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REVIEW ARTICLE

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Leflunomide: an immunosuppressive drug with multiple effects on T cell function

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Abstract Leflunomide has recently been introduced as a new treatment for rheumatoid arthritis. Although its immunosuppressive effect has been well demonstrated in experimentally induced autoimmune diseases and in organ transplant rejection in animal models, the exact mechanisms mediating its immunomodulatory effect are not fully understood. As T cells play a central role in the orchestration of immune responses in both physiological and pathological conditions, it has been proposed that the ability of leflunomide to suppress inappropriate and unwanted immunity is related to a functional inhibition of T cells. A precise knowledge of the mechanisms of leflunomide's action on T cells is therefore necessary. As the clinical effect of leflunomide has been well described elsewhere, this review will focus on, and will discuss, current data on the different aspects of leflunomide's effect on T cell function.

Key words Autoimmunity · Immunomodulation · Rheumatoid arthritis · T cells

Introduction

Leflunomide is an izoxazole pro-drug (n-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide) that is rapidly converted in the cytoplasm to an active compound, *N*-(4-trifluoromethylphenyl-2,2-cyano-3-hydroxycrotonamide), designated A77 1726, which is responsible for essentially all of the in vivo activities of the drug (Fig. 1). Recent studies have shown that leflunomide is a therapeutically effective immunosuppressant in animal models of autoimmune diseases such as spontaneous lupus in Mlp/lpr mice,[1] proteoglycan-induced arthritis,[2] the development of diabetes in NOD mice,[3] and in adjuvant[4] and collagen type II-induced arthritis in rats.[5] Moreover, leflunomide prevents the rejection of allo- and xenograft organ transplantations in animals.[6–8]

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Effect of leflunomide on T cell proliferation

An antiproliferative effect of leflunomide has been demonstrated in different in vitro systems where peripheral blood mononuclear cells were stimulated with mitogens such as monoclonal antibodies (mAb) to the CD3 complex and the costimulatory molecule CD28 (Fig. 2), with phorbol myristate acetate (PMA) or with phytohemagglutinin (PHA).[9–12] In these experiments, leflunomide inhibited T cell proliferation in a dose-dependent manner at concentrations above 25 μM leflunomide (Fig. 2). Chong et al.[13] have shown that the proliferation of the IL-2-dependent T cell line CTLL-4 was suppressed by different doses of leflunomide ranging from 1 μM to 100 μM. When the drug was present early after IL-2 supplementation in the culture, a maximum inhibition of cell proliferation was achieved, indicating that leflunomide interferes with early events involved in cell proliferation. In addition, a decrease in total RNA and DNA contents and an upregulation of the nuclear protein antigens proliferating-cell nuclear antigen (PCNA) and Ki-67 in leflunomide-treated, mitogen-stimulated rat spleen and human mononuclear cells has been reported, implying reduced proliferation in the presence of leflunomide.[11] Several studies have demonstrated that leflunomide causes the accumulation of T cells in the late G1 phase of the cell cycle, which results in a blockade of T cell proliferation.[11,14] Interestingly, the level of the tumor suppressor protein p53 in drug-treated T cells increased, suggesting that the cell cycle arrest might be assocciated with the activation of transcription factors and proteins involved in programmed cell death.[14,15] Activated p53 induces

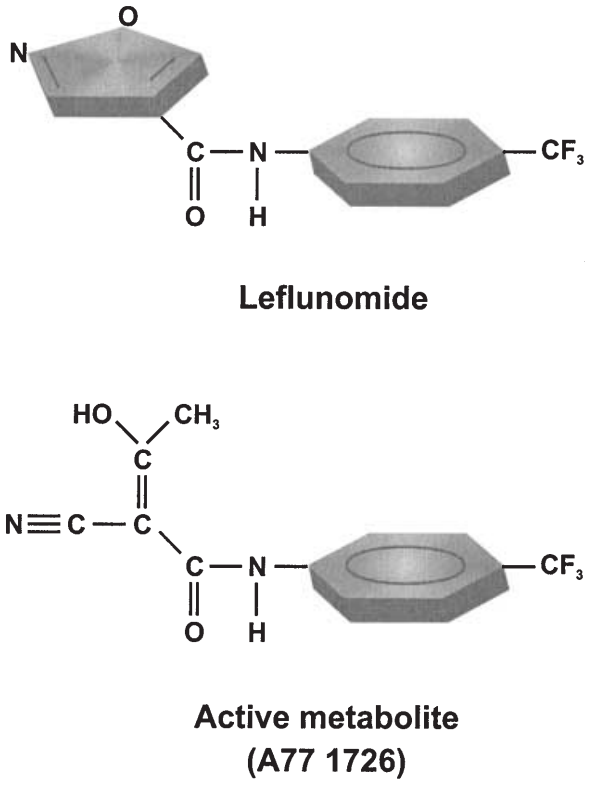

Fig. 1. Chemical structure of leflunomide and its active metabolite A77 1726

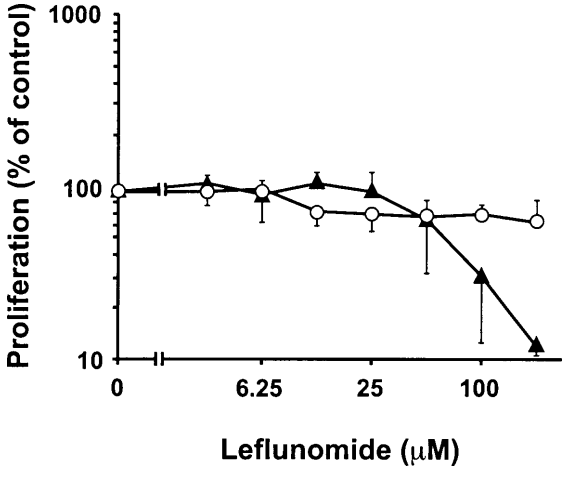

Fig. 2. Leflunomide inhibits the proliferation of human naive CD4 T cells. Naive CD4 T cells were isolated from cord blood and activated with mAbs to CD3 and CD28 in the presence of different concentrations of leflunomide and the presence (*open circles*) or absence (*closed triangles*) of uridine triphosphate for 5 days. ^{3}H-thymidine was added for the last 16 h of culture. The data represent mean ± SD of at least six individual experiments normalized to the control values in the absence of leflunomide

expression of the cyclin-dependent kinase (cdk) inhibitor protein p21$^{waf\text{-}1/cip\text{-}1}$ that suppresses the cyclin/cdk complex formation and the progression of the cell through the G1 to the S phase of the cell cycle, and might, thus, induce apoptosis.[15–18] Colic et al.[19] have shown that leflunomide induces the apoptosis of thymocytes and some T cell hydridomas, suggesting that the compound might have a strong potential to regulate inappropriate cell division characteristic of tumor cells,[20] and might also, by analogy, be important for the clearance of autoreactive T cells.

Effect of leflunomide on de novo pyrimidine biosynthesis

The proposed biochemical mechanism of the action of leflunomide is the inhibition of the de novo synthesis of pyrimidines. Leflunomide blocks the de novo pyrimidine biosynthetic pathway at the step of oxidation of dihydroorotate to orotate. Detailed analysis has demonstrated that the enzyme dihydroorotate dehydrogenase (DHODH), which catalyzes the oxidation of dihydroorotate to orotate, is the preferential target of the drug.[21–23] The reduced DHODH activity of mononuclear cells which infiltrate heart allograft tissue after leflunomide treatment,[24] and the accumulation of dihydroorotate in the serum of leflunomide-treated rats and in leflunomide-treated T lymphoblastoid cells in vitro,[25] are direct evidences for this proposed mode of action of leflunomide. Subsequent studies have shown that the active form of the drug, A77 1726, is a competitor of the ubiquinone binding site of human recombinant DHODH.[26] Liu et al.[27] have resolved the high-resolution crystal structure of human recombinant DHODH in complex with A77 1726. The analysis revealed that in the membrane-assocciated domain of the enzyme, a hydrophobic tunel leading to the redox site of the catalytic center is formed. The inhibitor, A77 1726, binds to a narrow region in this tunel, preventing the entry of quinone. These data clearly indicate that leflunomide inhibits the de novo biosynthetic pathway of pyrimidines by blocking DHODH activity. Although an inhibition of other ubiquinone binding proteins by A77 1726 has not formally been excluded, all the data imply that the inhibition of the ubiquinone binding site of DHODH is the major molecular activity of leflunomide, resulting in the inhibition of de novo pyrimidine synthesis.[21] Further support for this notion is derived from the observation that a dose-dependent inhibition of DHODH activity correlates with a dose-dependent inhibition of T cell proliferation.[22,26] Moreover, the blocking effect of leflunomide on T cell proliferation is reversible in vitro and in vivo by the addition of the exogenous pyrimidines uridine triphosphate (UTP) and cytidine triphosphate (CTP)[11,28] (see Fig. 2), but is not antagonized by the addition of the purines ATP and guanosine triphosphate (GTP),[11] confirming that the antiproliferative effect of the drug is related to the inhibition of the de novo pyrimidine pathway. As pyrimidine nucleosides serve as a substrate for RNA and DNA synthesis and are involved in several biochemical reactions related to lipid synthesis, protein glycosylation, and energy metabolism, the blockade of de novo pyrimidine synthesis has major implications on factors regulating cell proliferation. Importantly, resting T cells can use preformed nucleosides or nucleobases from the salvage path-

way of pyrimidines to meet their metabolic requirements, but activated lymphocytes are strongly dependent on the de novo biosynthesis pathway of pyrimidines[28,29] (see Fig. 2).

The leflunomide-induced depletion of the pyrimidine nucleotide pool,[11,30,31] however, might not only affect T cell proliferation, but might also alter T cell function via the modification of lipid synthesis[32] and protein glycosylation.[33,34] In relation to this, it has been reported that the expansion of UTP is important in order to provide enough substrates for the synthesis of UDP–Glu,[29,35] an essential intermediate for the glycosylation of adhesion molecules.[33,34] It is interesting to note that leflunomide down-modulates the expression of the adhesion molecules ICAM-1 and VCAM-1 on cells isolated from the synovium of leflunomide-treated patients with rheumatoid arthritis (RA),[36] as well as the expression of adhesion molecules P- and E-selectin on graft endothelial cells in the rat heart xenograft transplantation model.[37] Thus, by suppressing de novo pyrimidine synthesis and by depleting the nucleotide pool, leflunomide might influence cell migration and cell–cell contact.

CTP is an important substrate for the synthesis of cytidine diphosphate (CDP)-choline, an intermediate involved in the metabolism of inositol phosphates responsible for signal transduction events in activated T cells.[32,38] Thus, one might speculate that the blockade of the de novo synthesis of pyrimidines by leflunomide results in diminished T cell activation by altering T cell activation cascades.[38]

Recent studies have shown that leflunomide restricts (^{14}C)-hypoxanthine incorporation into GTP and ATP at concentrations above 100 μM, indicating that the purine salvage pathway in nonstimulated peripheral blood mononuclear cells and the de novo purine biosynthesis in mononuclear cells might also be compromised.[39] Depletion of ATP and GTP pools in peripheral blood mononuclear cells implies some serious consequences for the phosphorylation of ATP-dependent enzymes such as protein tyrosine kinases. Thus, leflunomide might cause a complete metabolic stasis of T cells, not only by inhibiting de novo pyrimidine synthesis, but also by restricting the purine pathways.[39]

Effect of leflunomide on T cell function

The full activation of T lymphocytes requires at least two distinct signals to proceed into the cell cycle.[40] The main signaling cascade is activated upon ligation of the T cell receptor (TCR) complex ("signal one"). Activation of a series of protein tyrosine kinases involved in different signaling pathways, including Zap70, Syk, p56^{Lck}, and p59^{Fyn}, occurs after TCR stimulation that results in an increase in intracellular Ca^{2+} concentration, the activation of transcription factors, and, ultimatively, gene transcription. A second set of signals is triggered by the ligation of nonpolymorphic cell surface receptors, for example, the costimulatory molecule CD28, and the activation of cytokine receptors by their cognate ligands.

Leflunomide inhibits the phosphorylation of some protein kinases belonging to the large family of cell surface receptors that is associated with intrinsic tyrosine kinase activity. These receptor kinases catalyze the transfer of the γ-phosphate of ATP to hydroxyl groups of tyrosines on target proteins.[41] Xu et al.[12] have shown that the active compound of leflunomide, A77 1726, reduces the intracellular levels of tyrosine phosphorylated proteins in LSTRA T cells. Furthermore, leflunomide is able to suppress the phosphorylation of p56^{Lck} and p59^{Fyn} in CTLL-4[42] and in Jurkat T cell lines.[43] Both kinases are closely associated with the ζ chain of the TCR, and once activated they recruite Zap70 to the aggregated receptor.[44] A critical threshold of Zap70 activity is required to sustain downstream signaling, which is initiated after TCR ligation.[45] Thus, leflunomide interferes with early events involved in T cell activation by influencing the phosphorylation rate of p56^{Lck} and p59^{Fyn}, and subsequently Zap70 activation. Importantly, as the presence of exogenous UTP during the stimulation of T cells was not able to restore the diminished phosphorylation of tyrosine kinases by leflunomide, the effect of the drug on tyrosine kinase phosphorylation appears to occur independently of the inhibition of the de novo pyrimidine synthesis pathway.[42,43]

Leflunomide inhibits the activation of the γ-isoform of the enzyme phospholipase C (PLC-γ1) that catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate to diacylglycerol and inositol 1,4,5-triphosphate, which induces a rapid increase in free cytosolic Ca^{2+}. Free Ca^{2+} binds to the ubiquitous Ca^{2+}-dependent regulatory protein calcineurin, which dephosphorylates the transcription factor NF-AT. Activated NF-AT translocates into the nucleus and binds to a consensus-binding sequence in the regulatory region of the IL-2, the IL-4, and other cytokine gene promotors, predominantly in association with the transcription factor AP-1.[46] Therefore, the initial blockade of PLC-γ1 activity by leflunomide in T cells that were stimulated via the CD3 complex critically influences all downstream events, including Ca^{2+} influx and the activation of NF-AT.[43,47] Some reports have suggested that leflunomide is also a potent inhibitor of the transcription factor AP-1.[47] AP-1 is composed of two proteins, Fos and C-jun. C-jun is phosphorylated by JNK kinases. It has been suggested that the inhibitory effect of leflunomide on AP-1 activity is mediated by the inhibition of JNK.[47] However, although JNK is critical for AP-1 activity, JNK has also an effect on the level of p53 by inhibiting its ubiquitin-mediated degradation.[48] Direct evidence that the increased level of p53 in leflunomide-treated T cells is associated with the inhibition of JNK activity however has not yet been established.

Together, the data imply that leflunomide might suppress early events after T cell receptor ligation, including the activation of tyrosine protein kinases, adaptor proteins, and transcription factors. Moreover, the drug might regulate the threshold of T cell activation, and therefore might influence the direction of an immune response. A functional consequence of the suppression of T cell activation is the reduced expression of T cell activation markers such as CD69, CD25, and MHC II molecules.[28,43,49] As the expres-

sion of these molecules is important for further interactions of T cells with B cells, dendritic cells, or macrophages, leflunomide might be able to modulate the development of an intact immune response.

In contrast to the modulation of "signal one," the effect of leflunomide on the signaling triggered via costimulatory molecules has not been clearly established, although some data imply that the CD28 signaling pathway might not be affected by leflunomide.[50]

Recent reports have addressed the influence of leflunomide on downstream events involved in cytokine receptor expression and cytokine production of T cells. Leflunomide inhibits IL-2 production and IL-2R-α (CD25) expression on stimulated T cells.[28,43,51] As IL-2 critically regulates the magnitude and duration of the proliferative expansion of T cells and controls cell differentiation and survival, the reduction of IL-2 production by leflunomide might result in a blockade of T cell proliferation.[52] The binding of IL-2 to its cognate receptor results in the activation of multiple transduction pathways, including the JAK/STAT pathways that are unique to cytokines.[53] Elder et al.[10] and Siemasko et al.[49] have demonstrated that leflunomide inhibits the phosphorylation of the JAK1 and JAK3 kinases and suppresses the activation of STAT5. As peripheral T cells from mice that lack the genes for both STAT5 forms (STAT5a and STAT5b) are unable to proliferate in response to TCR engagement even in the presence of IL-2,[54] the inhibition of STAT5 phosphorylation by leflunomide might correlate with the antiproliferative activity of the drug. Moreover, as the expression of the cell cycle genes cdk6, cyclin D2, and cyclin D3 is downmodulated in STAT5a/b-deficient T cells,[55] the suppression of STAT5 activition by leflunomide might result in a cell cycle arrest of activated T cells. Of note, some reports have failed to demonstrate that leflunomide suppresses IL-2 production and IL-2R-α expression.[56–58] The distinct durations and modes of T cell activation in the different in vitro systems employed might have contributed to these controversial results. Whatever the explanation, a recent detailed kinetic analysis of the effect of leflunomide on isolated human CD4 T cell subsets clearly established the ability of leflunomide to inhibit CD25 expression after T cell activation induced by physiological stimulation of T cells via the CD3 complex and CD28.[28]

Of great interest with respect to the clinical immunomodulatory effect of leflunomide is recent evidence suggesting that priming of human naive CD4 T cells in vitro with anti-CD3 and anti-CD28 mAbs in the presence of different concentrations of leflunomide results in an increase in the frequencies of immunomodulatory IL-4-producing T helper (Th) type 2 cells.[28] Importantly, leflunomide also significantly inhibited the generation of proinflammatory IFN-γ-producing Th1 effector cells. While Th1 effectors are critically involved in the pathogenesis of autoimmune diseases such as RA, an alteration of the Th cell balance in favor of anti-inflammatory Th2 effectors at the cost of proinflammatory Th1 cells would have a beneficial effect on the inflammatory activity promoting these diseases.[59] Thus, one might speculate that leflunomide exerts its immunomodulatory activity by inhibiting the activation and generation of Th1 effectors and by promoting the differentiation of uncommitted precursors in the Th2 direction. Decreased IFN-γ production of Th effectors by leflunomide would result in diminished activation and altered function of macrophages and fibroblasts. For example, IFN-γ mediates its effects, in part, by activating the transcrip-tion of enzymes required for the generation of reactive oxygen and nitrogen intermediates in macrophages and fibroblasts.[60] Leflunomide is able to inhibit IFN-γ-induced NO-synthase activity and NO-generation in fibroblasts and macrophages directly, and it has been suggested that this effect is mainly associated with the disruption of the MEK/MAP-kinase pathway in those cells.[61] Some reports have demonstrated an inhibitory effect of leflunomide on T cell-dependent B cell activation, proliferation, and antibody production.[62] Together, these data indicate that the alteration of T cell function by leflunomide might significantly change the direction of an immune response by altering multiple facets of cellular immunity.

Interestingly, leflunomide induces self-tolerance in rats and in Mlp/lpr mice after the transfer of splenocytes.[63] The speculation that drug treatment resulted in an increase in anergic T cells with a suppressive potential is partially confirmed by a report of Cao et al.[64] describing an up-regulation of TGF-β secretion in leflunomide-treated T cells. Unfortunately, no evidence for the effect of leflunomide on IL-10 production of T cells is available. Future investigations delineating the effect of leflunomide on the activation of the population of regulatory T cells will be important and of great interest.

As tumor necrosis factor-alpha (TNF-α) is involved in the pathological immune response characteristic of many autoimmune diseases,[65] agents that down-modulate TNF-α-mediated proinflammatory effector functions are among the most potent anti-inflammatory drugs currently available. Manna et al.[47,66] have demonstrated that leflunomide inhibits the activation of the transcription factor NF-κB induced by various stress stimuli, including TNF-α, PMA, LPS, H_2O_2, okadaic acid, or ceramide. Furthermore, the drug blocks TNF-induced phosphorylation of the inhibitory subunit of NF-κB, I-κBa, largely by suppressing the activation of IKK-β kinase.[66] Leflunomide also blocks TNF-α-induced lipid peroxidation and the generation of reactive oxygen intermediates.[47,66] Thus, the evidence implies that leflunomide interferes with TNF-α-induced signaling events in T cells.

In summary, leflunomide blocks T cell proliferation and induces several functional changes in T cells. Leflunomide may act by two different mechanisms: first, the inhibition of DHODH activity, which remains suppressed at low doses of leflunomide and is related to the antiproliferative effect of the drug, and second, the blockade of protein tyrosine phosphorylation, which is observed at concentrations above 100 μM leflunomide. The down-regulation of the expression of activation markers, adhesion molecules, and cytokine receptors are direct evidences that leflunomide interferes with events involved in T cell activation. Decreased IL-2 and IFN-γ production and increased production of IL-4 and

TGF-β suggest that leflunomide might also change the direction of an immune response by altering the effector functions of T cells. Thus, the therapeutic effects of leflunomide in several animal models of autoimmune diseases, and in RA in humans, might be related to the ability of the drug to modulate fundamental cellular processes, including cell proliferation and differentiation, cell cycle progression, migration, metabolism, and the survival of T cells.

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