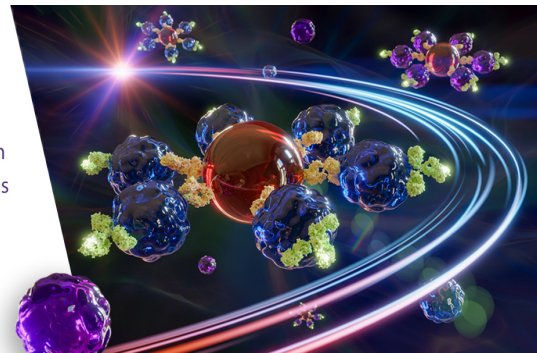


Discover More With Flow Cytometry— Quantify Cytokines With LEGENDplex™

- Pre-defined and custom panels with extensive bioassay validation
- Panels optimized for various immune cell types and research areas
- Setup manuals for flow cytometers
- Complimentary software suite for data analysis

Learn more ►



The Journal of Immunology

RESEARCH ARTICLE | NOVEMBER 01 2004

Targeting IL-15 Receptor-Bearing Cells with an Antagonist Mutant IL-15/Fc Protein Prevents Disease Development and Progression in Murine Collagen-Induced Arthritis¹ **FREE**

Sylvie Ferrari-Lacraz; ... et. al

J Immunol (2004) 173 (9): 5818–5826.

<https://doi.org/10.4049/jimmunol.173.9.5818>

Related Content

Targeting the IL-15 Receptor with an Antagonist IL-15 Mutant/Fcγ2a Protein Blocks Delayed-Type Hypersensitivity

J Immunol (June,1998)

An Antagonist IL-15/Fc Protein Prevents Costimulation Blockade-Resistant Rejection

J Immunol (September,2001)

Targeting a Mimotope Vaccine to Activating Fcγ Receptors Empowers Dendritic Cells to Prime Specific CD8⁺ T Cell Responses in Tumor-Bearing Mice

J Immunol (November,2009)

Targeting IL-15 Receptor-Bearing Cells with an Antagonist Mutant IL-15/Fc Protein Prevents Disease Development and Progression in Murine Collagen-Induced Arthritis¹

Sylvie Ferrari-Lacraz,^{2*} Eric Zanelli,[†] Manfred Neuberger,[†] Elina Donskoy,[†] Yon Su Kim,^{3*} Xin Xiao Zheng,^{*} Wayne W. Hancock,[‡] Włodzimierz Maslinski,^{4*} Xian Chang Li,^{*} Terry B. Strom,^{*} and Thomas Moll^{5†}

It has been suggested that the inflammatory cytokine IL-15 plays an important role in the development of several autoimmune diseases, including rheumatoid arthritis. We have generated a unique lytic and antagonistic IL-15 mutant/Fcγ2a fusion protein (CRB-15) that targets the IL-15R. In the present study we examined the effects of targeting the IL-15R on the prevention and treatment of collagen-induced arthritis (CIA) in mice and probed the possible mechanisms of action of this IL-15 mutant/Fcγ2a protein. Upon immunization with type II collagen, DBA/1 mice develop severe articular inflammation and destruction. Treatment of DBA/1 mice with a brief course of CRB-15 at the time of type II collagen challenge markedly inhibited the incidence and severity of arthritis. Moreover, in animals with ongoing established arthritis, treatment with CRB-15 effectively blocked disease progression compared with that in control-treated animals. The therapeutic effect of CRB-15 on either disease development or disease progression is remarkably stable, because withdrawal of treatment did not lead to disease relapse. A detailed analysis revealed that treatment with CRB-15 decreased synovitis in the joints; reduced bone erosion and cartilage destruction; reduced *in situ* production of the proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-17; and decreased the responder frequency of autoreactive T cells. Our study suggests that the effective targeting of IL-15R-triggered events with CRB-15 can be of therapeutic importance in the treatment of rheumatoid arthritis. *The Journal of Immunology*, 2004, 173: 5818–5826.

Rheumatoid arthritis (RA)⁶ is an autoimmune disorder characterized by synovial inflammation and the erosion of bone and cartilage, ultimately leading to the destruction of the affected joints. The dysregulated intra-articular expression of proinflammatory cytokines, in particular, TNF-α and IL-1β, plays a key role in the pathogenesis of RA (1), a finding that has been corroborated by the successful treatment of RA patients with therapeutic agents targeted at inhibiting the activity of either of these cytokines (2–7). Although often successful in clinical practice, blocking, for instance, TNF-α is not effective in all pa-

tients and does not lead to a remission of the disease. This may be due in part to the complexity of the cytokine network involved in inflammatory lesion formation, and it is likely that cytokines or growth factors other than TNF-α and IL-1β participate in the pathogenesis of RA. Recently, the proinflammatory cytokine IL-15 was identified as one of the factors likely to play an important role in RA disease progression (8).

IL-15 is a member of the four-α-helix bundle cytokine family with closest homology to IL-2 and IL-21 (9–11) and which, under physiological conditions, is involved in the differentiation and proliferation of NK and T cells (12, 13; for review, see Ref. 14). Furthermore, IL-15 is an important proinflammatory cytokine, produced, e.g., by activated macrophages (9, 15), that stimulates the activation, proliferation, survival, and effector functions of a variety of immune cells (14, 16–19). For instance, we have demonstrated that IL-15 is a critical growth factor for the proliferation of Ag-activated T cells in response to immunological stimuli *in vivo* (20), and increased IL-15 expression has been described in a variety of immunological disorders (14). Overexpression of IL-15 in patients with active RA was initially suggested by McInnes et al. (8), a finding since confirmed by various other groups (21, 22). These authors also demonstrated that in the rheumatoid lesion, IL-15 may act as a chemotactic and proliferation-inducing factor for synovial T cells and may increase synovial macrophage TNF-α production via a process requiring direct contact between T cells and macrophages (23, 24). These findings were extended by the observation that IL-15 mediates T cell extravasation into arthritic tissue *in vivo* (25). In addition, we have recently demonstrated that the activities of IL-15 in RA are not limited to T cells and macrophages, but also extend to synoviocytes, because IL-15 not only triggers IL-17 production (26), which, in turn, induces inflammatory cytokine expression by synoviocytes (27) and macrophages (28), but also directly

*Department of Medicine, Harvard Medical School, Division of Immunology, Beth Israel Deaconess Medical Center, Boston, MA 02215; [†]Cardion, Erkrath, Germany; and [‡]Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, PA 19104

Received for publication April 27, 2004. Accepted for publication August 19, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work is supported by a postdoctoral fellowship grant from the Fondation des Bourses en Médecine et Biologie and the Swiss National Science Foundation (to S.F.L.), Juvenile Diabetes Foundation International Grant 1-1999-317 (to X.X.Z.), and National Institutes of Health Grant RO1AI42298 (to T.B.S.).

² Current address: Division of Immunology and Allergy, Department of Medicine, University Hospital, Geneva, Switzerland.

³ Current address: Seoul National University, Boraman Hospital, 195 Shindaebang-dong, Tongjak-ju, Seoul 156-012, Korea.

⁴ Current address: Department of Pathophysiology and Immunology, Institute of Rheumatology, Spartanska 1, Warsaw, Poland.

⁵ Address correspondence and reprint requests to Dr. Thomas Moll at the current address: Telos Pharmaceuticals, 10150 Meanley Drive, San Diego, CA 92131. E-mail address: tmoll@forestlifesciences.com

⁶ Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; CII, type II collagen; PLN, peripheral lymph node cell; sIL-15Rα, soluble IL-15R α subunit.

stimulates the proliferation and survival of hyperproliferating primary human fibroblast-like synoviocytes (29).

In view of the large body of evidence suggesting an important role of IL-15 in RA disease progression, we hypothesized that therapeutic targeting of IL-15R⁺ cells should be beneficial for the treatment of RA. To test this, we have constructed a recombinant fusion protein consisting of a point-mutated IL-15 and the constant region of murine IgG2a. This IL-15 mutant/Fcγ2a fusion protein (CRB-15) binds the IL-15R with high affinity, but does not trigger signaling events, thereby blocking, e.g., IL-15-driven cell proliferation *in vitro* and acting as a receptor-specific antagonist (30). The Fc portion of this fusion protein confers *in vivo* longevity and is designed to target IL-15R-bearing cells, such as activated T cells, macrophages, and synoviocytes for deletion by the innate immune system. Previous studies using CRB-15 have demonstrated that this fusion protein is indeed remarkably effective in blocking delayed-type hypersensitivity responses and alloantigen-driven T cell proliferation *in vivo* (30). Also, CRB-15 can induce stable allograft survival in a murine model of islet transplant rejection (31). In the present study, using the murine model of type II collagen (CII)-induced arthritis (CIA), we examined the effect of CRB-15 on both the initial development and established ongoing arthritis and studied the possible mechanisms involved. We found that CRB-15 treatment not only could prevent the development of arthritis, but was also remarkably effective in blocking disease progression in a model of established ongoing disease. Intriguingly, we observed that the effects of CRB-15 treatment were sustained after discontinuation of treatment in both the disease onset and established disease models, suggesting a long term beneficiary effect of CRB-15 even after a short treatment regimen. We furthermore show that apart from reducing IL-1β, IL-6, IL-17, and TNF-α expression in the joints, CRB-15 treatment also decreases lymphocytic infiltration, reduces bone erosion and cartilage destruction, and decreases the responder frequency of Ag-reactive T cells.

Materials and Methods

Expression and purification of CRB-15 protein

The design and construction of the CRB-15 DNA construct were previously described (30). Briefly, glutamine residues 101 and 108 within the fourth α helix of IL-15 were mutated to aspartic acid via site-directed and PCR-assisted mutagenesis. This mutant IL-15 was then genetically linked to the constant region of murine IgG2a and cloned into an expressing vector. NS-1 cells (American Type Culture Collection, Manassas, VA) or, alternatively, CHO-K1 cells (DMSZ, Braunschweig, Germany) were stably transfected with a plasmid carrying the construct encoding the CRB-15 fusion protein (30). The transfectant cells were cloned and cultured in serum-free Ultraculture medium (BioWhittaker, Walkersville, MD) containing 100 μg/ml zeocin (Invitrogen Life Technologies, San Diego, CA). CRB-15 protein in the culture supernatant was purified by protein A affinity chromatography and, in some instances, ion exchange chromatography.

Induction of CIA

Male DBA/1 mice (The Jackson Laboratory, Bar Harbor, ME), 8–9 wk of age, were used in all experiments. CII derived from chicken sternal cartilage (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.1 M acetic acid at 2 mg/ml at 4°C overnight. The CII solution was then emulsified with an equal volume of CFA (1 mg/ml *Mycobacterium tuberculosis* (H37Ra, American Type Culture Collection 25177; Sigma-Aldrich) as previously described (32). To induce CIA, 200 μg of CII was injected intradermally at the base of the tail of DBA/1 mice. Twenty-one days after immunization, the animals were challenged with 200 μg of CII *i.p.* in PBS.

Treatment of animals

After immunization and subsequent challenge with CII, animals were divided into two groups: one group received a daily *i.p.* injection of IgG2a (BD Biosciences, San Jose, CA) at 1.5 μg/injection/day. The other group received a daily *i.p.* injection of CRB-15 (1.5 μg/injection/day). The data

shown are for a total of 38 CRB-15-treated and control-treated animals, respectively, and summarize five independent experiments. Treatment was given at the time of CII challenge and was continued for 10 consecutive days; no treatment was given thereafter. Mice were evaluated every day for signs of arthritis based on the following criteria: grade 0, normal joints and no swelling; grade 1, mild swelling and/or erythema; grade 2, pronounced edema or redness of the paw or several digits; and grade 3, severe swelling of entire paw and/or ankylosis. As each limb was individually graded, the maximal clinical score for each mouse was 12. Animals from each group were killed at different time points after the onset of arthritis (days 4, 7, 11, and 21) for histological and molecular analysis (*n* = 12). The remaining animals were followed until 42 days postchallenge.

Treatment of animals with established arthritis

DBA/1 mice were immunized and challenged with CII as described above and monitored every day for signs of arthritis based on the following criteria: grade 0, normal joints and no swelling; grade 1, mild swelling and/or erythema; grade 2, pronounced edema or redness of the paw or several digits; grade 3, severe swelling of entire paw; and grade 4, ankylosis. When animals developed overt arthritis with a minimal clinical score of 2, they were randomly assigned to treatment groups and given either IgG2a (5 μg/injection/day) or CRB-15 (5 μg/injection/day) for 14 consecutive days. Only animals developing arthritis were included in the analysis. On day 120 after the initial challenge, the paws of the animals were prepared for histological evaluation. In some instances DBA/1 mice were immunized with 100 μl of solution containing 100 μg of bovine CII in CFA containing 1 mg/ml *M. tuberculosis*, injected intradermally at the base of the tail. On day 21, all the animals received a boost immunization of 100 μl of solution containing 100 μg of CII in IFA, again intradermally at the base of the tail. Mice were subsequently scored daily for signs of arthritis. Similar results were obtained with this immunization protocol (data not shown).

Histology

Paws were removed postmortem, fixed in 1% paraformaldehyde for 3 days, and decalcified in 5% EDTA for 2 wk before being embedded in paraffin for sectioning. Serial sagittal sections were made, mounted on glass slides, and stained with H&E. Tissue sections were analyzed by light microscopy and graded blindly. For the quantification of joint inflammation and destruction in animals with established disease, joints were removed on day 120 after initial immunization, fixed, decalcified, and paraffin-embedded as described. Histopathologic changes in the joints were evaluated on five serial sections through the entire joint, spaced 70 μm apart. Tissue sections (5 μm thick) were stained with H&E. Synovial membrane thickness was scored from 0–4 (0 = normal thickness, 1 = minimal inflammatory infiltration, 2 = synovial thickness less than the depth of the epiphysis, 3 = equal, and 4 = more). Cartilage destruction was separately graded on the basis of chondrocyte degeneration, ruffling of cartilage surface, and/or dystrophic cartilage on a scale from 0 (normal cartilage) to 4 (complete loss of the articular cartilage). Bone erosion was scored on a scale of 0–4, ranging from no abnormalities to complete loss of cortical and trabecular bone (0 = no erosion, 1 = erosion <25% of the articular surface, 2 = 26–50%, 3 = 51–75%, and 4 = >75%). The joints studied were digits (front and hind paws), wrists, elbows, knees, and ankles. In some cases shoulders were also included. For each complex joint (digits, ankle, and wrist), a progression score was used, based on the number of joints (per complex joint) that showed disease: 0 = no joint involved; 1 = one joint involved; 2 = two joints involved; and 3, more than three joints involved. The progression score was used as a factor to calculate the total score per complex joint as follows: total score = progression score × histological score in the complex joint. The scores were used to compare treatment/control groups (maximum score = 12).

Immunohistology

The following rat anti-mouse mAbs and isotype-matched control mAbs were purchased from BD Pharmingen (San Diego, CA): anti-CD4 (H129.19), anti-CD8 (53-6.7), and anti-CD11b (M1/70). The mAbs against IL-1β (MAB401) and TNF-α (MP6-XT22) were obtained from R&D Systems (Minneapolis, MN). Cryostat sections of paws (four per group) were fixed in paraformaldehyde-lysine-periodate for analysis of leukocyte Ags or in acetone for localization of cytokines as previously described (33). Sections were briefly counterstained in hematoxylin and mounted for examination. Isotype-matched mAbs and control Abs were analyzed for endogenous peroxidase activity in each experiment. The specificity of cytokine labeling was controlled by overnight mAb absorption with cytokines (IL-1β and TNF-α) using Ag-coated ELISA wells (4°C; 1–5 μg/ml) before immunohistologic labeling. Sections were evaluated in a blinded manner, and the numbers of cytokine-positive cells in 10 consecutive high power

fields were enumerated, with data expressed as labeled cells per high power field (mean $\pm$ SD).

RNA isolation and RT-PCR

Paws of animals were collected for RNA analysis on days 3, 6, 9, 12, 15, 18, and 21 after initial disease development (eight per time point). Skin, muscle tissue, and other nonjoint bone materials were removed, and the samples were frozen in liquid nitrogen and stored at -80°C . Tissue samples were homogenized with a Polytron (KINEMATIKA, Lucerne, Switzerland), and total cellular RNA was extracted using RNeasy 60 (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. RNA was then quality-controlled for traces of chromosomal DNA by PCR analysis, and the concentrations of the RNA samples were determined with a spectrophotometer (DU 640; Beckman Coulter, Fullerton, CA). Two micrograms of RNA was reverse transcribed and quality controlled for the expression of the housekeeping gene GAPDH or, alternatively, cyclophilin (34). Subsequently, the relative abundance of inflammatory cytokines (IL-1 β , IL-6, IL-17, and TNF- α) was determined by TaqMan real-time PCR analysis with the ABI 7000 sequence detection instrument (Applied Biosystems, Foster City, CA) and normalized against the housekeeping gene cyclophilin. Primers and probes for IL-1 β , IL-6, and TNF- α were purchased from Applied Biosystems. Primers for IL-17, cyclophilin, and GAPDH were: IL-17 forward, GAAAGCTGGACCACCATGA; IL-17 reverse, GCTCTCAG GCTCCCTCTTCA; IL-17 probe, TTCTGTTCTCATCCAGCAAGAGATC CTGGT; cyclophilin forward, GCCTGGATGCTAACAGAAGGA; cyclophilin reverse, GTTCATCCCCTCGCTATGGT; cyclophilin probe, ATGACAAGGATGCCGGCAAGTGT; GAPDH forward, TGCACCAC CAACTGCTTAGC; GAPDH reverse, CCCTTCCACAATGCCAAAGT; and GAPDH probe, CCCCTGGCCAAGTCTATCCATG.

In vitro T cell proliferation assay

Peripheral lymph node cells (PLN) from DBA/1 mice immunized and challenged with CII and treated with IgG2a (1.5 $\mu\text{g}/\text{day}$) or CRB-15 (1.5 $\mu\text{g}/\text{day}$) for 10 days were removed, and single-cell suspensions were prepared. PLN from nonimmunized DBA/1 mice were used as controls. Cells were plated at 1×10^6 cells/well in U-bottom, 96-well plates in RPMI 1640, supplemented with 10% FCS, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 1% glutamine (BioWhittaker), and 2-ME (5×10^{-5} M; Sigma-Aldrich). Cells were incubated at 37°C for 84 h in plates coated with 100 μg of CII, followed by addition of 1 μCi of [^3H]TdR for an additional 16 h. Cells were then harvested and [^3H]TdR incorporation per well was measured and averaged for each triplicate.

CFSE labeling and analysis of T cell proliferation in vivo

Twenty-one days after immunization with CII, spleen cells from DBA/1 mice were harvested, and cell suspensions were prepared in HBSS (Bio-Whittaker). Hypotonic shock was used to lyse RBC, and mononuclear leukocytes were washed, resuspended in HBSS, and labeled with fluorochrome CFSE (Molecular Probes, Eugene, OR) at a final concentration of 5 nM, as described previously (35). Recipient DBA/1 mice were lethally irradiated (1000 rad; GammaCell irradiator; Ontario, Canada) before injection of the CFSE-labeled cells (30×10^6 cells/recipient) via the lateral tail vein. The same day, recipient mice received a boost of CII (an i.p. injection of 200 μg of CII in PBS) and daily treatment with 1.5 μg of control IgG2a or CRB-15 for 8 days. Adoptive transfer using naive host and recipient mice was also performed as a control. On day 8 recipient spleen cells were removed, and cell suspensions were processed as before.

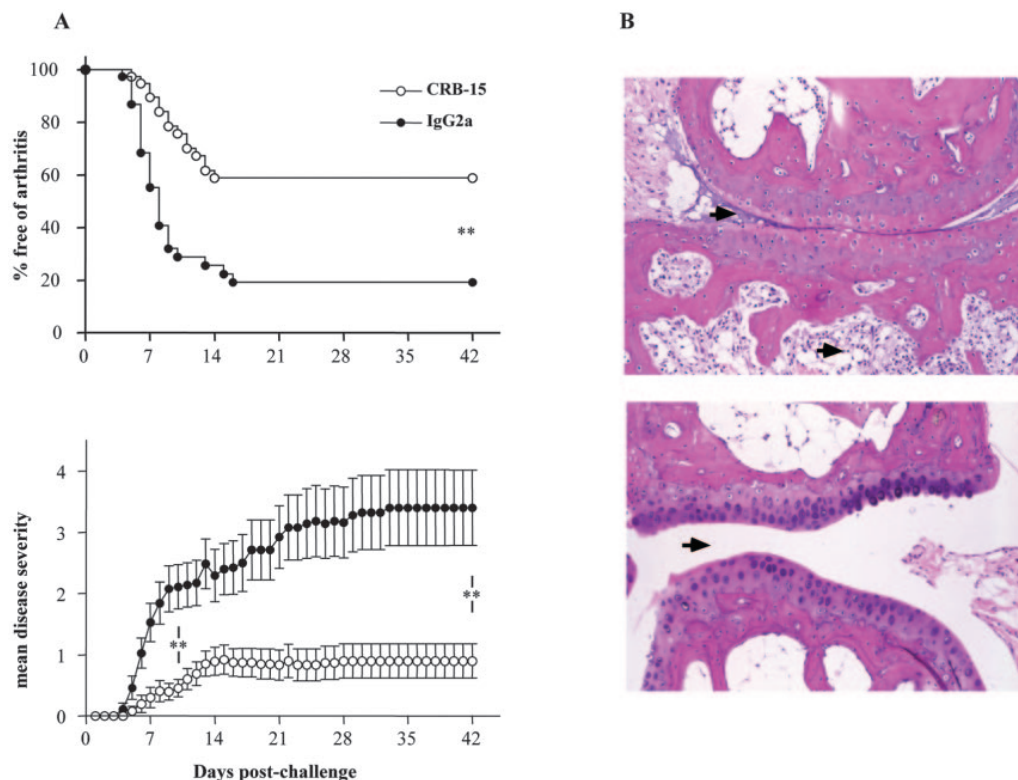


FIGURE 1. A, Short term treatment with CRB-15 decreases the incidence and severity of CIA. Mice were immunized with CII and after 21 days were challenged by i.p. readministration of CII. On the day of challenge, mice were randomly divided into two groups and were treated with either CRB-15 (1.5 $\mu\text{g}/\text{mouse}/\text{day}$; $\circ$) or control IgG2a (1.5 $\mu\text{g}/\text{mouse}/\text{day}$; $\bullet$) for a total of 10 days. Both the incidence (upper panel) and severity (lower panel; maximum score per animal, 12) of disease were monitored during treatment and for a period of 32 days after discontinuation of treatment. Treatment with CRB-15 prevents disease development and progression not only during treatment, but throughout the observation period (**, $p < 0.01$). The results shown are for a total of 38 CRB-15- and control-treated animals, respectively, and summarize five independent experiments. Mean disease severity scores $\pm$ SEM are given. B, CRB-15 treatment reduces articular inflammation and joint destruction. After development of the first symptoms of arthritis, joints from CRB-15- and IgG2a-treated animals ($n = 6$) were removed, formalin-fixed, and decalcified, and H&E stains were prepared. Representative sections of ankles from paws with a disease score of 2 on day 7 after disease onset are shown (magnification, $\times 40$). Whereas IgG2a-treated animals show severe joint cartilage erosion and joint space narrowing (upper panel), joints from CRB-15-treated animals show an essentially normal morphology (lower panel).

Cells were stained with anti-CD4-PE-conjugate (2 μ g/ml) or anti-CD8-PE-conjugate (2 μ g/ml; BD Pharmingen) for 30 min at 4°C. After staining, cells were washed once and resuspended in 0.5 ml of HBSS for analysis by flow cytometry using a FACS-Sort and CellQuest software (BD Biosciences, Mountain View, CA). Live events were collected and analyzed by gating onto CD4⁺CFSE⁺ or CD8⁺CFSE⁺ cells.

Calculation of the frequency of proliferating CD4⁺ T cells

Analysis of CD4⁺ T cell proliferation in response to Ag stimulation was performed according to Noorchashm et al. (36). With each round of cell division, the CFSE dye is divided equally between daughter cells. Using the FACS acquisition software (CellQuest), the total number of cells in each generation of proliferation can be established, and an estimate of the number of precursors that generated the daughter cells was determined using the following formula: $y/2^n$ (where y is the absolute number of cells in each peak, and n is the number of cell divisions). Calculation of the frequency of proliferating T cells was then analyzed by dividing the total number of precursors by the total CFSE-labeled cells under each peak.

Statistics

The incidence of arthritis (percentage) was analyzed using a Kaplan-Meier cumulative plot for the cumulative percentage free of arthritis and comparison between groups was performed using a log rank test for event time (Fig. 1A, upper panel). The disease severity of arthritis as well as the histological scores (Fig. 1A, lower panel, and Fig. 3) were analyzed using a two-tailed nonparametric Mann-Whitney U test. A value of $p < 0.05$ was defined as significant (StatView 5.1 (SAS Institute, Cary, NC) and PRISM 3.02 (GraphPad, San Diego, CA)). For gene expression analysis, cDNA samples were analyzed in triplicate by TaqMan real-time PCR. The automatic baseline was determined by the ABI 7000 sequence detection instrument, followed by manual quality control. The normalized calculated mean values for each sample were imported into the GraphPad PRISM software for statistical valuation, and linear regression analyses as well as two-tailed Pearson correlation analyses were performed.

Results

Treatment with CRB-15 decreased the incidence and severity of CIA

After initial immunization and subsequent challenge with CII 21 days later, development of a severe progressive form of arthritis was observed in a majority (81%) of the IgG2a-treated DBA/1 mice (Fig. 1A). The joints affected exhibited prominent swelling and edema, eventually resulting in restricted mobility and ankylosis. Histologically, the synovial membrane was heavily infiltrated with mononuclear leukocytes (see, e.g., Fig. 2); joint cartilage was severely eroded, and joint space narrowing, leading to direct bone apposition, was observed (Fig. 1B, upper panel). Treatment with 1.5 μ g of CRB-15 for 10 consecutive days and starting on the day of challenge with CII resulted in a marked decrease in the incidence of arthritis, with 59% of the treated mice free of clinical arthritis at the end of the observation period compared with only 19% of control IgG2a-treated mice ($p < 0.01$; Fig. 1A, upper panel). Also, the severity of arthritis was attenuated in mice treated with CRB-15 compared with that in the controls (Fig. 1A, lower panel). Even though treatment with CRB-15 was discontinued after 10 days, no relapse was observed in mice receiving the compound, and both disease incidence and severity remained low throughout the observation period (Fig. 1A). Interestingly, the disease progression and severity in CRB-15-treated animals that did develop disease (15 of 38 animals) were also significantly lower than those in control treated animals with disease, both at the end of treatment on day 10 (disease severity: CRB-15, 1.19 ± 0.27 ; IgG2a, 2.81 ± 0.39 ; $p < 0.01$) and at the end of the observation period on day 42 (disease severity: CRB-15, 3.00 ± 0.41 ; IgG2a, 5.00 ± 0.58 ; $p < 0.05$). Histological examination of CRB-15-treated animals confirmed these findings and revealed a strong reduction in articular inflammation and destruction. This was true even in animals that developed symptoms of disease (Fig. 1B, lower panel), and compared with the control-treated animals the

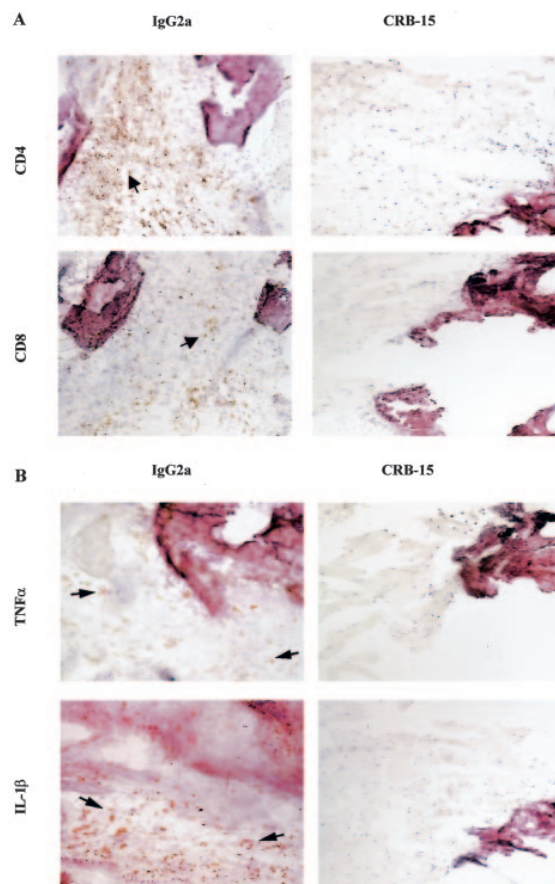


FIGURE 2. A, CRB-15 treatment reduces T cell infiltration into the joints. After development of the first symptoms of arthritis, joints from CRB-15- and IgG2a-treated animals ($n = 4$ /group) were removed, and immunohistologic stains were conducted on hematoxylin-counterstained cryostat sections. Representative sections of ankles from paws with a disease score of 2 on day 7 after disease onset are shown (magnification, $\times 100$). Animals treated with control IgG2a show a dense periarticular infiltrate containing CD4⁺ and CD8⁺ T cells. In contrast, CRB-15-treated animals present only rare periarticular CD4⁺ T cells and no CD8⁺ T cells. Large purplish areas are articular cartilage and bone fragments. B, CRB-15 treatment reduces articular IL-1 β and TNF- α protein expression after CIA induction. Representative sections of joints on day 7 after development of disease symptoms ($n = 4$ /group) are shown (magnification, $\times 100$). Serial sections show marked joint disruption, tissue infiltration by various mononuclear cells, and TNF- α (upper panel) as well as IL-1 β (lower panel) protein expression in mice treated with IgG2a. Animals receiving CRB-15 show almost normal histology, without mononuclear cell infiltration or expression of TNF- α or IL-1 β . Large purplish areas are articular cartilage and bone fragments. Arrows indicate positive cell staining for TNF- α and IL-1 β .

overall appearance of the joints on day 7 after disease induction was normal. Immunohistology also showed that infiltration by CD4⁺ and CD8⁺ T cells (Fig. 2A) was significantly reduced in mice treated with CRB-15 (Table I), as was the extent of cellular IL-1 β and TNF- α cytokine expression (Fig. 2B and Table I), compared with controls. Similar beneficial effects of CRB-15 vs control therapy were obtained throughout the first 3 wk after disease onset (data not shown).

Therapeutic effect of CRB-15 treatment on ongoing CIA

To determine the efficacy of CRB-15 on disease progression in animals with already established arthritis, treatment of immunized

Table I. Analysis of cell infiltration and cytokine expression in joints of CIA mice at day 7 of disease^a

	Control IgG2a	CRB-15	p
CD4 ⁺	219 ± 64	9 ± 7	<0.0001
CD8 ⁺	95 ± 48	3 ± 2	<0.0001
TNF-α ⁺	64 ± 23	3 ± 3	<0.0001
IL-1β ⁺	142 ± 69	2 ± 1	<0.0001

^a Immunohistology was performed on the joints of control IgG2a- and CRB-15-treated mice (n = 4/group) 7 days after first signs of disease were detected, as described in *Materials and Methods*. Sections were evaluated in a blinded manner, and the numbers of labeled cells in 10 consecutive high power fields were counted and expressed as cells per high power field (mean ± SD). Statistical evaluation was performed by unpaired *t* test; data are representative of three independent experiments.

mice was delayed until mice developed overt arthritis with a mean disease severity of at least 2 (mean disease severity, 3.1 ± 0.2 and 2.8 ± 0.3, respectively, for CRB-15 and IgG2a groups), and mice then received either control IgG2a (5 μg/day) or CRB-15 (5 μg/day) i.p. for 14 days. As shown in Fig. 3A, treatment with control IgG2a did not affect the progression of arthritis. In striking contrast, treatment with CRB-15 displayed a strong therapeutic effect, with a complete halt of disease progression during the course of treatment and reduced disease progression for a prolonged period after discontinuation of CRB-15 administration. This therapeutic effect was highly significant both at the end of the treatment window (day 14; n = 7; p < 0.01) and 5 wk after discontinuation of treatment (day 49; n = 5, p < 0.01). Histological examination and quantification of intra-articular disease 120 days after the initial immunization revealed that CRB-15 treatment led to a statistically significant reduction in synovial inflammation and bone and cartilage destruction (Fig. 3B). This therapeutic effect was realized despite the aggressive nature of the CIA model, in which synovial infiltration and bone erosion were observed 3 days after the disease was initially diagnosed (data not shown). Of note this disease model also involved strong osteoclastic activity (data not shown). Hence, treatment with CRB-15 is remarkably effective in both the prevention and the treatment of established arthritis in the CIA model.

Treatment with CRB-15 inhibited the expression of proinflammatory cytokines in joints of animals with established disease

In view of the finding that CRB-15 treatment reduced intra-articular TNF-α and IL-1β expression in the prophylactic treatment protocol (Fig. 2) and to further characterize the effects of targeting the IL-15R with CRB-15 on arthritis disease progression, we analyzed the effects of CRB-15 treatment on the *in situ* gene expression of the cytokines TNF-α, IL-1β, IL-6, and IL-17 in the paws of animals in which treatment was initiated only after the animals had developed overt arthritis. To do so, RNA was prepared from paws of mice at different time points after disease onset and initiation of treatment, and the cytokine expression profiles were analyzed by real-time PCR. Sampling time points ranged from days 3–21 after disease onset (n = 8 paws/time point; n = 56 total). As shown in Fig. 4A, the expression of the cytokines IL-1β, IL-6, and IL-17 was elevated in the joints of mice developing disease. Whereas we were not able to detect any temporal correlation in the cytokine expression patterns during the first 3 wk after disease onset (data not shown), we did observe a statistically significant linear correlation between disease severity in individual paws and cytokine expression levels in both the CRB-15 and control treatment groups. Given the overall reduced disease severity in the CRB-15-treated animals (see

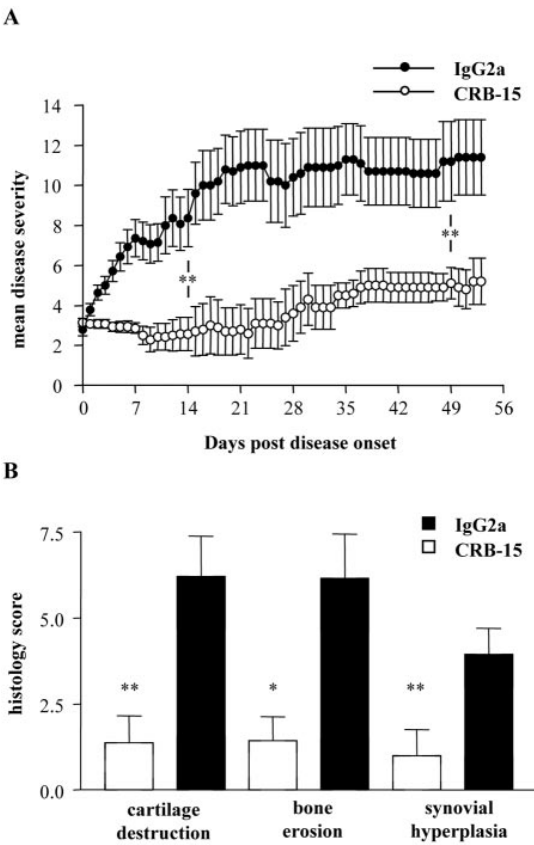


FIGURE 3. A, CRB-15 is effective in preventing disease progression in mice with established arthritis. After intradermal immunization and i.p. challenge with CII, mice were monitored daily for arthritis development. Treatment with either control IgG2a (5 μg/mouse/day; ●), or CRB-15 (5 μg/mouse/day; ○) was initiated as soon as a disease severity of at least 2 was scored in individual animals. The treatment was discontinued after 14 days, and disease progression was further monitored. Mean disease severity scores ± SEM are given. Treatment with CRB-15 prevents disease progression during the treatment period (n = 7; **, p < 0.01 on day 14). Interestingly, disease progression is reduced also after discontinuation of treatment, suggesting a long term effect of CRB-15 treatment (n = 5; **, p < 0.01 on day 49). Using a different immunization protocol involving intradermal immunization with CII and intradermal challenge with CII (compared with a challenge i.p.), very similar results were obtained (data not shown). The results shown are representative of at least three independent experiments. The maximum score per animal is 16 (see *Materials and Methods*). B, CRB-15 significantly reduces long term articular inflammation and destruction. H&E-stained paraffin sections from the joints of CRB-15- and IgG2a-treated animals in A were prepared 120 days after the initial immunization and were evaluated for synovial inflammation as well as bone and cartilage destruction. Quantification of the findings was performed as described in *Materials and Methods*. A 14-day course of treatment leads to a significant reduction of articular inflammation and joint destruction (n = 5 animals; *, p < 0.05; **, p < 0.01). Mean disease severity scores ± SEM are given.

Fig. 3A), we therefore observed an overall reduced expression of the inflammatory cytokines IL-1β, IL-6, and IL-17 in the CRB-15-treated animals. In paws of CRB-15-treated animals that did develop disease symptoms, the expression of these inflammatory cytokines was similar to that in controls. Intriguingly, a different expression pattern was observed for TNF-α. Although TNF-α expression correlated with disease severity in the paws of control-treated animals and was elevated

Downloaded from <http://journals.aai.org/immunol/article-pdf/173/9/5818/191232/5818.pdf> by guest on 06 September 2023

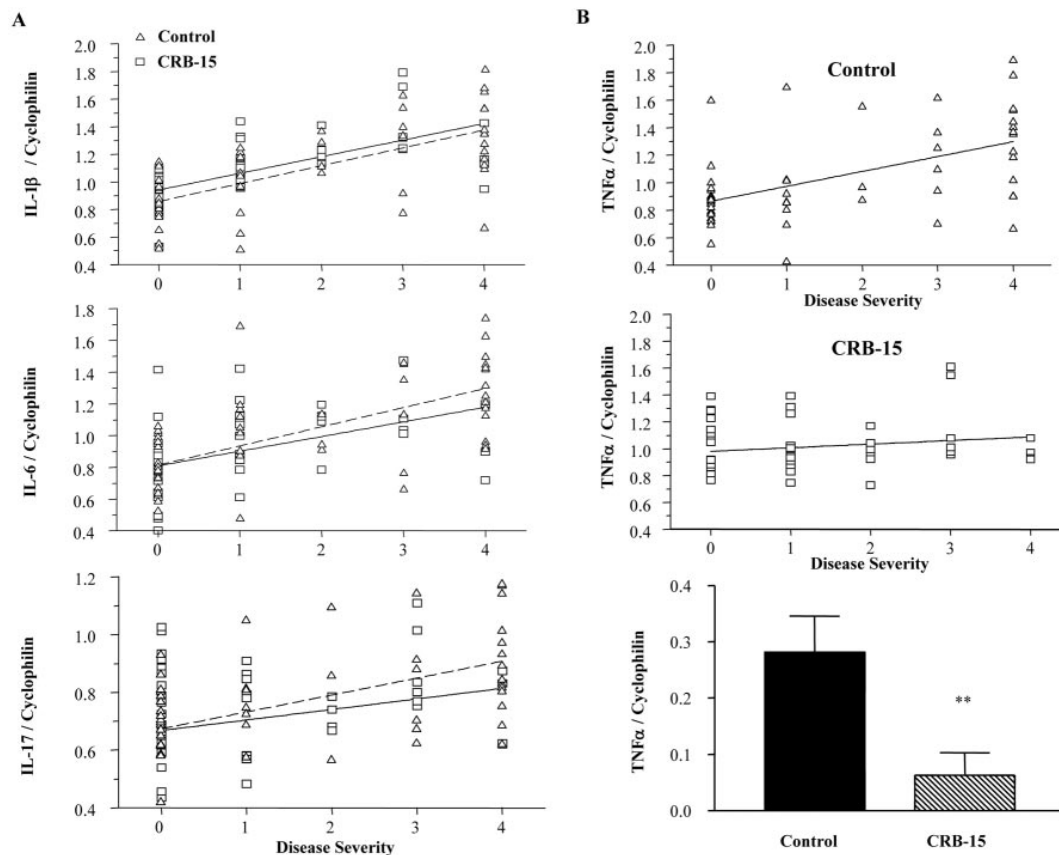


FIGURE 4. A, The articular expression of the inflammatory cytokines IL-1 β , IL-6, and IL-17 correlates with disease severity. RNA was prepared from paws of CRB-15- or control-treated arthritic mice at different time points during the treatment and until 7 days after discontinuation of treatment ($n = 8$ paws/time point; $n = 56$ total). Expression levels of IL-1 β , IL-6, and IL-17 were determined by RT and real-time PCR analysis and normalized against the housekeeping gene cyclophilin. The expression levels in individual legs are plotted against disease severity in those legs (maximum score per leg, 4). A statistically significant correlation between the expression levels of the inflammatory cytokines IL-1 β , IL-6, and IL-17 and disease severity is observed in both control (dotted line) and CRB-15-treated (solid line) animals (IL-1 β : $p = 0.02$; $r^2 = 0.87$; IL-6: $p = 0.01$; $r^2 = 0.91$; IL-17: $p = 0.004$; $r^2 = 0.96$). B, The articular expression of TNF- α correlates with disease severity in control animals, but is reduced in CRB-15-treated mice. Joints from CRB-15- or control-treated animals were analyzed for TNF- α mRNA expression as described in A, and the results were plotted against disease severity. The expression of the inflammatory cytokine TNF- α correlates well with disease severity in control-treated animals and is elevated in joints that develop disease ($p = 0.004$; $r^2 = 0.96$; top panel). In contrast, no correlation between disease severity and TNF- α expression is observed in CRB-15-treated animals (middle panel). Rather, TNF- α expression levels remain at the basal levels observed in legs with no apparent inflammation, even in animals with severe disease (slope_{lin, regr.} = 0.02 ± 0.02 ; $p = \text{NS}$), suggesting that CRB-15 treatment has a direct effect on TNF- α expression levels in the joints of treated animals. A direct comparison of the TNF- α expression levels in the paws of the animals with apparent symptoms of disease (paws with disease scores of 1–4), from either control- or CRB-15-treated animals, shows that TNF- α expression is strongly reduced in the CRB-15-treated animals (bottom panel; $p = 0.006$).

in the paws of control-treated animals, we found that in CRB-15-treated animals, the expression of TNF- α was reduced to the level seen in paws with no apparent disease, even in legs with severe arthritis (Fig. 4B), suggesting that a functional IL-15R may be required for TNF- α cytokine expression in the joints.

Effect of CRB-15 on immunological responses

Given the well-established function of IL-15 as a T cell activation and survival factor, the effects of targeting the IL-15R with CRB-15 on T cell responses to CII were analyzed both in vitro and in vivo. In a first set of experiments, peripheral lymph node cells from DBA/1 mice immunized and challenged with CII and treated with either CRB-15 (1.5 $\mu\text{g}/\text{day}$) or control IgG2a (1.5 $\mu\text{g}/\text{day}$) for 10 days were isolated and cultured in the presence of CII. As shown in Fig. 5, T cells from IgG2a-treated animals proliferated well in response to CII in vitro. In contrast, the proliferation of cells from CRB-15-treated animals was significantly reduced. Fur-

thermore, using an adoptive cell transfer system, splenocytes from DBA/1 mice immunized with CII were isolated, labeled with the long-lived fluorescent dye CFSE, and reinjected into lethally irradiated, CII-immunized, DBA/1 recipient mice. After 8 days, splenocytes were isolated from these recipient mice, and CD4 $^{+}$ and CD8 $^{+}$ T cells were monitored for their anticollagen proliferative response by FACS analysis. We observed that mice treated with CRB-15 displayed a decrease in the frequency of proliferating primed CD4 $^{+}$ and CD8 $^{+}$ T cells compared with control IgG2a-treated mice or untreated mice (Fig. 6). Although the responder levels observed in this type of in vivo assay system are generally very high, the elevated frequencies seen in this experiment are also supported by the fact that this experiment monitors a secondary recall response. Nevertheless, a significant reduction of the T cell response to type II collagen after treating CII-immunized mice with CRB-15 was observed both in vitro and in vivo. Hence, treatment with CRB-15 inhibits the development and progression of

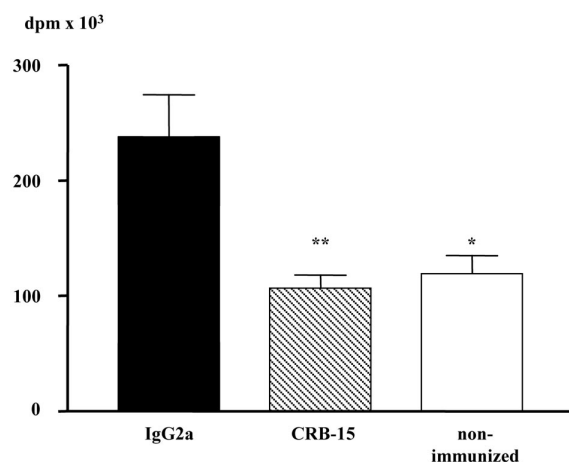


FIGURE 5. CRB-15 treatment reduces the proliferation of PLN in vitro. PLN from DBA/1 mice immunized and challenged with CII and treated with either CRB-15 (1.5 μ g/day) or control IgG2a (1.5 μ g/day) for 10 days were isolated, cultured in the presence of 100 μ g/well CII for 84 h, and then pulsed with [³H]thymidine for 16 h. Radioactivity (dpm $\times$ 10³) incorporated into proliferating cells from IgG2a- and CRB-15-treated, immunized animals is shown and plotted against PLN from nonimmunized, collagen-naïve animals. Cells from IgG2a-treated, immune animals proliferated well in response to CII in vitro (*, p = 0.01 vs naïve PLN). In contrast, the proliferation of cells from CRB-15-treated immune animals was strongly reduced (**, p = 0.005 vs IgG2a-treated PLN).

CIA and blunts the responder frequency of proliferating CD4⁺ and CD8⁺ T cells in the periphery.

Discussion

The proinflammatory and antiapoptotic cytokine IL-15 emerges as a factor that may play a multifaceted role in chronic inflammatory lesions, such as in RA. Not only does IL-15 stimulate the activation, proliferation, survival, and effector functions of a variety of immune cells (14, 16–19), but IL-15 is also a potent survival and proliferation-inducing factor for activated epitheloid cells expressing the IL-15R, such as synovial fibroblasts. In fact, we have recently shown that IL-15 stimulates the proliferation of primary human fibroblast-like synoviocytes expressing the IL-15R and also affects apoptosis in these cells by up-regulating Bcl-2 and Bcl-x_L (29). This suggests a function for IL-15 at different stages of RA disease progression. As TNF- α production in synovial macrophages is stimulated by T cells in an IL-15-dependent manner (24), and IL-15 also stimulates IL-17 production (26), IL-15 emerges as a cytokine playing a central and upstream role in the inflammatory cytokine cascade contributing to RA disease development. In addition, the findings that IL-15 is expressed by activated synovial macrophages and fibroblasts (21, 37) and that these cells, in addition to T cells, express the IL-15R and are responsive to IL-15 (24, 29), suggest that IL-15 may, in fact, act in an autocrine or paracrine fashion to sustain their continued survival and proliferation. IL-15 may therefore not only play a role in the development of RA, but may also be important for the progression and maintenance of the chronic inflammatory lesion. In view of this, it seemed of potential therapeutic relevance to investigate the effects of targeting the IL-15R and IL-15R⁺ cells on RA disease progression.

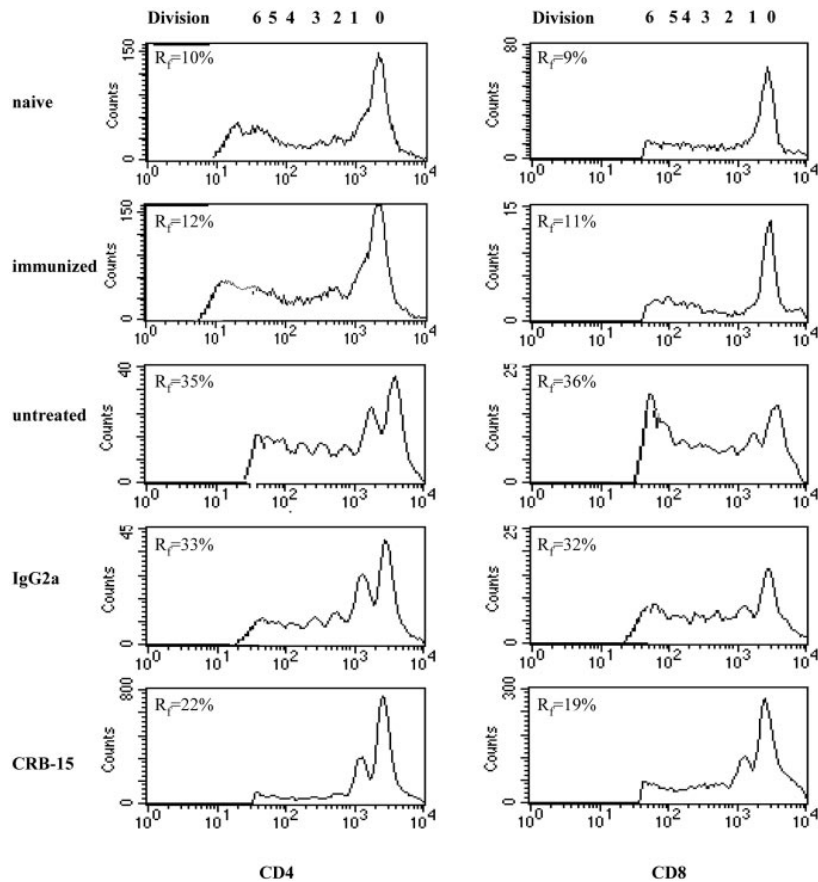
In the present report we have demonstrated that treatment with CRB-15 can block arthritis development and progression of ongoing disease in the murine model of CIA. Moreover, we have observed that targeting the IL-15R and IL-15R⁺ cells with CRB-15 produced a long-lived therapeutic effect, and there were

no relapses after discontinuation of treatment throughout the observation period in both a disease induction and an established disease model. Both the observed efficacy during treatment with CRB-15 and the long term beneficial effect seen after discontinuation of treatment are in line with a central role of the IL-15/IL-15R pathway not only in the inflammatory cytokine cascade, but also in sustaining a chronic inflammatory response, by supporting the survival and propagation of effector cells involved in lesion formation. We found that treatment with CRB-15 leads to a reduced expression of the inflammatory cytokines IL-1 β , TNF- α , IL-6, and IL-17. The effect on TNF- α is particularly striking, because treatment with CRB-15 strongly reduces expression of this cytokine in both the disease induction and the established disease models. Low levels of TNF- α expression are observed even in animals that do develop strong inflammation, joint destruction, and ankylosis. These results suggest that TNF- α expression in the joints may be dependent on a functional IL-15R pathway in vivo and extend the earlier in vitro findings in human cells (24). These results also suggest that arthritis development in the CIA model is not strictly TNF- α dependent. Indeed, CIA can be induced in TNF- α -deficient animals (38).

In addition to the reduction of inflammatory cytokine expression, we observed both a reduced T cell responsiveness to CII in vitro and in vivo and a reduced lymphocytic infiltration of the joints in CRB-15-treated animals. These results are consistent with previously reported data supporting a role for IL-15 in various aspects of T cell function (8, 10, 20, 30). Overall, treatment with CRB-15 led to a reduction in synovial inflammation, bone resorption, and cartilage destruction for prolonged periods of time also after discontinuation of therapy. The long term therapeutic effects seen in our studies may be attributed to a breaking of the positive feedback loop of cellular activation and inflammatory cytokine production, but may also be due to the use of a protein, CRB-15 (IL-15 mutant/Fc γ 2a), with not only antagonist properties, but also cytotoxic potential for cells bearing the IL-15R. Because the Fc γ 2a part of CRB-15 bears Fc sequences that support complement activation and fixation and the activation of FcR-bearing phagocytes, treatment may, in addition to creating receptor site blockade, destroy a portion of the IL-15R⁺ activated mononuclear leukocytes and synoviocytes in the inflamed joints. This would lead to a depletion of effector cells involved in disease progression, an effect that may be sustained after discontinuation of treatment.

In an earlier study, Ruchatz et al. (39) used a recombinant soluble IL-15R α subunit (sIL-15R α) to antagonize IL-15 and showed that IL-15 inhibition is effective in preventing disease development in the CIA model during the time window of treatment. During this period, inhibition of IL-15 prevented joint inflammation and destruction and reduced T cell and humoral responses against collagen. Our results, obtained by targeting the IL-15R, rather than IL-15, are consistent with these findings. This includes the effects of IL-15R blockade on T cell and humoral responses, because in our studies we observed a linear correlation between autoreactive anti-mouse collagen IgG2a Ab titers and disease severity in arthritic mice during treatment and up to 14 days after discontinuation of treatment (data not shown). We extended these results by showing that CRB-15 treatment leads to a reduction of expression of the inflammatory cytokines IL-1 β , TNF- α , IL-6, and IL-17, and that TNF- α expression in the inflamed joints may be dependent on a functional IL-15R. We furthermore demonstrated that CRB-15 is effective in preventing disease progression in established ongoing arthritis. These new findings are of particular relevance with respect to the potential utility of inhibiting the IL-15/IL-15R signaling pathway in human therapy, because they underscore the efficacy of IL-15R blockade in established disease,

FIGURE 6. Treatment with CRB-15 reduces the proliferative response of CD4⁺ and CD8⁺ T cells to CII in vivo. Twenty-one days after immunization with CII, splenic cells from DBA/1 mice were harvested, labeled with the fluorescent dye CFSE, and injected into lethally irradiated DBA/1 mice. The recipient mice received an injection of CII on day 1 and were either left untreated or treated with control IgG2a (1.5 μ g/injection/day) or CRB-15 (1.5 μ g/injection/day) for 8 days. As negative controls, CFSE-labeled splenocytes from nonimmunized DBA/1 mice (naïve) or immunized DBA/1 mice (immunized) were injected into irradiated DBA/1 host mice that did not receive a CII injection. After the end of treatment, splenic cells from the respective DBA/1 recipients were prepared and labeled with a PE-anti-CD4 or PE-anti-CD8 mAb, and the responder frequency of CFSE-labeled CD4⁺ T cells (*left panels*) and CD8⁺ T cells (*right panels*) was analyzed by flow cytometry. The percentage of cells in each individual cell division is presented on *top* of each panel. A strong proliferative response compared with the controls (*top and second rows*) is observed after exposure of collagen-primed lymphocytes to CII in vivo (*third and fourth rows*). The number of proliferating CD4⁺ and CD8⁺ T cells is reduced in CRB-15-treated animals (*bottom row*). The reduction of Ag-reactive CD4⁺ and CD8⁺ T cells in the CRB-15-treated animals may be due to proliferation inhibition, deletion of activated and proliferating cells, or both.



which is more relevant for human disease, and they demonstrate the effects of IL-15R blockade on TNF- α expression in an in vivo system. Interestingly, our findings are quite distinct from this earlier report with respect to disease progression after discontinuation of treatment. Although apparently discontinuation of IL-15 blockade with the sIL-15R α leads to a relapse and development of arthritis (39), we found that blockade of the IL-15R with CRB-15 for a brief period (10 or 14 days, respectively) could induce a stable, arthritis-free state in the disease induction model, and in the case of established ongoing arthritis led to a moderation of disease progression for several weeks after the end of treatment. The reasons for these differences are not resolved, but 1) the pharmacodynamics of antagonizing the IL-15R, rather than neutralizing IL-15, may be quite distinct; and 2) CRB-15 and sIL-15R α are likely to have different pharmacokinetic properties. In addition, CRB-15, through its Fc terminus, has the capacity to target macrophages, synovio-cytes, and T cells in the inflammatory lesion for destruction by complement or phagocytosis, an effect that would not be achieved with an IL-15-neutralizing agent. Again, these differences are highly relevant and have a clear impact on the design of potential therapeutics targeting the IL-15/IL-15R pathway for the treatment of disease in RA patients.

The therapeutic successes of TNF- α antagonists and, more recently, IL-1 antagonists have clearly outlined the importance of targeting inflammatory cytokines for the treatment of RA. At the same time, anti-TNF therapies that are effective in the majority, but by far not all, RA patients have suggested that other cytokines may contribute to RA disease progression. Our data support the idea that targeting of the IL-15R might be an attractive additional strategy for the treatment of RA.

Acknowledgments

We are grateful to S. L. Ferrari for his expertise and advice in statistical analysis.

References

- Choy, E. H. S., and G. S. Panayi. 2001. Cytokine pathways and joint inflammation in rheumatoid arthritis. *N. Engl. J. Med.* 344:907.
- Moreland, L. W., S. W. Baumgartner, M. H. Schiff, E. A. Tindall, R. M. Fleischmann, A. L. Weaver, R. E. Ettlinger, S. Cohen, W. J. Koopman, K. Mohler, et al. 1997. Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. *N. Engl. J. Med.* 337:141.
- Moreland, L. W., M. H. Schiff, S. W. Baumgartner, E. A. Tindall, R. M. Fleischmann, K. J. Bulpitt, A. L. Weaver, E. C. Keystone, D. E. Furst, P. J. Mease, et al. 1999. Etanercept therapy in rheumatoid arthritis: a randomized, controlled trial. *Ann. Intern. Med.* 130:478.
- Maini, R. N., F. C. Breedveld, J. R. Kalden, J. S. Smolen, D. Davis, J. D. Macfarlane, C. Antoni, B. Leeb, M. J. Elliott, J. N. Woody, et al. 1998. Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor α monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. *Arthritis Rheum.* 41:1552.
- Maini, R., E. W. St. Clair, F. Breedveld, D. Furst, J. Kalden, M. Weisman, J. Smolen, P. Emery, G. Harriman, M. Feldmann, et al. 1999. Infliximab (chimeric anti-tumor necrosis factor α monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomized phase III trial. ATTRACT Study Group. *Lancet* 354:1932.
- Jiang, Y., H. K. Genant, I. Watt, M. Cobby, B. Bresnihan, R. Aitchison, and D. McCabe. 2000. A multicenter, double-blind, dose-ranging, randomized, placebo-controlled study of recombinant human interleukin-1 receptor antagonist in patients with rheumatoid arthritis: radiologic progression and correlation of Genant and Larsen scores. *Arthritis Rheum.* 43:1001.
- Nuki, G., B. Bresnihan, M. B. Bear, and D. McCabe. 2002. Long-term safety and maintenance of clinical improvement following treatment with anakinra (recombinant human interleukin-1 receptor antagonist) in patients with rheumatoid arthritis: extension phase of a randomized, double-blind, placebo-controlled trial. *Arthritis Rheum.* 46:2838.
- McInnes, I. B., J. Al-Mughales, M. Field, B. P. Leung, F.-P. Huang, R. Dixon, R. D. Sturrock, P. C. Wilkinson, and F. Y. Liew. 1996. The role of interleukin-15 in T-cell migration and activation in rheumatoid arthritis. *Nat. Med.* 2:175.

9. Grabstein, K. H., J. Eisenann, K. Shanebeck, C. Rauch, S. Srinivasan, V. Fung, C. Beers, J. Richardson, M. A. Schoenborn, M. Ahdieh, et al. 1994. Cloning of a T cell growth factor that interacts with the β chain of the interleukin-2 receptor. *Science* 264:965.
10. Burton, J. D., R. N. Bamford, C. Peters, A. J. Grant, G. Kurys, C. K. Goldman, J. Brennan, E. Roessler, and T. A. Waldmann. 1994. A lymphokine, provisionally designated interleukin T and produced by a human adult T-cell leukemia line, stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. *Proc. Natl. Acad. Sci. USA* 91:4935.
11. Parrish-Novak, J., S. R. Dillon, A. Nelson, A. Hammond, C. Sprecher, J. A. Gross, J. Johnston, K. Madden, W. Xu, J. West, et al. 2000. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature* 408:57.
12. Lodolce, J. P., D. L. Boone, S. Chai, R. E. Swain, T. Dassopoulos, S. Trettin, and A. Ma. 1998. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* 9:669.
13. Kennedy, M. K., M. Glaccum, S. N. Brown, E. A. Butz, J. L. Viney, M. Embers, N. Matsuki, K. Charrier, L. Sedger, C. R. Willis, et al. 2000. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J. Exp. Med.* 191:771.
14. Fehninger, T. A., and M. A. Caligiuri. 2001. Interleukin 15: biology and relevance to human disease. *Blood* 97:14.
15. Doherty, T. M., R. A. Seder, and A. Sher. 1996. Induction and regulation of interleukin-15 expression in murine macrophages. *J. Immunol.* 156:735.
16. Carson, W. E., J. G. Giri, M. J. Lindemann, M. L. Linett, M. Ahdieh, R. Paxton, D. Anderson, J. Eisenmann, K. Grabstein, and M. A. Caligiuri. 1994. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J. Exp. Med.* 180:1395.
17. Alleva, D. G., S. B. Kaser, M. A. Monroy, M. J. Fenton, and D. I. Beller. 1997. IL-15 functions as a potent autocrine regulator of macrophage proinflammatory cytokine production. *J. Immunol.* 159:2941.
18. Kanegane, H., and G. Tosato. 1996. Activation of naive and memory T cells by interleukin-15. *Blood* 88:230.
19. Bulfone-Paus, S., D. Ungureanu, T. Pohl, G. Lindner, R. Paus, R. Rueckert, H. Krause, and U. Kunzendorf. 1997. Interleukin-15 protects from lethal apoptosis in vivo. *Nat. Med.* 3:1124.
20. Li, X. C., G. Demirci, S. Ferrari-Lacraz, C. Groves, A. Coyle, T. R. Malek, and T. B. Strom. 2001. IL-15 and IL-2: a matter of life and death for T cells in vivo. *Nat. Med.* 7:114.
21. Thirkow, E. W., I. M. Van Der Heijden, F. C. Breedveld, T. J. M. Smeets, M. R. Daha, P. M. Kluin, A. E. Meinders, and P. P. Tak. 1997. Increased expression of IL-15 in the synovium of patients with rheumatoid arthritis compared with patients with *Yersinia*-induced arthritis and osteoarthritis. *J. Pathol.* 181:444.
22. Ziolkowska, M., E. Kontny, J. Noworyta, A. Romicka, and W. Maslinski. 1997. IL-15 and proinflammatory cytokines (TNF α , IL-1 β , IL-6) are elevated in serum of rheumatoid arthritis and juvenile arthritis patients. *Immunol. Lett.* 56:410.
23. Wilkinson, P. C., and F. Y. Liew. 1995. Chemoattraction of human blood T lymphocytes by interleukin-15. *J. Exp. Med.* 181:1255.
24. McInnes, I. B., B. P. Leung, R. D. Sturrock, M. Field, and F. Y. Liew. 1997. Interleukin-15 mediates T cell-dependent regulation of tumor necrosis factor- α production in rheumatoid arthritis. *Nat. Med.* 3:189.
25. Oppenheimer-Marks, N., R. I. Brezinschek, M. Mohamadizadeh, R. Vita, and P. E. Lipsky. 1998. Interleukin-15 is produced by endothelial cells and increases the transendothelial migration of T-cells in vitro and in the SCID mouse-human rheumatoid arthritis model in vivo. *J. Clin. Invest.* 101:1261.
26. Ziolkowska, M., A. Koc, G. Luszczykiewicz, K. Ksiezopolska-Pietrzak, E. Klimczak, H. Chwalinska-Sadowska, and W. Maslinski. 2000. High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporine A sensitive mechanism. *J. Immunol.* 164:2832.
27. Fossiez, F., O. Djossou, P. Chomarat, L. Flores-Romo, S. Ait-Yahia, C. Maat, J.-J. Pin, P. Garrone, E. Garcia, S. Sealant, et al. 1996. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J. Exp. Med.* 183:2593.
28. Jovanovic, D. V., J. A. Di Battista, J. Martel-Pelletier, F. C. Jolicoeur, Y. He, M. Zhang, F. Mineau, and J.-P. Pelletier. 1998. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-1 β and TNF- α , by human macrophages. *J. Immunol.* 160:5313.
29. Kurowska, M., W. Rudnicka, E. Kontny, I. Janicka, M. Chorazy, J. Kowalczewski, M. Ziolkowska, S. Ferrari-Lacraz, T. B. Strom, and W. Maslinski. 2002. Fibroblast-like synoviocytes from rheumatoid arthritis patients express functional IL-15 receptor complex: endogenous IL-15 in autocrine fashion enhances cell proliferation and expression of Bcl-x_L and Bcl-2. *J. Immunol.* 169:1760.
30. Kim, Y., W. Maslinski, X. X. Zheng, G. H. Tesch, V. R. Kelley, and T. B. Strom. 1998. Targeting the IL-15 receptor with an antagonist IL-15/Fc protein blocks delayed type hypersensitivity. *J. Immunol.* 160:5742.
31. Ferrari-Lacraz, S., X. X. Zheng, Y. S. Kim, Y. S. Li, W. Maslinski, X. C. Li, and T. B. Strom. 2001. Treatment with an antagonist IL-15/Fc protein controls costimulation blockade resistant rejection. *J. Immunol.* 167:3478.
32. Courtenay, J. S., M. J. Dallman, A. D. Dayan, A. Martin, and B. Mosedale. 1980. Immunisation against heterologous type II collagen induces arthritis in mice. *Nature* 283:666.
33. Hancock, W. W., R. Buelow, M. H. Sayegh, and L. A. Turka. 1998. Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. *Nat. Med.* 4:1392.
34. Li, B., C. Hartono, D. Ruchuang, V. K. Sharma, R. Ramaswamy, B. Qian, D. Serur, J. Mouradian, J. E. Schwartz, and M. Suthanthiran. 2001. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. *N. Engl. J. Med.* 344:947.
35. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods* 171:131.
36. Noorchashm, H., Y. K. Lieu, S. Y. Rostami, H. K. Song, S. A. S. Greeley, S. Bazel, C. F. Barker, and A. Najj. 1999. A direct method for the calculation of alloreactive CD4⁺ T-cell precursor frequency. *Transplantation* 67:1281.
37. Harada, S., Y. Masahiro, H. Okamoto, Y. Morita, M. Kawashima, T. Aita, and H. Makino. 1999. Production of interleukin-7 and interleukin-15 by fibroblast-like synoviocytes from patients with rheumatoid arthritis. *Arthritis Rheum.* 42:1508.
38. Campbell, I. K. K. O'Donnell, K. E. Lawlor, and I. P. Wicks. 2001. Severe inflammatory arthritis and lymphadenopathy in the absence of TNF. *J. Clin. Invest.* 107:1519.
39. Ruchatz, H., B. P. Leung, X.-Q. Wei, I. B. McInnes, and F. Y. Liew. 1998. Soluble IL-15 receptor α -chain administration prevents murine collagen-induced arthritis: a role for IL-15 in development of antigen-induced immunopathology. *J. Immunol.* 160:5654.