2002-189-1

Longterm Protection of Mice Against Collagen-Induced Arthritis After Short-Term LF 15-0195 Treatment: Modulation of B and T Lymphocyte Activation

PATRICK DUCOROY, DANIEL de FORNEL, LAURENCE DUBREZ-DALOZ, ERIC SOLARY, and PATRICK DUTARTRE

ABSTRACT. ***Objectives***. LF 15-0195 is an immunosuppressive agent obtained by organic synthesis, currently under clinical development for the treatment of vasculitis. We define the effects of LF 15-0195 in the murine collagen-induced arthritis (CIA) model, an experimental model of human rheumatoid arthritis.

Methods. In our model, CIA was elicited in DBA/1 mice by immunization with bovine type II collagen (CII) in Freund's complete adjuvant, followed by a repeat injection 21 days later. Disease onset was observed 6 days after booster injection. In these experiments, mice were treated with 5 daily LF 15-0195 injections starting after the booster injection (days 21–25). The mice were observed for 40 days after the start of treatment, during which time arthritis was scored using clinical score and paw swelling assessment. Modulation of humoral immunity was documented by measuring the serum level of anti-CII IgG1 and IgG2a and cellular immunity by cytokines production by lymph node cells (LNC) and their proliferation *in vitro*.

Results. Short-term treatment of LF 15-0195 after booster injection prevented longterm development of CIA. LF 15-0195 inhibited B cell differentiation with a marked suppression of anti-CII IgG1 and IgG2a synthesis. Functional analyses of T lymphocytes showed that LF 15-0195 treatment reduces cytokine production by LNC after CII, anti-CD3, lipopolysaccharide stimulation.

Conclusion. LF 15-0195 treatment during a short time period prevented development of arthritis, inhibited humoral-specific response longterm, induced a decrease in the number of LNC, and decreased cytokine production of T LNC after *ex vivo* stimulation. (J Rheumatol 2003;30:918–25)

Key Indexing Terms:

AUTOIMMUNITY	IMMUNOSUPPRESSIVE AGENT	TH1/TH2
ARTHRITIS	IMMUNOGLOBULIN	MOUSE MODEL

Rheumatoid arthritis (RA) is a chronic disease that affects about 1% of the world population[1,2]. Treatments currently used are immunosuppressive agents and corticosteroids, which are now complemented with anti-tumor necrosis factor antibody therapies[3]. Induction of longterm remission or cure remains to be obtained, especially following short-term therapy.

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P. Ducoroy is supported by a CIFRE grant from Fournier SA Laboratories, the ANRT (Association Nationale de la Recherche Technique) and the Ministère de l'Education Nationale, de la Recherche et de la Technologie. L. Dubrez-Daloz is supported by a Post-Doctoral fellowship from the Conseil Regional de Bourgogne. E. Solary and group are supported by a special grant from the Ligue Nationale Contre le Cancer.

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Submitted February 27, 2002; revision accepted November 4, 2002.

Collagen type II-induced arthritis (CIA) is a well established model for human RA and has been extensively used to identify potential targets for therapeutic intervention[4,5]. CIA is induced in susceptible strains of mice by immunization with native bovine type II collagen (CII) in complete Freund's adjuvant[6-8]. Disease progression is associated with high levels of both cell-mediated and humoral immunity to collagen[9-11]. Arthritis can be transferred by both antibodies against CII and specific T cell line clones[12-14]. The development of CIA is known to depend on CD4+ T cell activation. Studies of cytokine production at different stages of the disease revealed that the Th1 cytokine profile [interleukin 2 (IL-2), interferon-γ, (IFN-γ)] predominates during induction and acute phases, whereas Th2 response (IL-4, IL-10) is associated with the remission phase[15,16]. A fine balance between Th1 and Th2 subset activity seems necessary for CIA development.

Many immunosuppressive agents have been reported to modify CIA development[17]. LF 15-0195 is a new immunosuppressive agent from a family of drugs that includes Treperimus and 15-deoxyspergualin[18] (Figure 1); LF 15-0195 was designed for increased stability in water solutions,

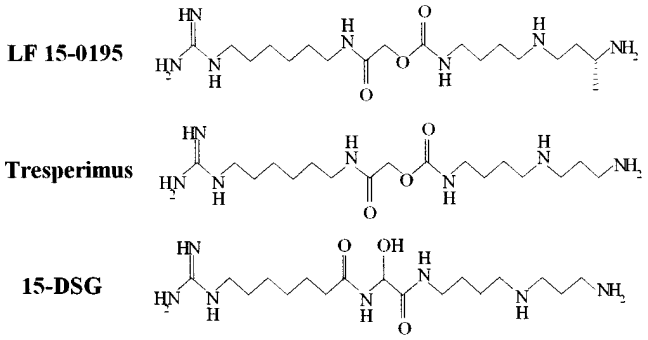

Figure 1. LF 15-0195. Chemical structure of LF 15-0195 and related compounds.

and resistance to *in vivo* oxidative metabolism. Its efficacy was demonstrated in preventing graft-versus-host disease[18], anti-glomerular basement membrane disease[19], in induction of allograft tolerance in a fully MHC-mismatched heart allograft model in the rat[20], and in the curative and preventive treatment of collagen type II-induced arthritis[21,22] in murine models. Studies have shown that LF 15-0195 binds to cytosolic heat shock protein; however, the precise molecular mechanism of its immunosuppressive activity remains poorly understood[23].

Our aim was to determine the modulation of humoral and cellular responses to CII induced by LF 15-0195 treatment, by measuring serum concentrations of anti-CII IgG1 and IgG2a and cytokine production by lymph node cells (LNC) *in vitro*. We observed that short-term pre-onset treatment with LF 15-0195 had a significant and longterm therapeutic effect on arthritis, associated with a strong suppression of anti-CII antibody production. Our results indicate that LF 15-0195 may prevent arthritis development by inhibition of T cell activation and cytokine production, leading to an inactivation of B cell and reduction of anti-CII antibody synthesis.

MATERIALS AND METHODS

Animals. Male DBA/1J mice (6 weeks old) were obtained from Bomholtgard-Mollegard (Ry, Denmark). They were allowed at least 2 weeks to adapt to their environment.

Induction of arthritis. Mice were immunized intradermally at several sites into the base of the tail with 100 µl emulsion that contained 100 µg of bovine type II collagen (Elastin Products, Owensville, MO, USA) in 0.01 M acetic acid, emulsified with an equal volume of Arthrogen-CIA Adjuvant (Morwell Diagnostics, Zurich, Switzerland). On Day 21, mice were boosted by intraperitoneal (ip) injection with 100 µg of bovine CII in 0.01 M acetic acid emulsified with an equal volume of incomplete Freund's adjuvant (Difco, Bonneuil sur Marne, France). Adjuvant was injected on the 2 sides of the base of the tail (2 × 50 µg bovine type II collagen).

Treatment. LF 15-0195 (Fournier Laboratories, Dijon, France) was prepared in saline solution, adjusted to pH 7.2, and 200 µl were administered subcutaneously (sc) at 2 mg/kg/day on Days 21–25; control mice received sc injection of saline alone.

Assessment of arthritis. From the 3rd week after initial immunization, mice were blindly monitored twice a week for signs of arthritis. The following clinical score system was used: 0 = normal; 1 = erythema, slight swelling; 2 = pronounced edematous swelling; 3 = pronounced edematous swelling plus deformity; 4 = joint rigidity. Each limb was graded separately with a maximum score of 16 for each mouse. Swelling was quantified by measuring the thickness of the foot with a micrometer caliper (Mitutoyo, Kanagawa, Japan).

Histology. Arthritic hind paws were removed *post mortem*, fixed in 10% (w/v) buffered formalin. The paws were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Anti-collagen antibody level. Measurement of anti-CII IgG1 and IgG2a subclasses was done by a modification of an ELISA as described[16]. Briefly, 96-well microtiter plates were coated overnight with 100 µl bovine type II collagen (10 µg/ml) dissolved in 0.05 M Tris-HCl pH 7.4 and 0.2 M NaCl, blocked and then incubated with serially diluted test sera. Titrated mouse anti-CII IgG1 and IgG2a (Lab Vision Corp., Fremont, CA, USA) were used as standards. Bound IgG1 and IgG2a were detected by incubation with peroxidase conjugated rat anti-mouse IgG1 or IgG2a (Biosource, Camarillo, CA, USA), followed by substrate (o-phenylenediamine dihydrochloride, Sigma-Aldrich Laboratories, St. Quentin Fallavier, France). Plates were washed between each step and absorbance was measured at 450 nm.

LNC preparation. Treated and control mice (n = 10) were sacrificed at different days, and inguinal lymph nodes were excised. For all experiments, excised bilateral nodes of each mouse were pooled. The lymph nodes of each mouse were teased apart to make a single cell suspension, which was then washed and enumerated. LNC solution was then separated in 2 parts for FACS analysis and cell culture.

FACS analysis. For each mouse, removed LNC (2.5×10^5) at the indicated time were resuspended in 200 µl of phosphate buffered saline (PBS, 1% bovine serum albumin, 0.1% azide) and then incubated on ice in the presence of specific antibodies for 30 min. Cells were then washed twice in PBS containing 0.1% sodium azide. The percentage of T lymphocyte subpopulations was determined using anti-CD4 and anti-CD8 antibody-labeled FITC and phycoerythrin (PE), respectively (clone H129.19 and clone 53-6.7, BD Biosciences, Heidelberg, Germany). The percentage of B lymphocytes was determined using anti-IgM FITC labeled antibody (clone 1B4B1, Southern Biotechnology, Birmingham, AL, USA) and granulocyte-macrophages using anti-Ly6+ and anti-CD11b antibodies labeled FITC and PE, respectively (clone RB6-8C5 and clone M1/70 B; BD Biosciences). Analyses were performed on a Coulter XL.

Cytokine and proliferation assays. LNC were cultured in RPMI 1640 containing 10% heat-inactivated FCS (v/v), 100 U/ml penicillin, 100 µg/ml streptomycin, 2×10^{-5} M 2-mercaptoethanol, 1% L-glutamine. Cells were cultured alone or in the presence of either CII (50 µg/ml) or lipopolysaccharide (LPS, 10 µg/ml) or anti-CD3 monoclonal antibody coated (10 µg/ml), at 37°C in 5% CO_2, in 96-well plates at a density of 1×10^6 cells/ml (200 µl/well) for 72 h. During the last 9 h, the cultures were pulsed with 1 µCi of [H^3]thymidine. After cultured cells were centrifuged, the supernatant was removed and cells were resuspended, harvested, and counted in a scintillation counter. The cytokine levels (IL-10, IFN-γ, TNF-α) in the supernatant were measured by a sandwich ELISA (Quantikine, R&D Systems, Abingdon, UK) according to the procedure recommended by the manufacturer.

Statistical analysis. The Mann-Whitney U test for nonparametric data was used to compare differences in the arthritis score between different populations of mice. Other data were compared using analysis of variance and Bonferroni test.

RESULTS

Effect of LF 15-0195 treatment after boost injection of type II collagen. As shown in Figure 2 and Table 1, a 5-day course of LF 15-0195 starting after boost injection induced significant inhibition of arthritis development. This inhibi-

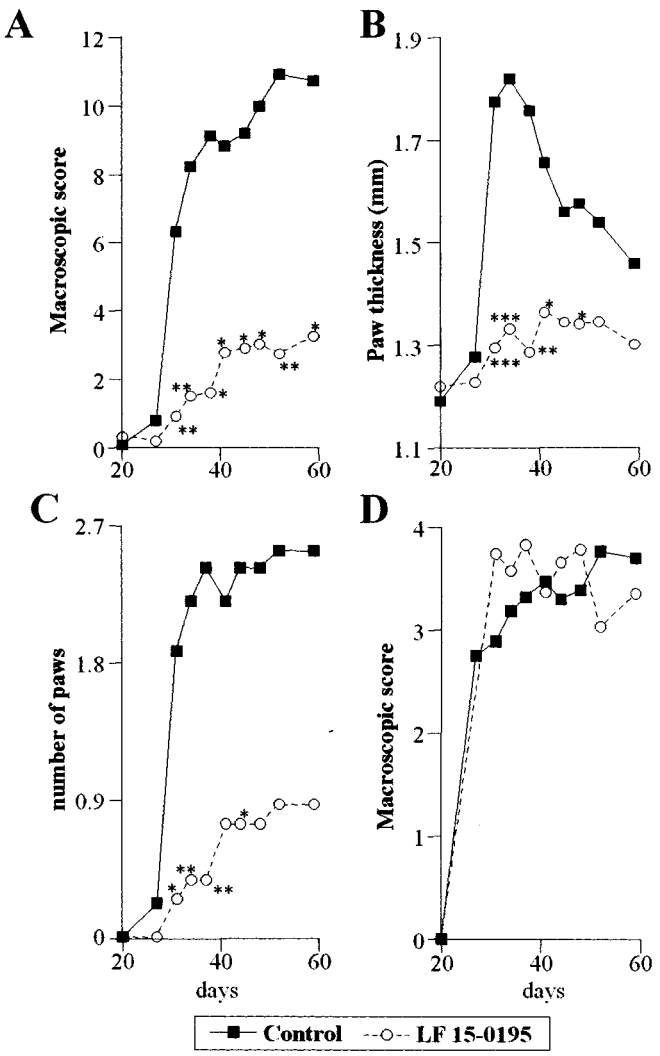

Figure 2. LF 15-0195 treatment after boost injection of type II collagen inhibits the onset of CIA (Experiment 1). Mice were treated daily with LF 15-0195 at 2 mg/kg/day on Days 21–25 or with saline alone for control. A. Clinical score. Each limb was graded, giving a maximum score of 16 per mouse. *p < 0.05, **p < 0.01, (Mann-Whitney U test, LF 15-0195 vs vehicle-treated animals). B. Paw swelling. Average paw width (mm). C. Average number of affected paws per mouse. *p < 0.05, **p < 0.01, ***p < 0.001 (Bonferroni test, LF 15-0195 vs vehicle-treated animals, group n = 8). D. Average clinical score per affected paw.

tion was observed during treatment and lasted until the end of the observation period. At Day 60 (Figure 2A), 25% of treated mice developed arthritis (87.5% for controls), with a mean clinical score of 2.5 (11 for controls). LF 15-0195 induced a reduction in the average paw swelling (Figure 2B) as well as in the average number of affected paws per mouse (Figure 2C). However, the clinical score of individual affected paws was identical to those of control mice (Figure 2D). This observation shows that LF 15-0195 treatment reduced the final number of affected joints; however, the macroscopic scoring of affected paws was not modified.

Histological examination of joints from LF 15-0195-treated mice (Figure 3B) showed a normal morphology with intact cartilage and absence of inflammatory cell infiltrate in most cases. Conversely, joints of control mice showed severe pathology with cartilage and bone degradation (Figure 3A).

Kinetic evolution of IgG1 and IgG2a anti-CII antibody levels. In control animals, a continuous increase of both Ig isotypes was observed until Day 38 post-first immunization (Figure 4). IgG2a anti-CII levels then decreased regularly until Day 52, while IgG1 anti-CII levels remained stable until the end of the experiment, with a switch towards IgG1 from Days 45 to 60. In LF 15-0195-treated mice a dramatic and statistically significant inhibition ($p < 0.001$) of synthesis of both Ig isotypes was observed until the end of the experiment (35 days after end of the treatment period). The IgG1/IgG2a ratio remained unchanged throughout the experiment.

Lymph node cell population analysis. An increase of LNC number in control and treated mice was observed after boost injection (Figure 5A). LF 15-0195 administration induced a dramatic decrease in number of LNC between Days 22 and 24. At Day 24, the number of LNC in treated mice was normalized, in comparison with mice that were not boosted, and remained stable until Day 33. In control mice, the number of LNC increased until Day 24, then slowly decreased until Day 28. Phenotypic analysis (Figure 5B) of LNC indicated that the percentages of macrophages and T and B cells were very similar in control and treated mice at all times of observation.

The lymph node cell function. Lymph node cells obtained from control or treated animals were stimulated *in vitro* by LPS, anti-CD3, and CII. For all stimuli, thymidine incorporation indicated similar proliferation of cells from both controls and LF 15-0195-treated animals (Table 2). In contrast to cell proliferation, significant differences between cells from control and treated animals were observed in cytokine production (Figure 6).

Cytokine production. Two periods were observed (Figure 6): during the first period (treatment period and immediately after, i.e., Days 22–28) production of IFN-γ after specific antigen (CII) (Figure 6A) or of IFN-γ and IL-10 after anti-CD3 (non-specific T activation) stimulation (Figure 6B and C), or of IL-10 and TNF-α after LPS (Figure 6D and E), was systematically lower in treated compared with untreated mice. The second period, at Day 33 (8 days after treatment discontinuation), we observed higher or identical production of IFN-γ, IL-10, and TNF-α in treated compared with control mice after activation by CII, anti-CD3, or LPS. This delayed and high level of cytokine secretion was not associated with an increase in arthritis symptoms. The level of IL-10 and IL-4 in supernatant after CII stimulation was very low and was below detection threshold in all types of cell

Table 1. Effect of LF 15-0195 on collagen-induced arthritis in DBA/1 mice (experiment 2). Animals were immunized at Days 0 and 21 with CII and monitored twice by week until Day 50 after the first immunization (control n = 17, LF 15-0195 n = 21).

Treatment[a]	Day of Onset	Affected Mice, %[b]			Clinical Variables: Clinical Score[6]			Clinical Variables: Paw Swelling, mm[d]		
		Day 30	Day 40	Day 50	Day 30	Day 40	Day 50	Day 30	Day 40	Day 50
Control	27.2 ± 1.4	58.8	76.5	76.5	2.7 ± 0.6	5.4 ± 1	6.4 ± 1.3	1.42 ± 0.05	1.51 ± 0.06	1.42 ± 0.04
LF 15-0195	40.3 ± 3.3**	9.5	23.8	38.1	0.4 ± 0.2***	1.3 ± 0.4***	2.3 ± 0.7**	1.21 ± 0.03***	1.23 ± 0.03***	1.31 ± 0.04

[a] At day 21, 21 treated and 17 control mice were injected daily during 5 days sc with, respectively, LF 15-0195 at 2 mg/kg and saline solution. [b] Incidence is expressed as percentage of animals per group having at least a score = 1. [c] Mean ± SEM, with a maximum cumulative value of 16 for all paws. ** $p < 0.01$; *** $p < 0.001$, Mann-Whitney U test, LF 15-0195 vs vehicle-treated animals. [d] Paw width was measured with a micrometer caliper. ** $p < 0.01$; *** $p < 0.001$, Bonferroni test, LF 15-0195 vs vehicle-treated animals. [e] Days after first immunization with bovine CII Day 0.

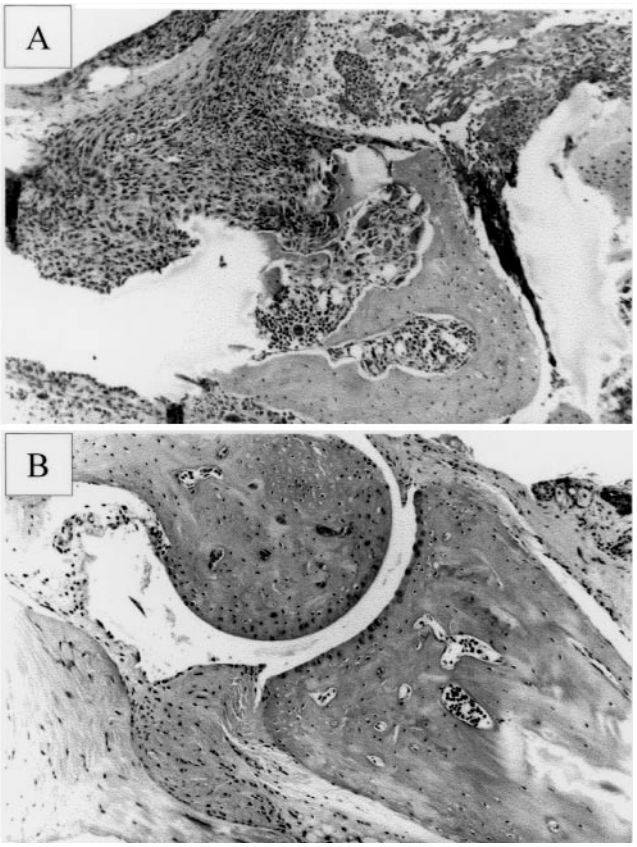

Figure 3. Histologic section of joints from treated and control mice. H&E stained sagittal sections of proximal interphalangeal joints from CIA mice. A. Control mouse shows severe arthritis with synovitis, erosion, and loss of joint integrity compared to LF 15-0195-treated mouse (2 mg/kg/day on Days 21–25). B. The majority of LF 15-0195-treated mouse joints examined had normal morphology, with smooth intact articulation cartilage and absence of inflammatory cell infiltrate. Original magnification × 100.

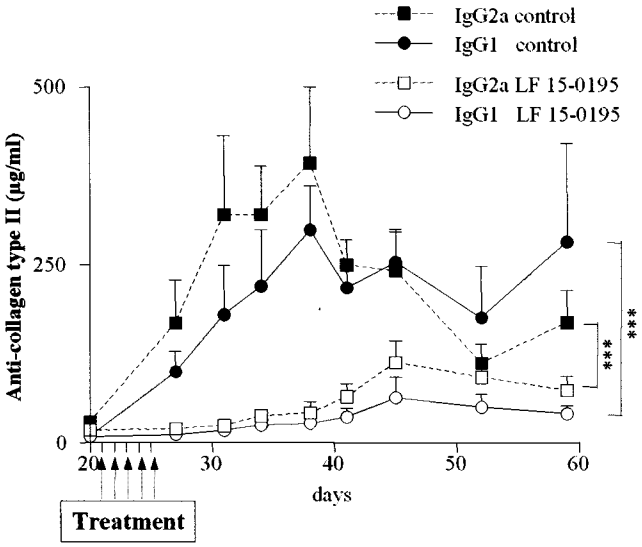

Figure 4. LF 15-0195 decreases the humoral response to CII. LF 15-0195 treatment inhibits the production of anti-CII IgG1 and IgG2a antibody. Blood was taken from each mouse at different times in the course of arthritis and serum levels of anti-CII IgG1 and IgG2a determined using sandwich ELISA. Results show average levels of antibodies for LF 15-0195 and vehicle-treated groups (n = 12 per group). ***$p < 0.001$.

cultures. The production of IL-4 by LNC obtained from treated and untreated mice at Days 28 and 49 after *ex vivo* anti-CD3 stimulation was not modified (data not shown).

DISCUSSION

These results indicate that LF 15-0195 administered during a short time period prevents the development of arthritis. Different timing of treatment showed that LF 15-0195 was most active when administered after boost injection. Five days of treatment after boost led to significant inhibition of CIA lasting at least 35 days. The main clinical effect was a reduction in the final number of affected paws per mouse. However, no reduction in inflammation scoring of affected paws was observed. The same activity profile was reported when LF 15-0195 was administered after the onset of clinical arthritis[19]. These observations suggest that LF 15-0195 strongly inhibited events occurring before joint inflammation rather than during progression of the inflammation.

We studied the effect of LF 15-0195 on both humoral and cellular responses to CII that are necessary for development of arthritis[9-11]. The anti-CII antibody levels have been described not to be correlated with disease severity in CIA[13] but play a crucial role in initiating joint injury[10,24-25]. Our

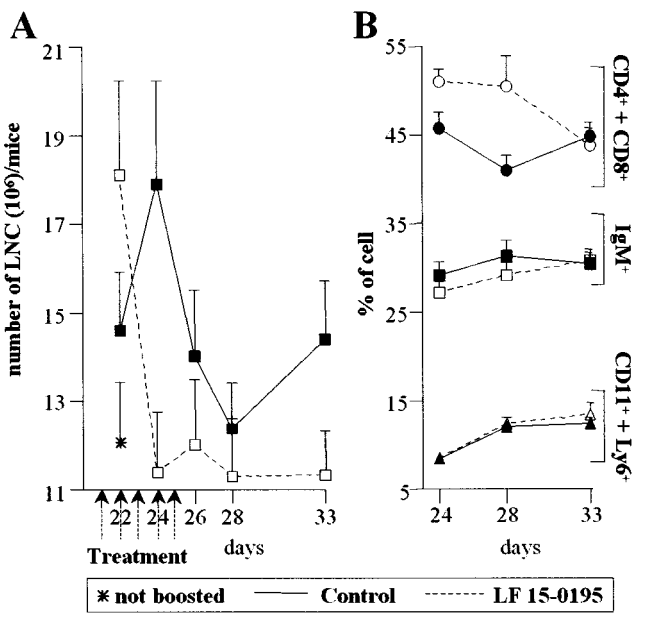

Figure 5. LF 15-0195 induced a decrease in number of lymph node cells without modifying the percentage of the subpopulation. Twelve mice from control and treated groups were sacrificed at Days 22, 24, 26, 28, 33, and their inguinal lymph nodes were excised. For each mouse LNC were enumerated (A, average of LNC number by mouse). B. Percentage of subpopulation of T lymphocytes ($CD4^+$ + $CD8^+$), B lymphocytes (IgM^+), and granulocyte-macrophages ($Ly6^+$ + $CD11b^+$) were measured as described in Materials and Methods. Values represent the mean of 12 mice per group.

study reveals marked inhibition of IgG1 and IgG2a anti-CII antibody synthesis in LF 15-0195-treated mice until the end of the observation period. Clinical improvement after LF 15-0195 administration on the initiation of inflammation may be the consequence of this inhibition.

At the cellular level, LF 15-0195 administration induced a dramatic decrease in the number of lymph node cells during the first 3 days of treatment, without modification of subpopulation percentage. Decrease in number of LNC after LF 15-0195 treatment was not associated with inhibition of LNC functionality. Indeed, no inhibition of *ex vivo* proliferation in response to specific stimulation by CII or nonspecific stimulation by anti-CD3 and LPS was observed. However, cytokine production by cells removed from treated mice was decreased. These data indicate that LF 15-0195 modified the *in vivo* response to antigen without inducing a complete inhibition of T and B cell response to different stimulation.

It is well established that the development of CIA depends on T cell activation, as arthritis can be prevented by treatment during the time of collagen immunization with antibodies against CD4, T cell receptor, and MHC[26-28]. CD4+ Th cells are divided into 3 subsets: Th1 cells (producing IL-2, IFN-γ) primarily associated with cell immunity and class switching to the IgG2a isotype, Th2 cells (secreting IL-4 and IL-10) mainly involved in humoral immunity and class switching to IgG1 isotype, and Th0 cells displaying a mixed lymphokine secretion pattern. It is generally accepted that CIA is a predominantly Th1 disease[15,16]. *In vitro* studies of LNC stimulation with specific (CII) and nonspecific antigen (anti-CD3) revealed that administration of LF 15-0195 decreases *ex vivo* IFN-γ and IL-10 cytokine production without modulating the IL-4 level. Moreover, the IgG1 and IgG2a anti-CII antibody production was blocked after LF 15-0195 treatment. Together these results indicate that LF 15-0195 did not modify the Th1/Th2 balance inducing a switch towards Th2 for preventing the onset of arthritis.

At Day 33, the 2-fold increase of IFN-γ production by LNC from treated mice after CII stimulation may illustrate Th1 response to CII but, surprisingly, marked clinical symptoms did not develop. It is generally accepted that IFN-γ plays a role during different phases of arthritis progression[15,16], but the exact role of IFN-γ in CIA is more controversial[29-31]. Indeed, it has been shown that IFN-α could have a biphasic effect during the development of CIA[32] and that anti-IFN-γ antibodies can exacerbate CIA in DBA/1 mice when administered after disease onset[33]. Moreover, Mauri, *et al* demonstrated that a nondepleting anti-CD4 monoclonal antibody treatment[34] induces anergy of CII-specific T cells, with production of a high level of IFN-γ after antigen stimulation. Results obtained after LF 15-0195 treatment suggest that inhibition of CIA is achieved by inhibition of

Table 2. *Ex vivo* cell proliferation after stimulation was not modified by LF 15-0195 treatment. Lymph node cells (LNC) were removed from control or LF 15-0195-treated mice and stimulated 72 h with CII (50 µg/ml) or anti-CD3 coated (10 µg/ml); cell proliferation was determined by thymidine incorporation during the last 9 h of culture. Values represent the mean value (x 10^3) of 12 mice per group ± SEM.

	Proliferative Response of LNC					
	Medium		CII		Anti-CD3	
	Control	LF 15-0195	Control	LF 15-0195	Control	LF 15-0195
Day 22[a]	26.2 ± 8.3	20.1 ± 6.9	40.2 ± 3.3	31 ± 3	119 ± 15	136 ± 12
Day 24	22 ± 5.9	21.6 ± 7.7	48.9 ± 6.9	48.4 ± 9.1	81 ± 13	91 ± 11
Day 28	23.3 ± 6.6	24.2 ± 10.5	37.3 ± 2.6	43.9 ± 5.1	81 ± 6	102 ± 4
Day 33	17.2 ± 8.3	12 ± 7.6	28.8 ± 4.1	23 ± 4.1	140 ± 24	153 ± 13

Bonferroni test, LF 15-0195 vs vehicle-treated animals. [a] Days after immunization with bovine CII.

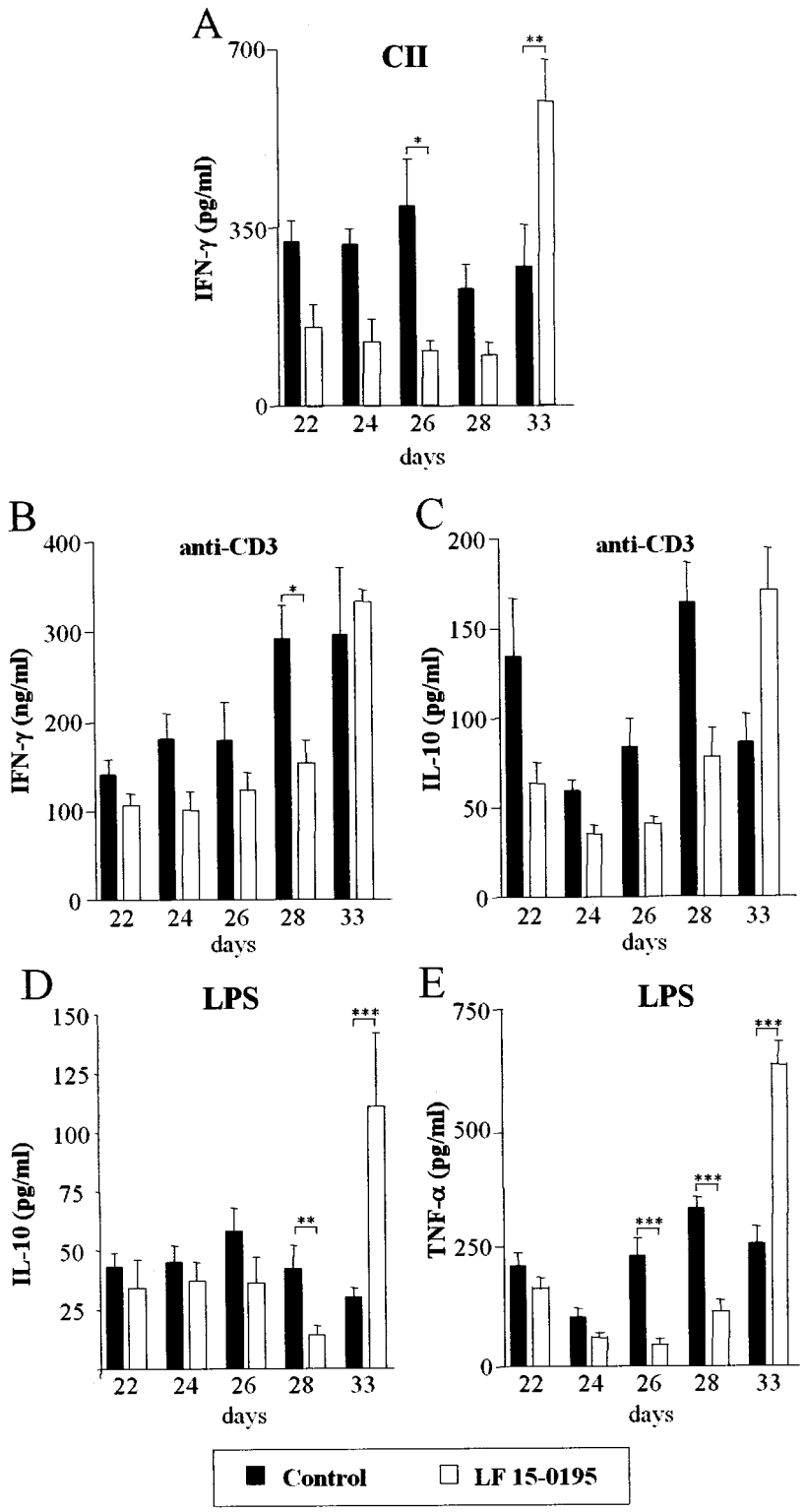

Figure 6. LF 15-0195 treatment-modified cytokine secretion without modification of lymph node cell proliferation. Lymph node cells were obtained from LF 15-0195 and vehicle-treated mice and stimulated 72 h with A: CII (50 µg/ml); or B, C: anti-CD3 coated (10 µg/ml); or D, E: LPS (10 µg/ml). Supernatants were collected after 72 h of culture and IFN-γ (A, B), IL-10 (C, D), and TNF-α (E) secretion levels were measured using sandwich ELISA. Values represent the mean of 12 mice per group SEM. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ (Bonferroni test, LF 15-0195 vs vehicle-treated animals).

IFN-γ-producing cells during the pre-onset phase of the disease in treated mice, followed by late overproduction of IFN-γ, which may participate in the longterm downmodulation of this pathology.

The results of our experiments revealed that LF 15-0195 treatment induced a transient inhibition of TNF-α production *ex vivo* by LNC after LPS stimulation, which was followed by an overproduction of TNF-α at Day 33, without induction of arthritis. Previous studies showed that TNF-α had a central role in joint inflammation with the induction of a proinflammatory cytokine cascade[35]. However, some data provide evidence for an immunomodulatory role of TNF-α during evolution of autoimmune responses[36,37] in contributing to T cell hyporesponsiveness in chronic inflammation[38]. These data and the inhibition of synovial cell infiltration may explain the absence of the disease induction by delayed overproduction of TNF-α.

Previous studies showed the importance of these different co-stimulation signals in the CIA model[39-42]. The results obtained on both humoral and cellular responses after LF 15-0195 treatment were similar to those obtained by Webb, *et al*[39] after blockage, before disease onset, of CD28 co-stimulation by CTLA-4 Ig treatment, with marked suppression of all anti-CII antibody isotype production, a decreased number of LNC, and inhibition of IFN-γ production by LNC after CII stimulation *in vitro*. We can postulate that LF 15-0195 prevents arthritis development by modulation of co-stimulation signals during lymphocyte activation, which may induce an incomplete differentiation of lymphocytes.

Different compounds were recently shown to block the development of experimental T cell-mediated autoimmune diseases by strongly enhancing CD95-induced apoptosis in murine-activated T cells[43,44]. Ogawa, *et al* demonstrated the therapeutic effect of anti-Fas antibody on a collagen-induced arthritis model[45] and we recently described that LF 15-0195 sensitized human peripheral T cells to activation induced cell death and enhanced activation-induced apoptosis by facilitating caspase-8 activation at the DISC level[46].

Our study confirms that LF 15-0195 treatment during a short period (5 days, starting after booster injection) prevents development of arthritis and reduces the final number of affected joints. LF 15-0195 inhibits B cell differentiation, leading to a marked suppression of both anti-CII IgG1 and IgG2a synthesis, and induces a diminution of lymph node cell number. LF 15-0195 decreases cytokine secretion of T lymph node cells after *ex vivo* stimulation. Our findings, together with previously published data, suggest that LF 15-0195 may prevent development of collagen type II-induced arthritis in mouse models by facilitating apoptosis of T cells activated after the proliferation stage and before the differentiation stage. New experiments are planned to test this hypothesis.

ACKNOWLEDGMENT

We thank Dr. Patrick Roignot for histological analysis; Florence Chirade, Paule Fontet, and Sylvaine Marie for technical assistance.

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