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ORIGINAL ARTICLE

Induction of Regulatory T Cells by Leflunomide in a Murine Model of Contact Allergen Sensitivity

Benno Weigmann[1], Elizabeth R. Jarman[1,2], Stephan Sudowe[1], Matthias Bros[1], Jürgen Knop[1] and Angelika B. Reske-Kunz[1]

Allergic contact dermatitis and contact hypersensitivity (CHS) are characterized by allergen-specific activation of $CD8^+$ and $CD4^+$ T cells and the production of cytokines resulting in an inflammatory response and tissue damage. We show here that the immunosuppressive compound leflunomide (*N*-[4-trifluoro-methylphenyl]-5-methylisoxazol-4 carboxamide, HWA 486) (LF) inhibited the contact allergic response induced in mice by epicutaneous application of the haptens dinitrofluorobenzene (DNFB) and oxazolone. The extent of ear swelling remained significantly reduced following repeated challenge with DNFB for up to 18 weeks. LF and DNFB had to be applied simultaneously for inhibition to occur. The loss of CHS responses was shown to be antigen-specific. Adoptive transfer of leukocytes from LF-treated mice into naïve mice resulted in a loss of CHS responsiveness. Transfer of both $CD4^+$ and $CD8^+$ cells was required for maximal loss of CHS responses, with $CD8^+$ cells playing a major role. Significantly enhanced levels of IL-10 mRNA were detected in $CD8^+$ T cells, but not in $CD4^+$ T cells, following LF treatment of mice. LF also suppressed CHS responses in mice previously sensitized and challenged with hapten, when administered together with the hapten. Our data suggest that LF induces a long-lived tolerance in mice by inducing $CD8^+$ and $CD4^+$ regulatory T cells.

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INTRODUCTION

Leflunomide (*N*-[4-trifluoro-methylphenyl]-5-methylisoxazol-4 carboxamide, HWA 486) (LF) is an immunoregulatory and anti-inflammatory compound, which is successfully used in the treatment of rheumatoid arthritis (Strand *et al.*, 1999). After administration, LF is chemically converted to the pharmacologically active metabolite A77 1726 (Figure 1). The mechanisms of action of A77 1726 *in vivo* remain to be clarified. *In vitro*, however, A77 1726 inhibits dihydroorotate dehydrogenase, an enzyme involved in the *de novo* synthesis of pyrimidines (Cherwinski *et al.*, 1995). As a consequence, proliferation of activated T and B cells, which depends on *de novo* synthesis of purine and pyrimidine nucleotides, is inhibited (Herrmann *et al.*, 2000). As a further consequence, A77 1726 inhibits tumor necrosis factor-α-induced cellular responses by blocking the activation of NF-κB (Manna *et al.*, 2000; Imose *et al.*, 2004). Both effects are reversed by the addition of uridine or cytidine (Cao *et al.*, 1995; Manna *et al.*, 2000). Besides inhibition of dehydroorotate dehydrogenase activity, A77 1726 directly inhibits the phosphorylation of Scr kinases $p56^{lck}$ and $p59^{fyn}$ (Xu *et al.*, 1995, 1996b) involved in T-cell receptor signal transduction, and of Janus kinase 1 and Janus kinase 3 (Elder *et al.*, 1997; Siemasko *et al.*, 1998), which play a role in IL-2 and IL-4 receptor signalling. However, as high concentrations of LF had to be used to reveal these activities in vitro, their relevance *in vivo* is unclear at present. Furthermore, an inhibitory effect on the activity of cyclooxygenase-2 was observed (Hamilton *et al.*, 1999). Reduced expression levels of the adhesion molecule intercellular adhesion molecule-1 by macrophages as well as decreased production of the cytokines tumor necrosis factor-α and IL-1 by macrophages and Kupffer cells after treatment with A77 1726 *in vitro* were reported (Cutolo *et al.*, 2003, Yao *et al.*, 2004).

Beside the potency of LF in the treatment of rheumatoid arthritis, this compound is also effective in inhibiting inflammatory responses in animal models of autoimmune diseases (Glant *et al.*, 1994; Korn *et al.*, 2004), transplantation (Williams *et al.*, 1994), and type I allergy (Jarman *et al.*, 1999). Experimental models of skin inflammation have shown that LF is effective in reducing edema formation, neutrophil infiltration, and the release of inflammatory mediators such as leukotriene B4 (Kurtz *et al.*, 1995). LF might

[1]*Clinical Research Unit Allergology, Department of Dermatology, Johannes Gutenberg-University, Mainz, Germany*

[2]*Current address: Wellcome Trust Research Laboratories, Blantyre-Liverpool, UK.*

Correspondence: Professor Dr Angelika B. Reske-Kunz, Clinical Research Unit Allergology, Department of Dermatology, Johannes Gutenberg-University, Obere Zahlbacher Str. 63, Mainz D-55131, Germany.
E-mail: a.reske-kunz@uni-mainz.de

Abbreviations: CHS, contact hypersensitivity; CMC, caboxymethyl-cellulose–sodium solution; DNFB, dinitrofluorobenzene; LF, leflunomide; OXA, oxazolone; Th, T-helper cells; Treg, regulatory T cell

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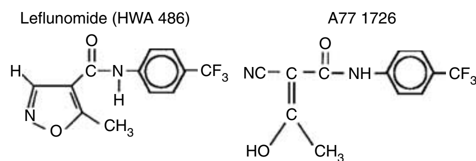

Figure 1. Structure of the prodrug LF and of its active metabolite A77 1726.

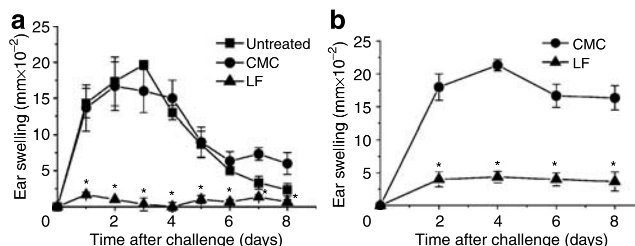

Figure 2. LF, administered orally, inhibits CHS responses to DNFB and OXA. Mice were orally administered LF in CMC for 6 days starting day −1 before sensitization. Control mice received CMC alone or, as a reference, were left untreated. (**a**) Mice ($n=3$) were sensitized with 25 μl of 0.5% DNFB on the shaved abdomen on day 0 and 1, and were challenged with 10 μl of 0.2% DNFB on each side of the right ear on day 4. (**b**) Mice ($n=3$) were sensitized with 25 μl of 3% OXA on the shaved abdomen on day 1 and challenged with 10 μl of 0.15% OXA on each side of the right ear on day 4. In all experiments, the left ear was treated with the vehicle for control. The increase in ear thickness was measured at the indicated time points following challenge. Data are presented as mean swelling values ± SEM and are representative of 12 independent experiments with three to eight mice per group for (**a**) and three independent experiments with three to six mice per group for (**b**). Asterisks denote statistically significant differences ($P<0.05$) as compared with mice receiving CMC.

therefore be effective in the treatment of T-cell-mediated skin diseases such as atopic dermatitis and psoriasis (Schmitt *et al.*, 2004).

Allergic contact dermatitis and contact hypersensitivity (CHS) are triggered by low molecular weight molecules or metal ions, which on penetrating the skin bind to both extracellular as well as intracellular proteins or even major histocompatibility complex-bound peptides expressed on the surface of antigen-presenting cells. These complexes are processed by Langerhans cells, which are the principle population of antigen-presenting cells residing in the epidermal layer of the skin, and are transported to regional lymph nodes. During this process, Langerhans cells undergo maturation, and once in the paracortical region of a lymph node, they present hapten-modified peptides in association with major histocompatibility complex class I and class II molecules to hapten-specific CD8$^+$ and CD4$^+$ T cells, respectively, resulting in their activation (sensitization phase). On subsequent re-exposure to the antigenic stimulus (challenge phase), memory effector T cells are recruited to peripheral tissues, where they interact with hapten-presenting cells to release lymphokines, thereby exerting cytotoxicity. This results in a localized inflammatory response and ensuing tissue damage. Major histocompatibility complex class I-restricted CD8$^+$ T cells are the principle effector cells involved in CHS responses to many types of haptens, with IL-4- and IL-10-producing CD4$^+$ T cells exerting a regulatory function (Bour *et al.*, 1995; Xu *et al.*, 1996a).

In this study, we have investigated the ability of LF to inhibit CHS responses induced in mice following the epicutaneous application of the hapten dinitrofluorobenzene (DNFB). Here, we show that LF abrogates the CHS response antigen specifically. Inhibition of the CHS response in mice administered LF was maintained over time, with the extent of ear swelling remaining significantly reduced on repeated re-challenge with DNFB, despite the absence of further LF treatment. The loss of CHS responsiveness to DNFB in LF-treated mice could be adoptively transferred to naïve recipients. Transfer of both CD4$^+$ and CD8$^+$ cells was required for maximal loss of DNFB-induced CHS responses in the recipients, with CD8$^+$ T cells expressing elevated levels of IL-10 mRNA playing a dominant role. Suppression of CHS responses following treatment with LF in the presence of hapten also occurred in previously hapten-sensitized mice.

RESULTS

LF inhibits the induction of a CHS response

Mice were orally administered LF dissolved in 1% carboxymethyl–cellulose–sodium solution (CMC) at a predetermined optimal dose of 40 mg/kg every 24 hours for 6 days (day –1 to day 4). Control mice received 1% CMC orally or were left untreated. Mice were sensitized by topical application of the contact allergens DNFB or oxazolone (OXA) to the shaved abdomen on days 0 and 1 (DNFB) or day 1 (OXA). Three days later, they were challenged by applying the allergen on the right ear and the vehicle on the left ear. Ear swelling was measured every 24 hours on subsequent days. Oral administration of LF at the time of sensitization significantly inhibited ($P<0.05$) the induction of a CHS response to DNFB (Figure 2a) and OXA (Figure 2b).

LF treatment provides long-lasting protection against the induction of CHS responses to DNFB

In order to determine whether the inhibitory effect of LF on CHS responses could be maintained in the absence of further treatment, groups of mice treated with LF in CMC or with CMC alone were sensitized and challenged with DNFB using the standard protocol. Efficient abrogation of the ear swelling response by LF was verified by ear swelling measurements (Figure 3a). On days 15 and 16, mice were again sensitized with DNFB, but this time in the absence of LF or CMC. Following challenge 3 days later, ear swelling responses were not detectable in the group of mice formerly treated with LF (Figure 3b). This finding suggests that during the second sensitization phase performed in the absence of LF induction of T effector cells could not take place, pointing towards the generation of regulatory T cells during the first round of sensitization with DNFB in the presence of LF.

In another type of experiment groups of mice treated with LF in CMC, with CMC alone or left untreated as a reference, were sensitized and then repeatedly challenged with DNFB on days 4, 18, 49, and 123 after the start of sensitization.

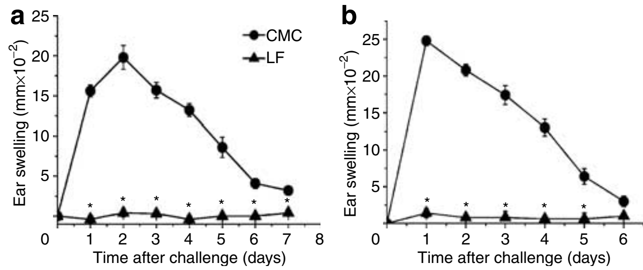

Figure 3. LF inhibits CHS responses induced by a second round of sensitization. (**a**) Groups of mice ($n=5$) were orally administered LF in CMC or CMC alone and were sensitized and challenged with DNFB as outlined in the legend to Figure 1a. (**b**) On days 15 and 16, mice were again sensitized with DNFB and challenged with this hapten 3 days later. The extent of ear swelling at the indicated time points following challenge is presented as mean swelling values ± SEM. Data are representative of two independent experiments with five mice per group. Asterisks denote statistically significant differences ($P<0.05$) as compared with mice receiving CMC.

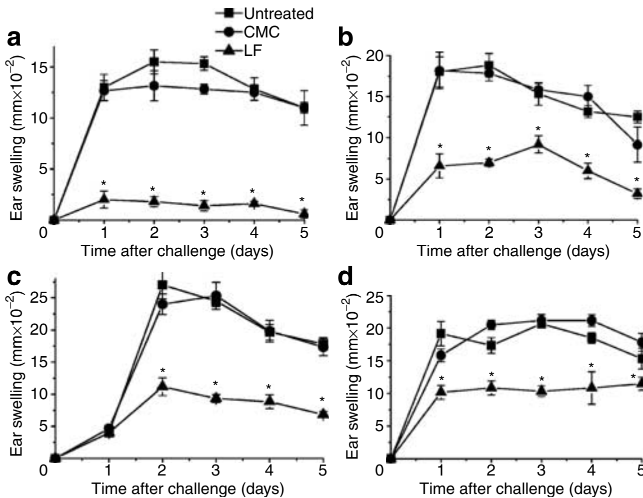

Figure 4. LF confers long-lasting protection against the induction of CHS responses to DNFB. Groups of mice ($n=6$) orally administered LF in CMC, CMC alone, or left untreated were sensitized with DNFB and then challenged as outlined in the legend to Figure 1a. Mice were rechallenged repeatedly at increasing intervals. LF significantly abrogated the inflammatory response as assessed by the extent of ear swelling, (**a**) during the initial challenge 3 days after sensitization, as well as during subsequent challenges on (**b**) day 18, (**c**) day 49, and (**d**) day 123. Data represent mean swelling values ± SEM and are representative of two independent experiments with six to eight mice per group. Asterisks denote statistically significant differences ($P<0.05$) as compared with mice receiving CMC.

Although the inhibitory effect of LF on CHS responses was most pronounced following the first localized challenge of mice with DNFB, inflammatory responses, as characterized by tissue swelling, were still significantly inhibited ($P<0.05$) on subsequent challenges at later time points (inhibition at the time point of maximal swelling, as compared with CMC-treated mice, for challenge on day 4: 84.2%; day 18: 63.7%; day 49: 63.2%; and day 123: 48.9%) (Figure 4). Thus, LF, when administered at the time of sensitization, can confer long-lasting protection against the induction of inflammatory responses induced by contact allergens.

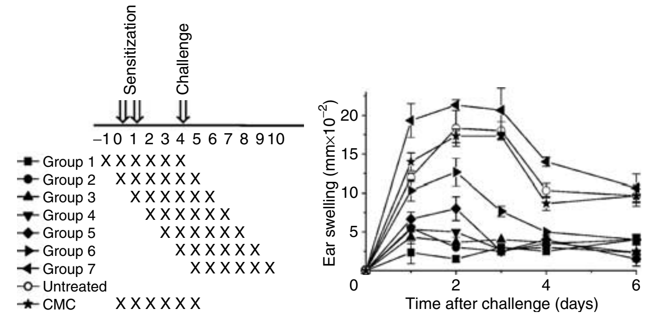

Figure 5. The inhibitory effect of LF on induction of CHS responses is optimal when the compound is applied during the sensitization phase. Groups of mice ($n=3$) were orally administered LF for 6 days. In each case, the starting point of administration was postponed by 1 day, as indicated. Control groups received CMC alone, starting on day −1, or were left untreated as a reference. All groups were sensitized and challenged with DNFB as outlined in the legend to Figure 1a. The extent of ear swelling is presented as mean ± SEM. Statistically significant differences ($P<0.05$) were found between values obtained with untreated mice and with mice of groups 1–5 throughout challenge, with mice of group 6 starting day 3 of challenge, and with mice of group 7 on days 1 and 4 of challenge.

LF confers protection against CHS responses to DNFB, when administered during sensitization and before subsequent challenge

We next analyzed the optimal time frame for the application of LF. Groups of mice were treated with LF for 6 consecutive days, whereby the groups differed with regard to the time point of first administration of LF as outlined in the schedule of Figure 5. The extent to which LF abrogated CHS responses, induced on challenge with DNFB, was most marked when administration started one day before sensitization (Figure 5). Nevertheless, while the suppressive capacity of LF decreased when administration was initiated at increasing time intervals post-sensitization, the levels of inhibition were still marked, provided that administration of LF to mice started before challenge (inhibition, as compared with untreated mice, on day 2 after challenge amounted for group 1 to 91.8%; for group 2 to 83.6%; for group 3 to 80.0%; for group 4 to 72.7%; for group 5 to 56.4%; for group 6 to 30.9%). The treatment of mice with LF one day after challenge resulted in an enhancement of the CHS response. Mice given LF for 6 days starting on day –12, that is, before sensitization with DNFB, developed normal CHS responses (data not shown). These data indicate that LF and DNFB had to be applied simultaneously for effective inhibition to occur.

The inhibitory effect of LF is antigen-specific

To determine whether the inhibitory effect of LF on CHS responses was antigen-specific, re-challenge experiments were performed. Briefly, groups of mice treated with LF or CMC as a control were sensitized and challenged with DNFB, using the standard protocol. The ear swelling response was efficiently inhibited by LF as shown in Figure 6a. On day 13, mice were sensitized with OXA on the back skin. Five days later, challenge was performed by applying DNFB to the right ear and OXA to the left ear. Measurements

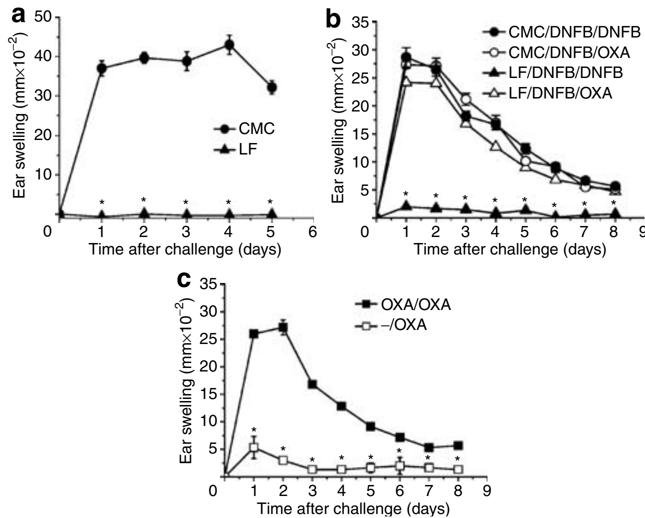

Figure 6. Antigen specificity of the inhibitory effect of LF. Groups of mice ($n=6$) were orally administered LF or CMC for 6 days starting day −1. (**a**) Mice were sensitized and challenged with DNFB as outlined in the legend to Figure 1a, and ear swelling was measured. On day 13, mice were sensitized with 25 μl of 3% OXA on the shaved back. Five days later, mice were rechallenged with 10 μl of 0.2% DNFB on both sides of the right ear and with 10 μl of 0.15% OXA on both sides of the left ear. The increase in ear thickness is shown in (**b**). (**c**) Further control mice were solely sensitized and challenged with OXA or were only ear challenged with OXA without prior sensitization. Data represent mean swelling values ± SEM and are representative of two experiments with six mice per group. Asterisks denote statistically significant differences ($P<0.05$) as compared (**a**, **b**) with mice treated with CMC or (**c**) with sensitized mice.

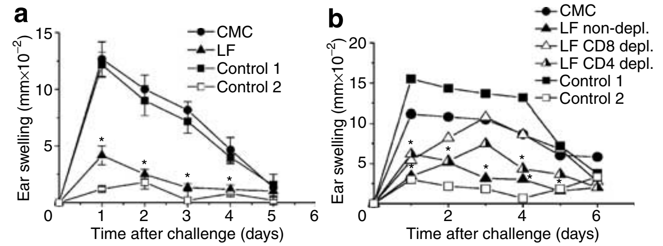

Figure 7. Suppression of CHS responses upon adoptive transfer of leukocytes obtained from secondary lymphoid organs of LF-treated mice. Mice were orally administered LF or, as a control, CMC alone on days −1 to 4, and were sensitized and challenged with DNFB as described in the legend to Figure 1a. On day 7, spleen and draining (cervical, axillary, and inguinal) lymph node cells were prepared. (**a** and **b**) Unseparated cells or (**b**) cell suspensions depleted of $CD4^+$ or $CD8^+$ cells were transferred intravenously into syngeneic recipients (10^8 cells per recipient; $n=6$ per group). After 24 and 48 hours, recipient mice were sensitized and were then challenged with DNFB following the standard protocol. The left ear was treated with vehicle. Two groups of control mice, which did not receive donor cells, were set up in parallel. Group 1 mice were sensitized and challenged with DNFB. Group 2 mice were solely challenged with DNFB without prior sensitization. Data represent mean increases in ear swelling ± SEM in (**a**); for the sake of clarity, error bars were omitted in (**b**). Asterisks denote statistically significant differences ($P<0.05$) of mice receiving cells from LF-treated donors as compared with mice receiving cells from CMC-treated donors. Data are representative of three and two independent experiments with five to six mice per group for (**a**) and (**b**), respectively.

of ear thickness were performed immediately before challenge and on the indicated time points after challenge, and were used to calculate ear swelling. Whereas the extent of ear swelling remained substantially reduced ($P<0.05$) in the LF-treated group on re-challenge with DNFB, the response of the group challenged with OXA was not efficiently inhibited, when compared with the group receiving CMC (Figure 6b) and with a control group solely sensitized and challenged with OXA (Figure 6c). This finding suggests that the observed inhibitory effect of LF on secondary challenge was dependent on prior administration at the time of antigen-specific priming of effector T-cell responses.

LF-induced suppression of CHS can be adoptively transferred to naïve recipients

To determine whether the inhibitory effect of LF on the induction of CHS responses was owing to the induction of tolerance, we performed adoptive transfer experiments, in which leukocytes isolated from secondary lymphoid tissues of LF-treated mice were transferred to naïve recipient mice. Mice exposed to LF or CMC were sensitized and challenged with DNFB. The ear swelling response was completely inhibited by LF treatment (data not shown). Seven days after the start of sensitization, spleen and regional lymph node cells from both groups of mice were obtained and transferred intravenously into naïve syngeneic recipients. The recipient mice were sensitized with DNFB 24 and 48 hours after cell transfer and then challenged after another 3 days. The extent of tissue swelling observed after challenge with DNFB was significantly reduced ($P<0.05$) in recipient mice, which had received cells from LF-treated donors as compared with recipients that had received cells from CMC-treated mice, or the control group in which no cells were adoptively transferred (Figure 7a).

In a further step, naïve recipient mice were adoptively transferred with whole mononuclear cell populations or with $CD4^+$ and $CD8^+$ cell-depleted populations, respectively, obtained from secondary lymphoid organs of mice, treated with LF at the time of sensitization with DNFB. Depletion was performed by immunomagnetic separation using standard procedures and the efficiency of depletion was verified by flow cytometric analysis. In the control group of mice that did not receive cells by adoptive transfer, but were sensitized and challenged with DNFB using the standard protocol, tissue swelling reached a plateau at between 24 and 96 hours following challenge (Figure 7b). Mice receiving unseparated cells from LF-treated mice exhibited a significant inhibition ($P<0.05$) in the inflammatory response induced by DNFB challenge, as determined by the extent of tissue swelling, which was maintained throughout the challenge period. Both groups of recipient mice that had received $CD4^+$ cell-depleted or $CD8^+$ cell-depleted leukocyte populations from LF-treated mice exhibited comparable levels of suppression of tissue swelling at 24 hours following challenge. In mice receiving $CD8^+$ cell-depleted cells, there was a delayed increase in the swelling response, which first reached levels

comparable to those observed in mice, which had received cells from CMC-treated donors, at 72 hours following challenge. In contrast, recipient mice that had received $CD4^+$ cell-depleted leukocyte populations exhibited a sustained inhibition of the inflammatory response. The marked reduction in swelling observed at 24 hours was maintained for the duration of the challenge period. These data suggest that although both $CD4^+$ and $CD8^+$ cells play a role in suppression of the CHS response to antigen, $CD8^+$ cells are predominantly responsible for the observed immunoregulation.

$CD8^+$ suppressor T cells were reported to express CD25 on the cell surface and to lack CD28 (Liu *et al.*, 1998). $CD4^+$ regulatory T cells (Treg), on the other hand, are characterized by constitutive expression of CD25, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), and glucocorticoid-induced tumor necrosis factor receptor (GITR) (Fehervari and Sakaguchi, 2004). The T cells to be transferred were thus analyzed for the expression of these cell surface markers using flow cytometric analysis. We did not observe an increase either in $CD4^+CD25^+CTLA\text{-}4^+GITR^+$ cells or in $CD8^+CD25^+CD28^-$ cells (data not shown).

Foxp3 is a transcription factor characteristically expressed by naturally occurring $CD4^+$ Treg as well as Treg induced on contact with tumor growth factor-β (Chen *et al.*, 2003; Fehervari and Sakaguchi, 2004). The transcription factor repressor of GATA (ROG) and the regulator of G-protein signalling (RGS1) have also been reported to be specifically expressed in induced Treg (Cobbold *et al.*, 2003). Induction of CD5 expression on activated T cells by steady-state dendritic cells was reported to result in antigen-specific T-cell unresponsiveness (Hawiger *et al.*, 2004). Lymphocyte activation gene-3 (LAG-3) is expressed on the surface of induced Treg and contributes to their suppressive activity (Huang *et al.*, 2004). Moreover, induced Treg are characterized by secretion of high amounts of IL-10 (Tr1) and tumor growth factor–β (T-helper type 3 cells (Th3)), respectively (Levings *et al.*, 2002). Using real-time reverse transcriptase-PCR, we quantified the level of the respective mRNA in purified $CD4^+$ and $CD8^+$ T cells within the draining lymph node and spleen cell populations transferring suppression. Significantly ($P<0.05$) enhanced levels of IL-10 mRNA were detected in $CD8^+$ T cells, but not in $CD4^+$ T cells following the treatment of mice with LF (Figure 8). The mRNA levels of Foxp3, ROG, RGS1, tumor growth factor–β1, CD5, and LAG-3 were not augmented.

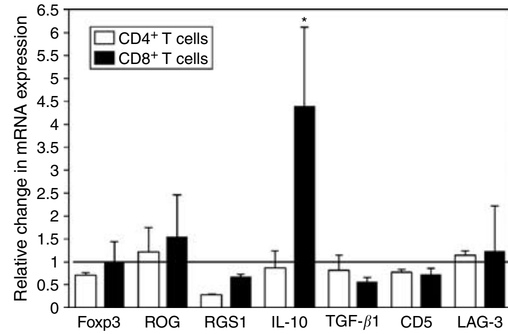

Figure 8. Expressional changes of markers of induced regulatory T cells. Mice ($n=3$ per group) were orally administered LF or, as a control, CMC alone, and were sensitized and challenged with DNFB as described in the legend to Figure 1a. On day 7, spleen and draining (cervical, axillary, and inguinal) lymph node cells were prepared and pooled. Real-time PCR analysis was performed with mRNA obtained from $CD4^+$ and $CD8^+$ T-cell populations positively enriched by immunomagnetic separation. Data indicate the relative changes in mRNA expression in the $CD4^+$ and $CD8^+$ T-cell populations of LF-treated *versus* CMC-treated mice. Data represent the mean ± SEM obtained from two separate experiments with three mice per group performed in duplicate.

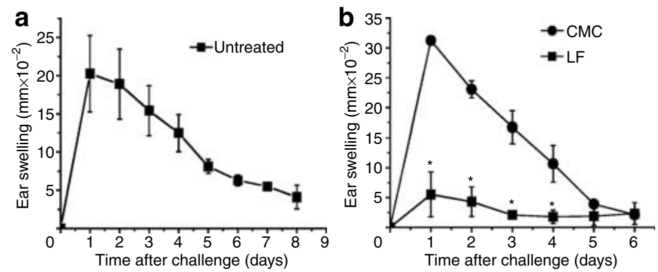

Figure 9. Inhibition of the CHS response by LF administered to hapten-presensitized mice. Mice ($n=20$) were sensitized with 25 μl of 3% OXA on the shaved abdomen on day 0 and challenged with 10 μl of 0.15% OXA on each side of the right ear on day 5. The left ear was treated with the vehicle for control. The mean increase in ear thickness ± SEM is depicted in (**a**). Half the mice were orally administered LF in CMC for 6 days starting day 18 of sensitization, whereas the other half received CMC alone. On day 19, both groups of mice were again sensitized with OXA on the back and challenged 5 days later. Mean values of ear swelling ± SEM are shown in (**b**). Data are compiled from two independent experiments with eight and 12 mice for (**a**) and four and six mice per group for (**b**). Asterisks denote statistically significant differences ($P<0.05$) as compared with mice treated with CMC.

LF abrogates CHS responses when administered to previously sensitized mice

The question arose whether LF would also be effective in inhibiting already existing CHS responses and might thus be of use for the treatment of allergic contact dermatitis. Mice were sensitized with OXA once on the abdomen in the absence of LF and were ear challenged 5 days later, resulting in an efficient ear swelling response (Figure 9a). On day 19, half the mice were again sensitized and challenged with OXA, but this time in the presence of LF, or CMC for control. As depicted in Figure 9b, the extent of ear swelling was substantially reduced ($P<0.05$) in the LF-treated group as compared with the CMC-treated group, pointing to therapeutic potential of LF in allergic contact dermatitis.

DISCUSSION

In this study, we used a mouse model of allergic contact dermatitis to evaluate whether the immunomodulatory compound LF inhibited a contact allergic response induced by epicutaneous application of the haptens DNFB and OXA. We demonstrate that the inhibition of CHS responses by LF is antigen-specific and long-lasting. This compound induced regulatory T cells that could transfer hapten-specific tolerance to naïve recipient mice. Inhibition was also observed,

when LF was administered together with the hapten to hapten-presensitized mice.

The observed suppression of CHS responses in LF-treated mice suggested that expansion of contact allergen-specific effector T cells was inhibited by LF and/or that T cells with counter-regulatory potential had developed. LF may inhibit activation of hapten-specific effector T cells owing to its effect on *de novo* pyrimidine biosynthesis. The active metabolite of LF, A77 1726, was shown to bind with high affinity to dehydro-orotate dehydrogenase, the rate-limiting enzyme in the *de novo* biosynthetic pathway of pyrimidines, inhibiting its enzymatic activity and thus causing a loss of *de novo* pyrimidine synthesis (Williamson *et al*., 1995). During T-cell activation, the pyrimidine pool expands about eight-fold, and the expression of T-cell effector functions is dependent on *de novo* biosynthesis of pyrimidines (Fairbanks et al., 1995). Therefore, as a result of LF action, proliferation of T cells is suppressed (Ruckemann *et al*., 1998; Herrmann *et al*., 2000). Another consequence of the inhibitory activity of LF on dehydro-orotate dehydrogenase is inhibition of NF-κB-associated kinases (Manna *et al*., 2000). Several cytokine genes, for example, the IFN-γ gene, harbor NF-κB binding site(s) in their promoter region (Sica *et al*., 1997). The production of IFN-γ by myelin-basic protein-specific T-cell lines stimulated with their cognate antigen *in vitro* was reported to be inhibited by LF (Korn *et al*. 2004). In the CHS model used here, we observed reduced production of IFN-γ in draining lymph node cells from LF-treated *versus* CMC-treated mice isolated 24 hours after challenge of CHS and stimulated *in vitro* with DNFB (data not shown). This finding is in line with the notion that expansion of hapten-specific T cells was inhibited by the treatment of mice with LF.

The loss of CHS responsiveness was long-lasting and could be transferred to naive recipient mice by regional lymph node and spleen cells from mice previously treated with LF. Transfer of both CD4$^+$ and CD8$^+$ cells was required for maximal inhibition of allergic responses in the recipient mice, with CD8$^+$ cells playing a major role. These findings indicate that cells with counter-regulatory activity were induced as a consequence of LF treatment of mice. The most likely explanation would be that CD4$^+$ and CD8$^+$ T lymphocytes were responsible for transferring tolerance. Th2 cells have previously been shown to regulate CHS responses to DNFB (Xu *et al*., 1996a). LF was reported to promote Th2 differentiation from uncommitted precursors (Dimitrova *et al*., 2002). Increased levels of antigen-specific IgG1 antibodies were observed in mice immunized with keyhole limpet hemocyanin in the presence of LF, which correlated with an increase in IL-4 and a decrease in IFN-γ production by splenocytes obtained from these mice on restimulation with keyhole limpet hemocyanin *in vitro*. Contrary to this report, we had previously shown that LF suppressed ovalbumin-specific IgG1 and IgE as well as IgG2a antibody responses, in a murine model of type I allergy (Jarman *et al*., 1999). Similarly, ovalbumin-stimulated splenocytes obtained from mice sensitized with ovalbumin adsorbed to aluminum hydroxide, in the presence of LF, produced reduced levels of the Th2 cytokines IL-4 and IL-5 as compared with splenocytes from CMC-treated mice. In the experiments outlined here, we could not detect elevated levels of IL-4 or IL-5 in supernatants obtained from DNFB-stimulated spleen and lymph node cells isolated from LF-treated mice, 24 hours after challenge with DNFB (data not shown).

Treg have been shown to exert suppressive activity for CD4$^+$ Th1 and Th2 as well as CD8$^+$ T-cell responses in several experimental systems and disease states. Treg expressing CD4$^+$ and CD8$^+$ phenotypes have been described (Fehervari and Sakaguchi, 2004; Faunce *et al*., 2004). Naturally occurring CD4$^+$CD25$^+$ Treg seem to suppress T-cell responses by cell-to-cell contact, whereas the inhibitory activity of induced Treg is dependent on the secretion of soluble factors like IL-10 (Tr1) or tumor growth factor–β (Th3). Induced CD4$^+$ Treg were shown to express high levels of CD25, CTLA-4, and GITR as well as the transcription factors Foxp3 and ROG, and the regulator of G-protein signalling, RGS1 (Cobbold *et al*., 2003; Fehervari and Sakaguchi, 2004). We did not find an enhanced level of CD4$^+$CD25$^+$ cells co-expressing CTLA-4 or GITR in spleen and lymph node cell populations obtained from LF-treated and DNFB-challenged mice by cytofluorometry (data not shown). Nor could we detect by real-time PCR elevated levels of mRNA for Foxp3, ROG, RGS1, or for IL-10 and tumor growth factor–β1 in the CD4$^+$ T-cell population isolated from the leukocyte preparation shown to transfer tolerance. We did not observe increased expression of the cell surface markers CD5, reported to be associated with T-cell unresponsiveness (Hawiger *et al*., 2004), or LAG-3, which contributes to the suppressor activity of induced Treg (Huang *et al*., 2004). These findings, however, do not exclude that a minor population of DNFB-specific CD4$^+$ Treg was induced by LF, which was not detectable owing to the presence of a dominant population of natural Treg.

CD8$^+$ Tregs have not been as intensely studied as their CD4$^+$ counterparts. The most extensively characterized population of induced CD8$^+$ Treg are the human CD8$^+$CD28$^-$ suppressor T cells, which express Foxp3 and induce a cell-to-cell contact-dependent tolerogenic phenotype in antigen-presenting cells owing to downregulation of costimulatory molecules and an upregulation of the inhibitory receptors ILT3 and ILT4 (Manavalan *et al*., 2004). A second distinct population of CD8$^+$CD28$^-$ suppressor T cells has been shown to exert inhibitory activity on CD4$^+$ and CD8$^+$ T cells in an antigen-nonspecific manner, through the release of soluble factors (Filaci *et al*., 2004). We did not observe an increase in the frequency of CD8$^+$CD28$^-$ cells, as determined by cytofluorometric analysis, in the leukocyte population capable of transferring suppression (data not shown). Nor did we detect an increment in Foxp3 transcripts in CD8$^+$ T cells isolated from this population. Again, a minor population of DNFB-specific CD8$^+$ Treg with these characteristics might have escaped from detection. A separate study described CD8$^+$ Treg induced on stimulation with allogeneic plasmacytoid dendritic cell, which inhibited CD4$^+$ as well as CD8$^+$ T cells via IL-10 secretion (Gilliet and Liu, 2002). In our study, CD8$^+$ T cells from LF- and

DNFB-treated mice similarly expressed significantly augmented levels of IL-10 mRNA. Therefore, it is conceivable that these cells represent the Treg population. In accordance with these findings, we demonstrated that spleen and draining lymph node cells from LF- and DNFB-treated mice depleted of $CD4^+$ cells efficiently inhibited CHS responses to DNFB after transfer into naïve mice, whereas following depletion of $CD8^+$ cells, the resulting leukocytes exerted less efficient suppressive capacity. Nevertheless, both $CD8^+$ and $CD4^+$ cells were required for optimal suppression, as transfer of the non-depleted leukocyte population was required for maximal suppression.

The mechanisms leading to induction of $CD8^+$ T suppressor cells are not known. As an explanation, one may invoke a different sensitivity of effector T cells and Treg towards LF. It was shown recently that LF was extruded from cells by the action of multidrug-resistant pumps (van der Heijden *et al.*, 2004). Different T-cell subsets, namely resting Th1 and Th2 cells, were reported to differ with respect to the activity of these pumps (Lohoff *et al.*, 1998). The possibility therefore exists that Treg are superior to effector T cells in their capacity to extrude LF, rendering T effector cells more prone to the detrimental effects of the compound.

Treg cells are known to be induced by stimulation with their cognate antigen presented by dendritic cells matured in the presence of exogenous IL-10 Steinbrink *et al.*, 1997; Müller *et al.*, 2002) or secreting endogenous IL-10 (Wakkach *et al.*, 2003). In a recent report, LF was demonstrated to enhance production of IL-10 in LPS-stimulated microglia (Korn *et al.*, 2004). It remains to be tested whether LF has a similar effect on dendritic cell function.

The inhibitory effect of LF on CHS responses was even observed when mice presensitized with hapten were treated with LF in the presence of the hapten. This finding may suggest that Treg induced by LF treatment efficiently inhibited pre-existing effector T cells of CHS responses. Further studies are required to verify that LF-induced Treg indeed are responsible for suppression of effector T cells in this setting. The capacity of Treg to efficiently suppress CHS effector T cells was recently demonstrated using Treg induced by epicutaneous application of haptens to UV-exposed skin (Schwarz *et al.*, 2004). These Treg efficiently inhibited the elicitation of CHS, when injected into the ears of sensitized mice, but were not effective, when injected intravenously. On the other hand, intravenously injected Treg were capable of inhibiting the induction of CHS. Expression of the lymph node homing receptor L-selectin, but not of the ligands for the skin-homing receptors E- and P-selectin by Treg was the basis of this finding. It will be interesting to test whether LF-induced Treg express ligands for E- and P-selectin.

Taken together, our findings imply that short-term administration of LF at the time of encounter with a contact allergen leads to a long-lasting inhibition of CHS reactivity despite renewed encounter with the hapten and to inhibition of a pre-existing CHS response. The use of LF may thus provide new opportunities for the treatment of allergic contact dermatitis.

MATERIALS AND METHODS

Mice

BALB/c mice were bred and maintained under specific pathogen free conditions on a standard diet. They were used at 8–16 weeks of age. The ''Principles of Laboratory Animal Care'' (NIH publication no. 85–23, revised 1985) were followed. The experiments were approved by the Ethics Commission according to the German Law on the Protection of Animals.

Induction of CHS and treatment with LF

CHS was induced as described (Weigmann *et al.*, 1997) with modifications. Mice were sensitized on days 0 and 1 by applying 25 μl of a 0.5% solution of DNFB (Sigma, Deisenhofen, Germany) in acetone/olive oil (4:1) to the shaved abdomen. On day 4, mice were challenged by applying 10 μl of 0.2% DNFB solution to both sides of the right ear. When OXA was used as a contact allergen, mice were sensitized once with 25 μl of 3% OXA (Sigma) in acetone/olive oil (4:1) and challenged with 10 μl of 0.15% OXA on both sides of the right ear 5 days later, unless indicated otherwise. In all cases, 10 μl of the vehicle acetone/olive oil were applied to both sides of the left ear. Measurements of ear thickness were performed blindly using an engineer's micrometer (Mitutoyo, Japan) both before and at the indicated time points following challenge. Ear swelling was calculated by subtracting the thickness of the vehicle-challenged contralateral ear ($\leqslant$0.02 mm) from the swelling recorded for the hapten-challenged ear in sensitized animals. Age-matched mice that were challenged without previous sensitization exhibited ear swelling $\leqslant$0.03 mm for DNFB and $\leqslant$0.05 for OXA.

LF HWA486 was a generous gift from Aventis Pharma (Frankfurt am Main, Germany). Mice received 40 mg/kg body weight of LF in 1% CMC (100 μl) via a stomach tube daily for 6 days starting on day –1 before sensitization. The solution was prepared shortly beforehand by sonication to emulsify LF in CMC. The prodrug LF is rapidly converted to the active metabolite A77 1726. The structures of LF and A77 1726 are depicted in Figure 1.

Adoptive transfer of lymphocytes

CHS was induced in LF- and CMC-treated mice, respectively, as described above. Spleens and draining (cervical, axillary, and inguinal) lymph nodes were prepared 7 days after the start of sensitization with DNFB and were pooled. Single-cell suspensions were prepared by teasing the tissue apart and passing it through a fine wire mesh to remove cell debris. For depletion of T-cell subsets, the cells were incubated with anti-CD4 monoclonal antibody clone GK1.5 (Dialynas *et al.*, 1983) or anti-CD8 monoclonal antibody clone 53.6.72 (Ledbetter and Herzenberg, 1979). Subsequently, $CD4^+$ and $CD8^+$ cells were depleted by immunomagnetic separation using sheep anti-rat IgG Dynabeads (Dynal, Oslo, Norway) as recommended by the manufacturer. The efficiency of depletion was determined by flow cytometric analysis: $1.7 \pm 1.5\%$ $CD4^+$ cells remained in the CD4-depleted cell suspension and $2.0 \pm 1.6\%$ $CD8^+$ cells remained in the CD8-depleted cell suspension. Unseparated leukocytes or leukocyte preparations depleted of $CD4^+$ and $CD8^+$ cells, respectively, were injected intravenously (10^8 cells) into naive syngeneic recipients. After 24 and 48 hours, recipient mice were sensitized, and 3 days later, challenged with DNFB as described. One group of mice did not receive donor cells, but was sensitized and challenged with DNFB in parallel with the

Table 1. Primers used for real-time PCR

Gene	Sequence
Ubiquitin C	
Forward	5′-GTCTGCTGTGTGAGGACTGC-3′
Reverse	5′-CAGGGTGGACTCTTTCTGGA-3′
Foxp3	
Forward	5′-CTTATCCGATGGGCCATCCTGGAAG-3′
Reverse	5′-TTCCAGGTGGCGGGGTGGTTTCTG-3′
ROG	
Forward	5′-CCCACTGACCTTGAACCACT-3′
Reverse	5′-GCCCAGACGTTTCTATCTGC-3′
RGS1	
Forward	5′-TTGGAATGGACGTGAAAACA-3′
Reverse	5′-CCTCACAAGCCAACCAGAAT-3′
IL-10	
Forward	5′-CCAAGCCTTATCGGAAATGA-3′
Reverse	5′-TTTTCACAGGGGAGAAATCG-3′
TGF-β1	
Forward	5′-TTGCTTCAGCTCCACAGAGA-3′
Reverse	5′-TGGTTGTAGAGGGCAAGGAC-3′
CD5	
Forward	5′-AGGAGCCCTACACCGATCTT-3′
Reverse	5′-AAGTGGGCAGCACTCAAAGT-3′
LAG-3	
Forward	5′-TCCTGTTACTGCCCCAAGTC-3′
Reverse	5′-CACTTGGCAATGAGCAAAGA-3′

recipient mice. A separate group was challenged with DNFB alone without prior sensitization and cell transfer. The increase in ear thickness was measured at the indicated time points following challenge.

Positive isolation of CD4$^+$ and CD8$^+$ T cells

CD4$^+$ and CD8$^+$ T cells in draining lymph node and spleen cell suspensions from LF-treated mice as well as CMC-treated control mice, sensitized and challenged with DNFB, were enriched by magnetic separation as described previously (Sudowe *et al.*, 2000). The purity of positively selected T-cell populations was verified by cytofluorometric analysis and constituted 87.0±3.7% for CD4$^+$ T cells and 91.0±2.0% for CD8$^+$ T cells.

Real-time reverse transcription-polymerase chain reaction-PCR analysis

To assess transcriptional changes of markers of induced regulatory T cells at the mRNA level, CD4$^+$ and CD8$^+$ T cells from mice treated with LF or CMC as control before and following elicitation of CHS were isolated on day 7 as described above. Total RNA was isolated from at least 4×10^5 cells per group by using the RNeasy MiniPrep kit (Qiagen, Hilden, Germany) and performing on-column DNase treatment (Qiagen) according to the protocol recommended by the manufacturer. Ten microliters of eluted RNA was reverse-transcribed applying 1 μl of a 1:1 mix of Oligo-dT and random hexamer primers and using the Reverse-IT RTase Blend kit (ABgene, Hamburg, Germany) as recommended by the manufacturer. Primer sequences were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; Rozen and Skaletsky, 2000), and primer pairs bridging intron–exon bounderies were chosen (Table 1). Primers were purchased from MWG Biotech (Ebersberg, Germany). Reaction mixtures for real-time PCR had a final volume of 25 μl and included 200 ng of cDNA, 12.5 μl of SYBRGreen mastermix (Absolute QPCR SYBRGreen Fluorescein Mix; ABgene), and 1.75 μl of premixed forward and reverse primer. PCR conditions were: 15 minutes at 95°C, and 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each sample was run in duplicate. The housekeeping gene ubiquitin C served as control and was used for subsequent normalization. Real-time PCR was performed in an iCycler iQ Multicolor Real-Time PCR system (Bio-Rad, München, Germany) and analyzed by using the iCycler iQ Optical System Software vers. 3.0a (Bio-Rad). Specificity of product amplification was confirmed by automated melting curve analysis. Relative quantification of gene expression was performed by applying the comparative threshold cycle method (Winer *et al.*, 1999).

Statistical analysis

Statistical evaluation of the experimental data was performed by Student's *t*-test. A value of $P<0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Bour H, Peyron E, Gaucherand M, Garrigue JL, Desvignes C, Kaiserlian D *et al.* (1995) Major histocompatibility complex class I-restricted CD8+ T cells and class II-restricted CD4+ T cells, respectively, mediate and regulate contact sensitivity to dinitrofluorobenzene. *Eur J Immunol* 25:3006–10

Cao WW, Kao PN, Chao AC, Gardner P, Ng J, Morris RE (1995) Mechanism of the antiproliferative action of leflunomide. A77 1726, the active metabolite of leflunomide, does not block T-cell receptor-mediated signal transduction but its antiproliferative effects are antagonized by pyrimidine nucleosides. *J Heart Lung Transplant* 14:1016–30

Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N *et al.* (2003) Conversion of peripheral CD4+CD25− naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198:1875–86

Cherwinski HM, Byars N, Ballaron SJ, Nakano GM, Young JM, Ransom JT (1995) Leflunomide interferes with pyrimidine nucleotide biosynthesis. *Inflamm Res* 44:317–22

Cobbold SP, Nolan KF, Graca L, Castejon R, Le Moine A, Frewin M *et al.* (2003) Regulatory T cells and dendritic cells in transplantation tolerance: molecular markers and mechanisms. *Immunol Rev* 196:109–24

Cutolo M, Sulli A, Ghiorzo P, Pizzorni C, Craviotto C, Villaggio B (2003) Anti-inflammatory effects of leflunomide on cultured synovial macrophages from patients with rheumatoid arthritis. *Ann Rheum Dis* 62:297–302

Dialynas DP, Wilde DB, Marrack P, Pierres A, Wall KA, Havran W *et al.* (1983) Characterization of the murine antigenic determinant, designated L3T4a, recognized by monoclonal antibody GK1. 5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. *Immunol Rev* 74:29–56

Dimitrova P, Skapenko A, Herrmann ML, Schleyerbach R, Kalden JR, Schulze-Koops H (2002) Restriction of *de novo* pyrimidine biosynthesis inhibits Th1 cell activation and promotes Th2 cell differentiation. *J Immunol* 169:3392–9

Elder RT, Xu X, Williams JW, Gong H, Finnegan A, Chong AS (1997) The immunosuppressive metabolite of leflunomide, A77 1726, affects murine T cells through two biochemical mechanisms. *J Immunol* 159:22–7

Fairbanks LD, Bofill M, Ruckemann K, Simmonds HA (1995) Importance of ribonucleotide availability to proliferating T-lymphocytes from healthy humans. Disproportionate expansion of pyrimidine pools and contrasting effects of *de novo* synthesis inhibitors. *J Biol Chem* 270: 29682–9

Faunce DE, Terajewicz A, Stein-Streilein J (2004) *In vitro*-generated tolerogenic APC induce $CD8^+$ T regulatory cells that can suppress ongoing experimental autoimmune encephalomyelitis. *J Immunol* 172:1991–5

Fehervari Z, Sakaguchi S (2004) CD4+ Tregs and immune control. *J Clin Invest* 114:1209–17

Filaci G, Fravega M, Fenoglio D, Rizzi M, Negrini S, Viggiani R *et al.* (2004) Non-antigen specific CD8+ T suppressor lymphocytes. *Clin Exp Med* 4:86–92

Gilliet M, Liu YJ (2002) Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. *J Exp Med* 195:695–704

Glant TT, Mikecz K, Brennan F, Negroiu G, Bartlett R (1994) Suppression of autoimmune responses and inflammatory events by leflunomide in an animal model for rheumatoid arthritis. *Agents Actions* 41:C267–70

Hamilton LC, Vojnovic I, Warner TD (1999) A771726, the active metabolite of leflunomide, directly inhibits the activity of cyclo-oxygenase-2 *in vitro* and *in vivo* in a substrate-sensitive manner. *Br J Pharmacol* 127:1589–96

Hawiger D, Masilamani RF, Bettelli E, Kuchroo VK, Nussenzweig MC (2004) Immunological unresponsiveness characterized by increased expression of CD5 on peripheral T cells induced by dendritic cells *in vivo*. *Immunity* 20:695–705

Herrmann ML, Schleyerbach R, Kirschbaum BJ (2000) Leflunomide: an immunomodulatory drug for the treatment of rheumatoid arthritis and other autoimmune diseases. *Immunopharmacology* 47:273–89

Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G *et al.* (2004) Role of LAG-3 in regulatory T cells. *Immunity* 21:503–13

Imose M, Nagaki M, Kimura K, Takai S, Imao M, Naiki T *et al.* (2004) Leflunomide protects from T-cell-mediated liver injury in mice through inhibition of nuclear factor κB. *Hepatology* 40:1160–9

Jarman ER, Kuba A, Montermann E, Bartlett RR, Reske-Kunz AB (1999) Inhibition of murine IgE and immediate cutaneous hypersensitivity responses to ovalbumin by the immunomodulatory agent leflunomide. *Clin Exp Immunol* 115:221–8

Korn T, Magnus T, Toyka K, Jung S (2004) Modulation of effector cell functions in experimental autoimmune encephalomyelitis by leflunomide – mechanisms independent of pyrimidine depletion. *J Leukocyte Biol* 76:950–60

Kurtz ES, Bailey SC, Arshad F, Lee AA, Przekop PA (1995) Leflunomide: an active antiinflammatory and antiproliferative agent in models of dermatologic disease. *Inflamm Res* 44:S187–8

Ledbetter JA, Herzenberg LA (1979) Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol Rev* 47:63–90

Levings MK, Bacchetta R, Schulz U, Roncarolo MG (2002) The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells. *Int Arch Allergy Immunol* 129:263–76

Liu Z, Tugulea S, Cortesini R, Suciu-Foca N (1998) Specific suppression of T helper alloreactivity by allo-MHC class I-restricted $CD8^+CD28^-$ T cells. *Int Immunol* 10:775–83

Lohoff M, Prechtl S, Sommer F, Roellinghoff M, Schmitt E, Gradehandt G *et al.* (1998) A multidrug-resistance protein (MRP)-like transmembrane pump is highly expressed by resting murine T helper (Th) 2, but not Th1 cells, and is induced to equal expression levels in Th1 and Th2 cells after antigenic stimulation *in vivo*. *J Clin Invest* 101:703–10

Manavalan JS, Kim-Schulze S, Scotto L, Naiyer AJ, Vlad G, Colombo PC *et al.* (2004) Alloantigen specific $CD8^+CD28^-Foxp3^+$ T suppressor cells induce $ILT3^+ILT4^+$ tolerogenic endothelial cells, inhibiting alloreactivity. *Int Immunol* 16:1055–68

Manna SK, Mukhopadhyay A, Aggarwal BB (2000) Leflunomide suppresses TNF-induced cellular responses: effects on NF-kappa B, activator protein-1, c-Jun N-terminal protein kinase, and apoptosis. *J Immunol* 165:5962–9

Müller G, Müller A, Tüting T, Steinbrink K, Saloga J, Szalma C *et al.* (2002) Interleukin-10-treated dendritic cells modulate immune responses of naive and sensitized T cells *in vivo*. *J Invest Dermatol* 119:836–41

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics methods and protocols: methods in molecular biology* (Krawetz S, Misener S, eds), Totowa, NJ: Humana Press, 365–86

Ruckemann K, Fairbanks LD, Carrey EA, Hawrylowicz CM, Richards DF, Kirschbaum B *et al.* (1998) Leflunomide inhibits pyrimidine *de novo* synthesis in mitogen-stimulated T-lymphocytes from healthy humans. *J Biol Chem* 273:21682–91

Schmitt J, Wozel G, Pfeiffer C (2004) Leflunomide as a novel treatment option in severe atopic dermatitis. *Br J Dermatol* 150:1182–5

Schwarz A, Maeda A, Wild MK, Kernebeck K, Gross N, Aragane Y *et al.* (2004) Ultraviolet radiation-induced regulatory T cells not only inhibit the induction but can suppress the effector phase of contact hypersensitivity. *J Immunol* 172:1036–43

Sica A, Dorman L, Viggiano V, Cippitelli M, Ghosh P, Rice N *et al.* (1997) Interaction of NF-κB and NFAT with the interferon-gamma promoter. *J Biol Chem* 272:30412–20

Siemasko K, Chong AS, Jack HM, Gong H, Williams JW, Finnegan A (1998) Inhibition of JAK3 and STAT6 tyrosine phosphorylation by the immunosuppressive drug leflunomide leads to a block in IgG1 production. *J Immunol* 160:1581–8

Steinbrink K, Wolfl M, Jonuleit H, Knop J, Enk AH (1997) Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* 159:4772–80

Strand V, Cohen S, Schiff M, Weaver A, Fleischmann R, Cannon G *et al.* (1999) Treatment of active rheumatoid arthritis with leflunomide compared with placebo and methotrexate. Leflunomide Rheumatoid Arthritis Investigators Group. *Arch Intern Med* 159:2542–50

Sudowe S, Arps V, Vogel T, Kölsch E (2000) The role of interleukin-4 in the regulation of sequential isotype switch from immunoglobulin G1 to immunoglobulin E antibody production. *Scand J Immunol* 51: 461–71

van der Heijden J, de Jong MC, Dijkmans BA, Lems WF, Oerlemans R, Kathmann I *et al.* (2004) Acquired resistance of human T cells to sulfasalazine: stability of the resistant phenotype and sensitivity to non-related DMARDs. *Ann Rheum Dis* 63:131–7

Wakkach A, Fournier N, Brun V, Breittmayer JP, Cottrez F, Groux H (2003) Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation *in vivo*. *Immunity* 18:605–17

Weigmann B, Schwing J, Huber H, Ross R, Mossmann H, Knop J *et al.* (1997) Diminished contact hypersensitivity response in IL-4 deficient mice at a late phase of the elicitation reaction. *Scand J Immunol* 45:308–14

Williams JW, Xiao F, Foster P, Clardy C, McChesney L, Sankary H *et al.* (1994) Leflunomide in experimental transplantation: control of rejection and alloantibody production, reversal of acute rejection, and interaction with cyclosporine. *Transplantation* 57:1223–31

Williamson RA, Yea CM, Robson PA, Curnock AP, Gadher S, Hambleton AB *et al.* (1995) Dihydroorotate dehydrogenase is a high affinity binding protein for A77 1726 and mediator of a range of biological effects of the immunomodulatory compound. *J Biol Chem* 270: 22467–72

Winer J, Jung CK, Shackel I, Williams PM (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes *in vitro*. *Anal Biochem* 270:41–9

Xu H, DiIulio NA, Fairchild RL (1996a) T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon gamma-producing (Tc1) effector $CD8^+$ T cells and interleukin (Il) 4/Il-10-producing (Th2) negative regulatory $CD4^+$ T cells. *J Exp Med* 183:1001–12

Xu X, Williams JW, Bremer EG, Finnegan A, Chong AS (1995) Inhibition of protein tyrosine phosphorylation in T cells by a novel immunosuppressive agent, leflunomide. *J Biol Chem* 270:12398–403

Xu X, Williams JW, Gong H, Finnegan A, Chong AS (1996b) Two activities of the immunosuppressive metabolite of leflunomide, A77 1726. Inhibition of pyrimidine nucleotide synthesis and protein tyrosine phosphorylation. *Biochem Pharmocol* 52:527–34

Yao HW, Li J, Chen JQ, Xu SY (2004) A 771726, the active metabolite of leflunomide, inhibits TNF-α and IL-1 from Kupffer cells. *Inflammation* 28:97–103