effective protection from AA. (J Rheumatol 2006;33:110–8)

Key Indexing Terms:
EXPERIMENTAL ARTHRITIS IMMUNOTHERAPY LYMPHOCYTE
INFLAMMATION CYTOKINES ANTIBODIES

Adjuvant arthritis (AA) is a rat model of human rheumatoid arthritis (RA) that has been widely used to elucidate pathogenic mechanisms and to identify potential targets for therapeutic intervention. The key features of this model are joint inflammation, paw swelling, and weight loss in response to a single injection of heat-killed mycobacteria emulsified in a lipid carrier.

Chronic inflammatory joint disease probably begins with the activation of synovial lining cells, which produce chemokines that attract monocytes and lymphocytes into the joint. At this time, it is clear that T lymphocytes contribute significantly to AA[1-5] and RA[6,7]. When T cells recognize an antigen in the articular tissues, they are retained and inflammation ensues[8]. Thus, the initial activation of arthritogenic T cells may be secondary to the inappropriate attachment of danger signals to self-antigens presented by resident cells in the synovium[4].

Therapeutic studies using T cell-specific monoclonal antibodies (Mab) established a role for CD4+ α/ß T cells in AA development. Thus, this disease is treated or prevented with a Mab specific for the coreceptor CD4[9-11] or for a constant determinant of the α/ß T cell receptor (TCR)[12]. In contrast, Mab specific for γ/δ T cells exacerbate, rather than ameliorate, the course of the disease[13]. Further, arthritis can be transferred passively with CD4+ T cell lines and clones specific for mycobacterial antigens, which may crossreact with self-antigens[1,3,14].

After activation, CD4+ T cells differentiate into func-

F*rom the Department of Physiology, Faculty of Pharmacy, University of Barcelona, Barcelona, Spain; and the Institute for Virology and Immunobiology and the Neurological Clinic, University of Würzburg, Würzburg, Germany.*

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M. Rodríguez-Palmero, PhD; T. Hünig, PhD, Professor of Immunology, Department of Immunology; C. Kleinschnitz, MD, Professor of Neurology; G. Stoll, MD, Professor of Neurology, Department of Neurology, University of Würzburg; A. Franch, PhD, Professor of Physiology; M. Castell, PhD, Professor of Physiology; C. Pelegrí, PhD, Professor of Physiology; F.J. Pérez-Cano, PhD, Assistant Professor of Physiology; C. Castellote, PhD, Professor of Physiology, Department of Physiology, Faculty of Pharmacy, University of Barcelona.

M. Rodríguez-Palmero and A. Franch contributed equally to this study.

Address reprint requests to Prof. C. Castellote, Departament de Fisiologia, Facultat de Farmàcia, Av. Joan XXIII s/n, edifici B, 3a planta, E-08028 Barcelona, Spain. E-mail: cristinacastellote@ub.edu

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tionally distinct subsets[15]. Thus, Th1 cells promote cell-mediated immunity and inflammation, mainly by producing cytokines with proinflammatory activities, most notably interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). These cells are believed to play a critical role in inflammatory conditions such as arthritis. In contrast, Th2 cells produce cytokines that promote B cell activation and direct antibody class switching while counteracting the development of Th1 cells. Again, the characteristic cytokines produced by Th2 cells, interleukin 4 (IL-4), IL-5, IL-10, and IL-13, are crucial for these functions. In autoimmune-inflammatory disease models like AA, it has been proposed that the balance between Th1 and Th2 cells is the prime indicator of tolerance, and its breakdown triggers disease development, although experimental data supporting this hypothesis are controversial[5,16].

More recently, the role of "regulatory" T cells (T*reg* cells) in the control of autoimmunity and inflammation has been extensively described in rodents and humans[17,18]. These cells are a subset of CD4+ lymphocytes with an antigen-experienced phenotype that typically express CD25, the IL-2 receptor α chain[17], CD152, a key negative regulator of T cell activation[19], the TNF-receptor (TNF-R) family member GITR (glucocorticoid-induced TNF-R family-related gene)[20,21], and the transcription factor Foxp3[21]. In humans, T*reg* cells can be isolated from the joints of arthritic patients, where they are thought to fight inflammation[22-24]. RA is associated with the reduction in T*reg* cells in peripheral blood[23-26], and the protective role of these cells has been reported in mouse models of autoimmunity and inflammation[18], including arthritis models[27-30].

In rats, the expression of high versus low levels of the high molecular weight isoform of CD45, CD45RC, can be used to further subdivide CD4+ T cells. Functional studies have shown that Th1 cells are found within the CD4+CD45RC^{high} subset, and Th2 cells within the CD4+CD45RC^{low} subset[31,32]. The former but not the latter induce graft versus host disease and produce IL-2 and IFN-γ *in vitro* in response to allostimulation[33]. CD45RC expression on CD4+ T cells increases in association with streptococcal cell wall-induced arthritis in both blood and lymph nodes[34]. The effectiveness of the anti-CD4 therapy in AA is associated with the specific decrease in blood CD4+CD45RC^{high} cells[35]. However, the downregulation of CD45RC is also correlated with the transition from a naive to an antigen-experienced state[36], and the CD45RC^{low} subset also contains T*reg* cells[37]. These observations are consistent with the hypothesis that these are self-reactive suppressor cells constantly confronted with their antigens.

The signals induced in CD4+ T cells (Th1 or Th2) upon antigen recognition are amplified and modulated by costimulation. Of the increasing family of costimulatory molecules, CD28 is a potent and probably the most important cell-surface receptor that mediates costimulation[38]. Two natural ligands, CD80 and CD86, are expressed on antigen-presenting cells and are active in costimulation[39]. CD28 signaling promotes sustained T cell growth *in vitro*, mostly by enhancement of IL-2 synthesis, but studies in IL-2-deficient mice indicate that additional growth-promoting signals are induced by costimulation[40]. The balance between TCR and CD28 signals in CD4+ T cell activation contributes to their differentiation to a Th1 or Th2 phenotype. Thus, weak TCR signals combined with strong CD28 signals promote Th2 differentiation, while strong TCR signals suppress Th2 development in favor of a Th1 fate[41-44].

In rats, 2 functionally distinct anti-rat CD28 Mab have been described, JJ316 and JJ319[45,46]. Both antibodies react with virtually all T lymphocytes bearing α/ß TCR, with most γ/δ TCR-bearing cells, and with a subset of natural killer cells. JJ319 is a "conventional" CD28-specific Mab that costimulates anti-TCR-triggered proliferation *in vitro*, and leads to transient CD28 modulation *in vivo*. In contrast, JJ316 is a CD28 "superagonistic" Mab that induces polyclonal T cell proliferation *in vitro* and *in vivo* and does not require TCR ligation[46], a property associated with the binding to a distinct region of the CD28 molecule[47]. The *in vivo* response to JJ316 results not only in blastogenic transformation of peripheral CD4+ T cells and dramatic lymphocytosis, but also in a shift toward Th2 responses and marked induction of IL-10[44], which was recently ascribed to a disproportionate "overactivation" of T*reg* cells during the expansion phase[37]. To investigate further the effect of JJ316 on T lymphocytes, we applied preventive immunotherapy with this antibody against AA, a CD4+ T cell-associated experimental autoimmune inflammatory disease.

MATERIALS AND METHODS

Animals. Female Wistar rats (Charles River, Criffa, Barcelona, Spain) weighing 200–220 g were housed 3 per cage and given food and water ad libitum. Temperature (20°C ± 2°C), humidity (55%), and light cycle (on from 8:00 AM to 8:00 PM) were controlled. Housing conditions were clean but not pathogen-free. The animals were allowed 2 weeks to adjust to the conditions before experiments began. Studies were performed in accord with the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation of the University of Barcelona.

Induction and assessment of AA. Rats were injected intradermally into the tail base with 0.5 mg of heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI, USA) in 0.1 ml of liquid vaseline. AA was assessed using an arthritic score and by increases in hindpaw volume and body weight. Arthritic score was established by grading each paw from 0 to 4 according to the extent of erythema and edema of the periarticular tissue (maximal score per animal was 16). Hindpaw edema was measured using a water plethysmometer (LI 7500, Letica, Barcelona, Spain). Left and right hindpaw volumes were summed and expressed as the percentage of increase compared with Day 0. Area under the curve (AUC) was calculated for the 3 variables measured between Days 8 and 28 after induction of AA. All scoring was performed in a blinded manner.

Immunotherapy of AA. JJ316 (mouse IgG1, anti-rat CD28), JJ319 (mouse IgG1, anti-rat CD28), and 124 1D1 (mouse IgG1, anti-human CD7 as control isotype Mab) hybridomas were used. After culturing the hybridomas, ascites was induced in Balb/c mice (Charles River) by standard methods.

IgG from ascitic liquid was purified by protein-A column and its concentration was quantified spectrophotometrically. All Mab obtained were assayed for endotoxin contamination using a standard limulus assay (Escherichia Toxate; Sigma) and contained < 0.01 ng/ml endotoxin, the lower limit of detection of the assay. Antibodies were administered using phosphate buffered saline (PBS) as a vehicle, and the delivery volume was 2 ml per injection.

Immunotherapy was administered on Day 0, before arthritis induction, and on Day 9 postinduction. Rats were distributed in 4 groups that were intraperitoneally treated with PBS (AA control), 0.5 mg/rat/day of control isotype Mab (AA+IgG1), 0.5 mg/rat/day of conventional anti-CD28 Mab (AA+JJ319), or 0.5 mg/rat/day of stimulatory anti-CD28 Mab (AA+JJ316). An additional group of untreated animals were used as healthy controls.

Determination of anti-M. butyricum antibodies. Anti-*M. butyricum* antibody levels in sera from Days 14, 21, and 28 postinduction were determined by an indirect ELISA technique as described[48]. Polystyrene micro-ELISA plates (Nunc Maxisorp, Wiesbaden, Germany) were incubated with a soluble protein fraction of *M. butyricum* in PBS (3 μg/ml). Peroxidase-conjugated goat anti-rat Ig (1/2000) (PharMingen, San Diego, CA, USA) was used as secondary antibody. Since standards were not available, several dilutions of pooled sera from arthritic control animals were added to each plate. This pool was arbitrarily assigned 8000 U/ml.

Immunofluorescence staining and flow cytometry. On Days 14, 21, and 28 postinduction, peripheral blood samples were collected from the tail vein into EDTA-coated tubes (Sardstedt, Canovelles, Spain). Before staining, erythrocytes were eliminated by osmotic lysis[9]. Two-color immunofluorescence staining was performed with the mouse anti-rat Mab OX19 (anti-CD5), W3/25 (anti-CD4), and OX22 (anti-CD45RC) (all from PharMingen). Mononuclear cells (2×10^5) were incubated with the unlabelled OX19 or OX22 Mab for 20 min in ice. Phycoerythrin-labeled goat anti-mouse IgG (Sigma, Alcobendas, Spain), preadsorbed with 2% rat serum, was used as secondary antibody. Cells were then incubated with normal mouse IgG (10 μg/ml) for 10 min to block residual anti-mouse Ig binding sites. Finally, cells were washed and incubated with FITC-conjugated W3/25 Mab. Cells were fixed with 1% paraformaldehyde and stored at 4°C in the dark until analysis. For each animal, negative control staining was included using an isotype-matched Mab. Viable cells with the forward and side-scatter characteristics of lymphocytes were analyzed on an Epics Elite flow cytometer (Coulter, Hialeah, FL, USA).

Results are expressed as percentages of stained cells compared with total lymphocyte population. To distinguish CD4+ cells with high and low expression of CD45RC, the regions analyzed were carefully set on OX22/W3/25 histograms.

Assessment of cytokine mRNA expression. On Days 9 and 28 postinduction, samples of ankle joint synovial membrane from several control, IgG1-administered and JJ316-treated arthritic rats were dissected, snap-frozen in 500 μl guanidinium thiocyanate buffer[49], and kept at –80°C until processing.

Tissue homogenization and RNA isolation were performed as described with slight modifications[50]. In brief, total RNA from pooled synovia (n = 3) was prepared using TRIzol reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions and was quantified spectrophotometrically. Homogenization was performed with a Miccra D-8 power homogenizer (ART, Mühlheim, Germany) at 39,000 RPM for 25 s. Relative levels of cytokine mRNA were quantified by real-time quantitative polymerase chain reaction (RT-PCR) using fluorescent TaqMan technology. A quantity of 250 ng total RNA was reverse-transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystems, Weiterstadt, Germany) following the manufacturer's protocol using random hexamers. The resulting cDNA was measured spectrophotometrically.

PCR primers and probes specific for rat IL-2, IL-10, and IFN-γ and 18s rRNA were obtained as TaqMan Predeveloped Assay Reagents for gene expression (Applied Biosystems). The 18s rRNA was used as an endogenous control to normalize the amount of sample RNA. PCR was performed with equal amounts of cDNA in the GeneAmp 7700 sequence detection system (Applied Biosystems) using the TaqMan Universal PCR Master Mix (Applied Biosystems) following the manufacturer's instructions. The PCR reaction mixtures (total volume 50 μl) were incubated at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Water controls were included to ensure specificity. Each sample was measured in triplicate and data points were examined for integrity by analysis of the amplification plot.

The comparative Ct method was used for relative quantification of gene expression[51]. The amount of target mRNA, normalized to an endogenous control (18s rRNA) and relative to a calibrator (tissue samples from AA controls), is given by $2^{-\Delta\Delta Ct}$, where Ct is the cycle number at which the fluorescence signal of the PCR product crosses an arbitrary threshold set within the exponential phase of the PCR, and

$$\Delta\Delta Ct = [(Ct_{\text{target (unknown sample)}} - Ct_{\text{end. control (unknown sample)}})] - [(Ct_{\text{target (calibrator sample)}} - Ct_{\text{end. control (calibrator sample)}})].$$

Statistical analysis. Differences between groups were analyzed using the nonparametric Mann-Whitney U-test from the Statistica 6.0 program (StatSoft, Melbourne, Australia). For PCR data, statistical analysis was performed by the nonparametric Friedmann test followed by Dunn's post hoc test to correct for multiple comparisons, using GraphPad Prism software 3.0 (GraphPad Prism, San Diego, CA, USA). Differences were significant for $p < 0.05$.

RESULTS

Effect of Mab treatment on arthritis development. AA controls developed a full arthritic syndrome, i.e., increased arthritic scores and hindpaw volumes and marked loss of body weight, which remained from Day 14 postinduction until the end of the study on Day 28 (Figure 1A-1C). AA rats treated with an irrelevant IgG1 Mab showed a very similar time course of the disease (Figure 1A-1C).

Administration of the conventional anti-CD28 Mab JJ319 did not ameliorate the inflammatory signs of AA because it did not affect arthritic scores, hindpaw volumes, or loss of body weight (Figure 1), and even exacerbated the disease when the arthritic score AUC was considered ($p < 0.05$; Figure 1D). The weight increase of the JJ319-treated group was similar to that of AA controls (Figure 1C and 1F).

In contrast, the administration of the superagonistic anti-CD28 Mab JJ316 on Days 0 and 9 efficiently diminished articular swelling from Day 14 until the end of the study. Animals in this group showed lower arthritic scores (Figure 1A) and paw volume increases (Figure 1B) than AA controls from Day 16 until the end of the study ($p < 0.05$). When we summarized these results as AUC, the JJ316 group showed lower values than the AA controls ($p < 0.05$; Figures 1D, 1E). Moreover, the JJ316 group followed a body weight increase pattern similar to that of healthy animals, with significant differences from the AA control group, in particular on Days 16–28 ($p < 0.05$; Figure 1C) and globally in the AUC ($p < 0.05$; Figure 1F).

JJ319 Mab did not improve the inflammation of arthritic animals, and so we did not obtain samples from these rats.

Anti-M. butyricum antibody levels. Serum anti-*M. butyricum* antibody levels are shown in Figure 2. Values from the AA+IgG1 group followed a time course similar to

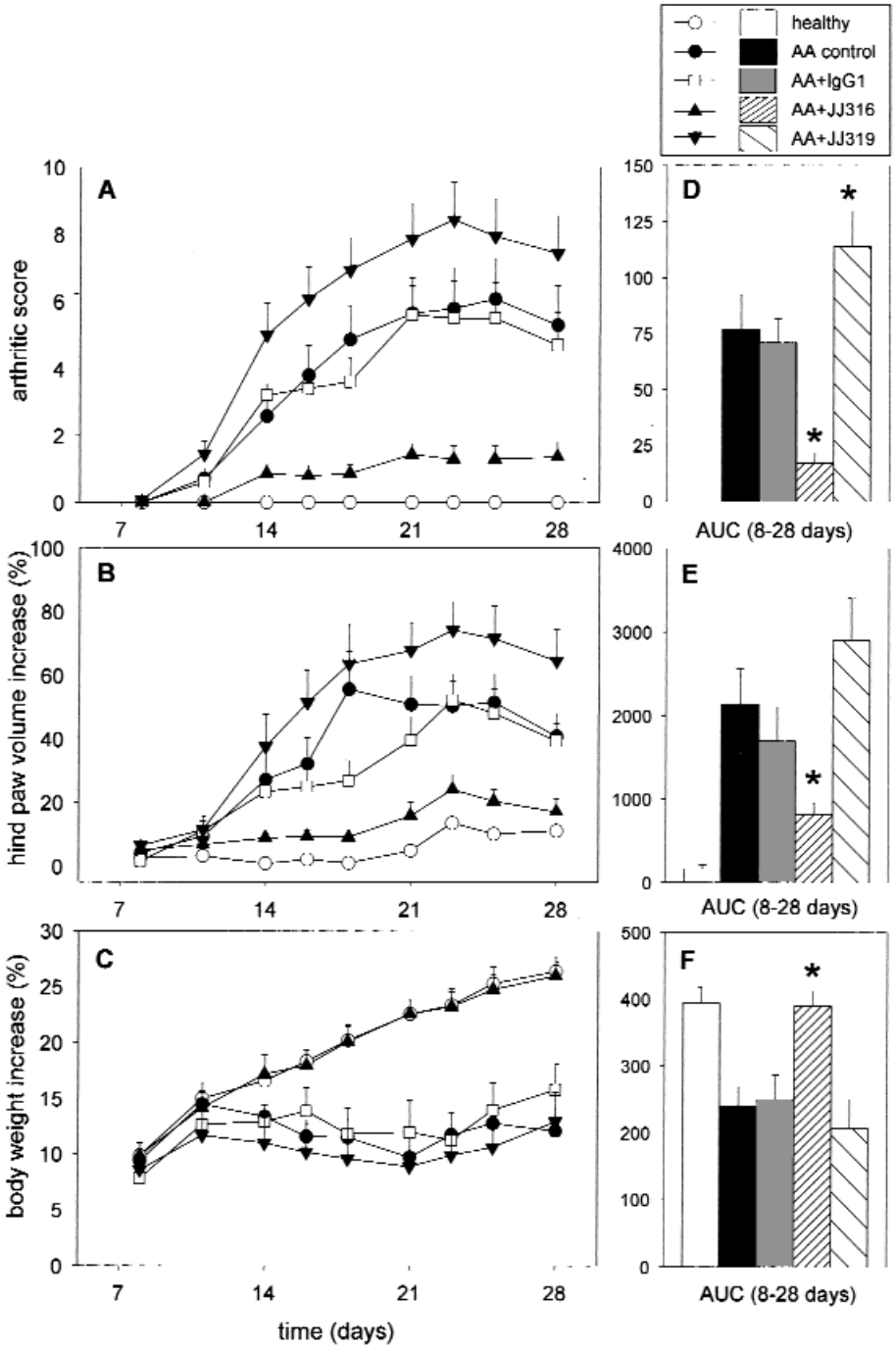

Figure 1. Arthritis assessment. Time course of arthritic score (A), hindpaw volume increase (B), and body weight increase (C) in the study groups. Area under the curve (AUC) from arthritic score (D), hindpaw volume increase (E), and body weight increase (F) determined between Days 8 and 28 after arthritis induction. Values are summarized as mean ± SEM of 6 rats. *$p < 0.05$ compared with AA control rats.

AA controls throughout the study. Treatment with JJ316 reduced specific antibody synthesis and thus significantly decreased anti-*M. butyricum* antibody levels compared with the AA group on Days 21 and 28 (Figure 2).

Expression of CD45RC on CD4+ T cells. The percentages of blood CD5+, CD4+, CD4+CD45RC^{high}, and CD4+CD45RC^{low} cells are shown in Figure 3. Results from the AA+IgG1 group were similar to those of AA controls. JJ316 Mab did not modify the percentages of CD5+ and CD4+ T lymphocytes compared with the healthy and AA

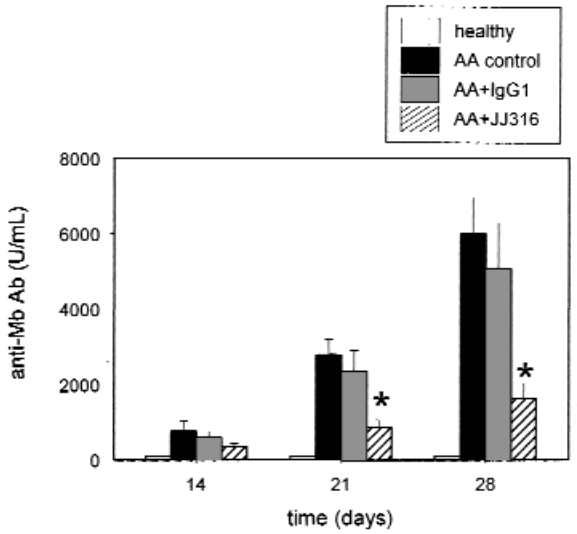

Figure 2. Time course of specific anti-*M. butyricum* antibody levels in healthy, arthritic controls (AA control), arthritic isotype controls (AA+IgG1), and arthritic rats treated with JJ316 Mab (AA+JJ316). Values are summarized as mean ± SEM of 6 rats. *p < 0.05 compared with AA control rats.

control groups in peripheral blood, indicating that this Mab did not cause T lymphocyte deletion. Regarding CD45RC expression on CD4+ T cells, 2 populations have been established, with high and low expression of CD45RC[35,36]: the CD45RC^{high} population, which includes proinflammatory Th1 cells, and the CD45RC^{low} population, which includes the antiinflammatory Th2 and T*reg* subsets[37]. On Day 14 postinduction, when the inflammatory process had been established, rats treated with the stimulatory JJ316 Mab showed a significant decrease in CD4+CD45RC^{high} T cells (Figure 3C and 3F), which was accompanied by a parallel increase in CD4+CD45RC^{low} T cells compared with AA controls (Figure 3D and 3E). Therefore, the CD4+CD45RC^{high}/CD4+CD45RC^{low} T cell ratio, which was 1.13 ± 0.07 (mean ± SEM) on Day 14 in AA control rats, fell to 0.44 ± 0.16 in the AA+JJ316 group ($p < 0.05$).

On Day 21, although the JJ316 group and AA controls did not differ significantly, the same tendency was observed. On Day 28 postinduction, i.e., 19 days after the last dose of JJ316, all groups showed similar percentages of CD4+CD45RC^{high} and CD4+CD45RC^{low} T cells.

Expression of cytokine mRNA in the synovial membrane. To characterize the disease process of the synovia by means of pro- and antiinflammatory cytokines, RT-PCR was performed on RNA extracted from frozen joint synovial tissue obtained on Days 9 and 28 after disease induction (Figure 4). On Day 9, i.e., in the ascending phase of disease development, the most prominent effect was 80% inhibition of IFN-γ mRNA levels in JJ316-treated animals (Figure 4B). On Day 28, after the disease maximum, the JJ316-treated group displayed strong local expression of the antiinflammatory cytokine IL-10 (Figure 4A), which may indicate the *in situ* effect of regulatory T cells. Administration of the isotype control antibody causes some statistically nonsignificant effects in IFN-γ and IL-2 expression.

DISCUSSION

Current therapeutic approaches to autoimmune and inflammatory diseases are usually aimed at interrupting stimulatory pathways or at blocking effector systems of the immune system. In mycobacteria-induced AA in the rat, these approaches have been successfully applied using pharmacological inhibitors of inflammation such as corticosteroids and Mab that target whole T cell subsets[10,12,35]. In human RA, antiinflammatory and immunosuppressive drugs and blockade of the proinflammatory effector cytokine TNF-α have proved useful[52]. An attractive alternative is to enhance naturally occurring mechanisms that counteract immunopathology. As for polarized immune responses that involve specific damaging effector mechanisms, this can be accomplished by inducing immune deviation toward the opposite response, i.e., by tipping the balance from a Th1-dominated proinflammatory response toward one dominated by Th2. In a more attractive scenario, stimulation of T*reg* cells, which are natural guardians against autoimmunity and the hyperreactivity of both cell-mediated and antibody-mediated responses[18,53], may counteract immunopathology without causing general immunosuppression.

Stimulation of the T cell compartment with a "superagonistic" anti-CD28 Mab may be beneficial, while administration of a conventional anti-CD28 Mab does not protect against an inflammatory response. JJ316 has proven successful following a preventive protocol as described here, and also when following a curative protocol (unpublished results). Moreover, JJ316 Mab prevent and reduce inflammation in experimental autoimmune neuritis[54] and encephalomyelitis[55]. The clear protective effect of JJ316 is probably due to the induction of immune deviation from a Th1 to a Th2 response and/or to the induction of T*reg* cells. The protection achieved with this activating approach is as strong as that obtained using CD4 or α/ß TCR-specific Mab[9,12], which indicates that the enhancement of antiinflammatory regulatory mechanisms is a viable alternative to immunosuppressive protocols.

AA is accompanied by an increase in the blood lymphocyte count[56] and JJ316 also induces lymphocytosis[44]. In our study, total blood lymphocyte count was not analyzed, but the proportions of CD5+ and CD4+ lymphocytes were not modified by AA or JJ316 treatment. However, considering the proportions of CD4+ T cells that coexpressed CD45RC, anti-CD28 immunotherapy caused a switch toward a higher blood CD4+CD45RC^{low} subset, which may reflect the expansion of Th2 cells[31,32]. Moreover, the study of cytokine expression in ankle synovial membrane revealed an early decrease in the expression of IFN-γ mRNA in the anti-CD28-protected group, and a late increase in IL-10 mRNA

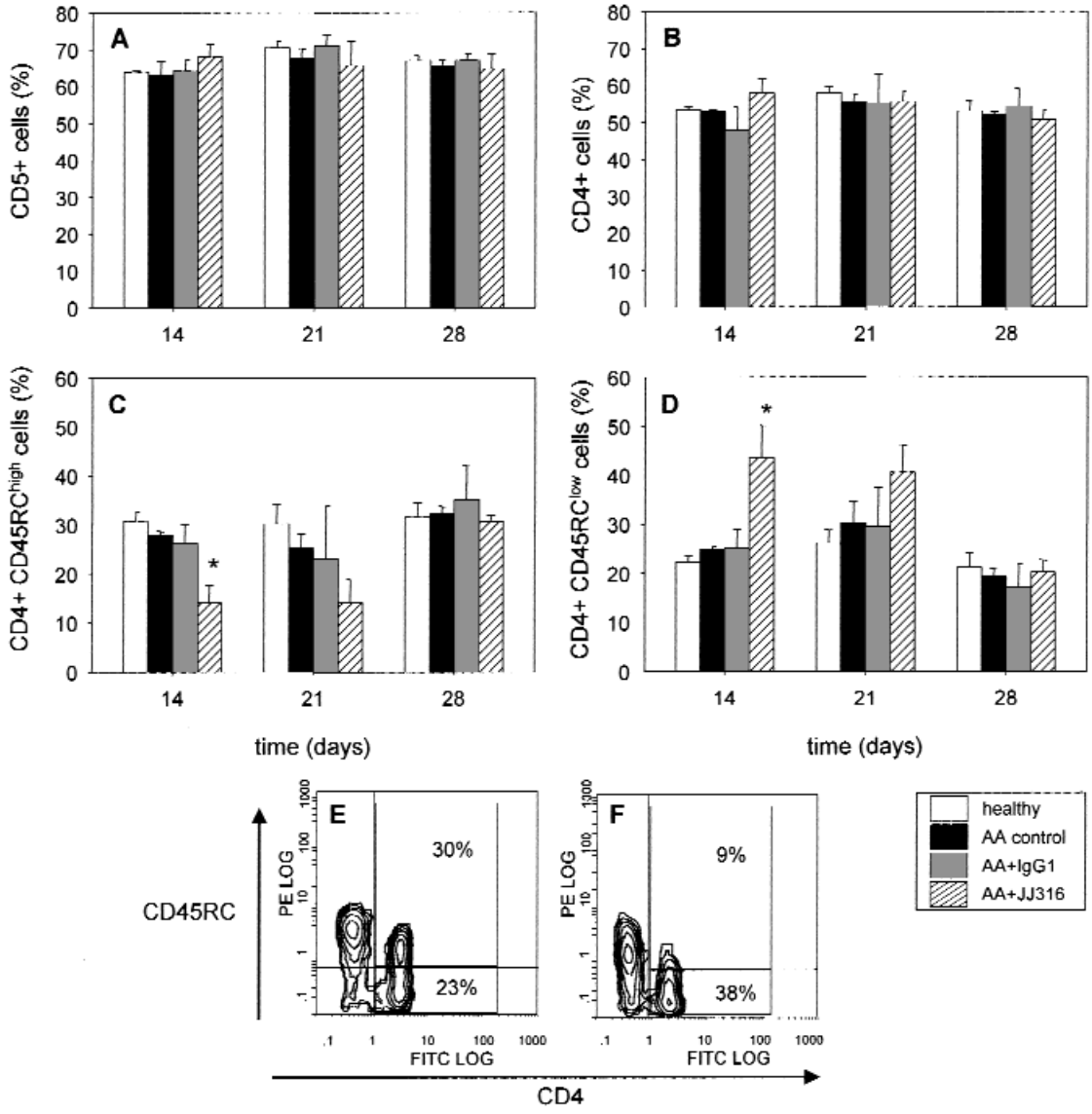

Figure 3. Blood lymphocyte subsets. Time course of percentages of CD5+ (A), CD4+ (B), CD4+CD45RC^{high} (C), and CD4+CD45RC^{low} (D) cells in healthy, arthritic controls (AA control), arthritic isotype controls (AA+IgG1), and arthritic rats treated with JJ316 Mab (AA+JJ316). Values are summarized as mean ± SEM of 6 rats. *$p < 0.05$ compared with AA control rats. Representative histogram frequency distribution of fluorescence intensity after staining with FITC-anti-CD4 and phycoerythrin anti-CD45RC Mab peripheral blood lymphocytes from an arthritic control animal (E) and a JJ316 animal (F) on Day 14 postinduction.

expression. JJ316 injection is accompanied by the induction of IL-4 and especially IL-10, whereas proinflammatory cytokines are not induced[44]. Recently, we identified T*reg* cells as the source of IL-10 and showed that this cell type is transiently overrepresented in the CD28-stimulated pool of CD4+ T cells. Overall, T*reg* cell numbers increased by 20-fold at the peak of the response to treatment with the CD28 superagonist[37]. Although not directly addressed in the present study, these findings suggest that the main protective effect of the anti-CD28 superagonist in AA is mediated by the expanded pool of T*reg* cells. The strong synovial expression of IL-10 may indicate a possible *in situ* effect of T*reg*. In humans, T*reg* cells home in on the inflamed joint, where they may counteract the inflammatory process, as recently reported[22,23].

Further, production of *M. butyricum*-specific antibody was reduced by treatment with JJ316. A similar result was obtained after effective anti-CD4 therapy[9,10,35]. On the other hand, *in vivo* stimulation with the anti-CD28 superagonist JJ316 enhances immunoglobulin production of all major isotypes, in particular IgM, IgG1, and IgE[44]. This was attributed to the non-antigen-specific assistance provided by CD28-activated CD4+ T cells to B cells that have previously encountered an antigen. The decrease in anti-*M.*

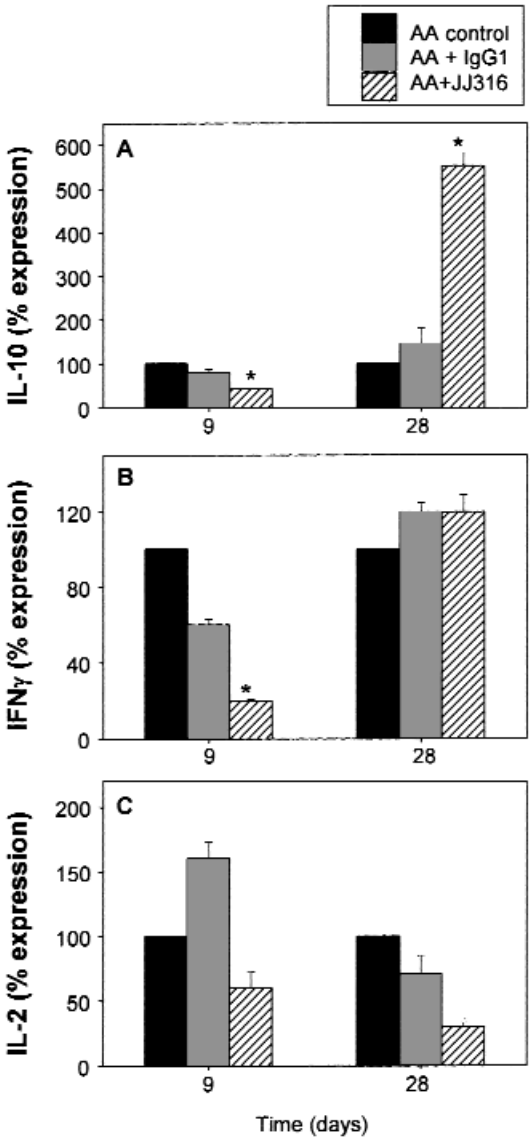

Figure 4. Cytokine expression of IL-10 (A), IFN-γ (B), and IL-2 (C) mRNA levels in synovial tissue from rats undergoing AA as measured by RT-PCR. Each data point corresponds to the mean ± SEM of 3–4 values. Each value was obtained from 3 synovial tissues. Results are given as relative changes compared with the AA control group. *$p < 0.05$.

butyricum antibodies in the JJ316-treated group suggests that the stimulation of antigen-specific helper T cells required to support the response of B cells to newly introduced *M. butyricum* antigen may be impaired, perhaps due to competition under the dramatic increase in total B cell number, which by far exceeds that observed in any antigen-driven response and which would hamper further expansion by individual clones. The exact mechanism remains to be elucidated.

In addition, the lack of protective effects and even a possible exacerbation observed with the conventional CD28-specific Mab JJ319 are expected, as this Mab does not activate T cells[46] or induce Th2 or T*reg* cytokines[44]. Both JJ316 and JJ319-enhanced TCR triggered proliferation *in vitro* to a similar extent, while activation in the absence of TCR triggering occurred only with JJ316, both *in vitro* and *in vivo*[46]. In other systems[42,43], a reduction in the costimulatory signal favored the development of Th1 cells. Large amounts of JJ319 *in vivo* produce downmodulation of surface CD28 expression[57]; thus, in our study administration of this antibody could lead to a reduction of its own costimulatory signal, favoring Th1 development.

Taken together, these results show that JJ316, a stimulatory CD28-specific Mab that promotes Th2 function and expansion of T*reg* cells *in vivo*, provides protection from arthritis induced by mycobacterial antigens. In further experiments, we shall attempt to identify the T cell subset that mediates protection and to elucidate the events that interfere with the inflammatory process. Whatever the outcome, it seems clear that antiinflammatory mechanisms of the immune system can be addressed via the CD28 molecule, providing a potential strategy to treat autoimmune-inflammatory joint disease.

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