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Dose-dependent enhancing and inhibitory effects of A77 1726 (leflunomide) on cytotoxic T lymphocyte induction

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Abstract

Leflunomide is an immunosuppressive prodrug which prevents allograft rejection in several animal model systems and may, therefore, have clinical application in organ transplant recipients. Although cytotoxic T lymphocytes (CTL) are an important component of the allograft rejection response, the effect of leflunomide on CTL development has not been thoroughly explored. In this study we have determined the effect of A77 1726, the active metabolite of leflunomide, on CTL induction in C57BL/6 mouse T cell cultures stimulated with anti-CD3 monoclonal antibody. Conjugate formation with P815 target cells, granzyme B enzymatic activity in CTL lysates, and P815 cytolysis in a ^{51}Cr-release assay were used as determinants of in vitro CTL function. At high concentrations (10–20 μM), A77 1726 strongly inhibited CTL generation. In contrast, a low concentration (0.5 μM) of A77 1726 promoted CTL development. These dose-dependent opposing effects of A77 1726 on CTL induction could not be attributed to alterations in $CD8^+$ lymphocyte percentages, interleukin-2 or CD25 expression, or the ability to conjugate with P815 target cells. However, both interferon-γ and granzyme B expression were significantly decreased when CTL were induced in the presence of 10-20 μM A77 1726, and were slightly, but not always significantly, elevated in the presence of 0.5 μM A77 1726. We conclude that at high concentrations A77 1726 is a potent inhibitor of CTL induction, but a low concentration of A77 1726 enhances CTL development. © 1998 International Society for Immunopharmacology. Published by Elsevier Science Ltd

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1. Introduction

Although current immunosuppressive drugs such as cyclosporine have been remarkably successful at preventing acute allograft rejection in transplant recipients, their side-effects and failure to

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control chronic rejection have created interest in new immunosuppressive agents. Leflunomide is an isoxazol derivative with potent immunosuppressive and anti-inflammatory activity (Bartlett et al., 1991) which has recently been shown to prevent and reverse acute rejection of cardiac allografts and inhibit experimental chronic vascular rejection in rats (Williams et al., 1994; Swan et al., 1995). The primary metabolite of leflunomide, A77 1726 (Bartlett et al., 1991), inhibits in vitro T cell proliferation by the dual mechanisms of blocking pyrimidine biosynthesis and interfering with the enzymatic activity of protein tyrosine kinases involved in interleukin (IL)-2 receptor signal transduction (Elder et al., 1997). B cell proliferation and cell cycle progression in vitro is also inhibited by A77 1726, acting primarily at the level of pyrimidine biosynthesis (Siemasko et al., 1996).

Acute rejection of transplanted allogeneic tissues and organs is a cell-mediated event involving alloantigen-specific T helper cells and cytotoxic T lymphocytes (CTL), as well as nonspecific inflammatory cells such as macrophages (Hutchinson, 1991). Destruction of target cells by CTL is mediated largely by granzyme B and perforin molecules that are exocytosed from the allospecific CTL following cognate binding to the target cell (Podack, 1995). Indeed, perforin and granzyme B expression by T lymphocytes infiltrating transplanted tissues is an indication of imminent graft rejection (Legros-Maida et al., 1994). Although CTL play an important role in acute rejection responses, the effect of leflunomide on CTL induction has not been studied. Here, we describe the effect of A77 1726, the active metabolite of leflunomide, on CTL development in primary mouse T cell cultures following stimulation with anti-CD3 monoclonal antibody (mAb), which is known to induce nonspecific cytotoxicity in polyclonal $CD8^+$ T cell populations (Kaiser and Hoskin, 1992).

2. Experimental procedures

2.1. Animals

Six-week-old female C57BL/6 mice were purchased from Charles River Canada (Lasalle, PQ) and maintained on standard mouse chow and water in our animal care facilities. The care and use of animals in experimentation was in accordance with institutional guidelines.

2.2. Generation of anti-CD3-activated cytotoxic T cells

Spleens were removed aseptically from mice at sacrifice and single cell suspensions were prepared by mechanical dissociation of spleens in cold phosphate buffered saline, pH 7.2. Erythrocytes were depleted by osmotic shock and spleen cells were passaged through nylon wool (Cellular Products, Buffalo, NY) columns to enrich for T lymphocytes. Natural killer cells were removed from the nylon wool nonadherent spleen cells by a two-step treatment with anti-asialoGM1 antiserum (1/40; Wako Pure Chemicals, Richmond, VA) and rabbit complement (1/12; CedarLane Laboratories, Hornby, ON). The resulting T cell-enriched preparation was cultured in RPMI 1640 medium supplemented with 100 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (all ICN Biomedicals Canada, Mississauga, ON), 5 mM HEPES buffer (Sigma Chemical, St. Louis, MO), 5% heat-inactivated (at 56°C for 30 min) fetal calf serum (Gibco, Burlington, ON), and containing

anti-CD3 mAb (1/20 final dilution of culture supernatant from hybridoma 145-2C11; kindly provided by Dr. Bluestone, University of Chicago, IL), with or without graded concentrations of A77 1726 (a generous gift from Hoechst, Wiesbaden). T cell cultures were performed at 1.25×10^6 cells/ml (0.2 ml volume) in 96-well round-bottom microtiter plates (proliferation assay) or at 4×10^6 cells/ml (2 ml volume) in 24-well flat-bottom plates (all other assays) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cell-free supernatants were collected from 24-well plates at 48 h of culture and stored at −70°C for subsequent measurement of interferon-γ (IFN-γ) and IL-2 levels.

2.3. Lymphoproliferation assay

T cell proliferation in response to anti-CD3 mAb stimulation was measured at 48 h of culture by tritiated thymidine ([^{3}H]TdR) incorporation as previously described (Fitzpatrick et al., 1996). Briefly, 0.5 μCi [^{3}H]TdR (specific activity 65 Ci/mmol; ICN) was added to each well of the microtiter plate during the last 6 h of culture. Cultures were then harvested onto glass fiber mats and [^{3}H]TdR incorporation was determined by scintillation counting. Data from quadruplicate cultures are expressed as mean counts per minute (cpm) plus or minus the standard deviation (SD).

2.4. ^{51}Cr-release cytotoxicity assay

Cytolytic activity in 48 h anti-CD3-activated mouse T cell cultures was determined by ^{51}Cr-release assay as described earlier (Fitzpatrick et al., 1996). Briefly, P815 mastocytoma target cells were labeled with 100 μCi [^{51}Cr]sodium chromate (specific activity 250–500 mCi/mg Cr; ICN) and combined with anti-CD3-activated T cells in wells of a 96-well microtiter plate at various effector:target (E:T) cell ratios. Following a 4 h incubation at 37°C in a humidified atmosphere containing 5% CO_2, 0.1 ml of supernatant was collected from the wells and % ^{51}Cr released from lysed P815 cells was determined by γ-counting. Data are presented as the mean % ^{51}Cr released from triplicate samples ($\pm$SD).

2.5. ELISA

IFN-γ and IL-2 levels in supernatants from 48 h cultures of anti-CD3-activated T cells were measured by sandwich ELISA using paired mAb, recombinant cytokines and protocols supplied by Pharmingen Canada (Mississauga, ON).

2.6. Flow cytometric analysis

The percentage of $CD8^+$ and $CD25^+$ T lymphocytes in 48 h cultures of anti-CD3-activated T cells was determined by flow cytometric analysis using a standard protocol (Fitzpatrick et al., 1996) and fluorescein isothiocyanate-conjugated mAb to mouse CD8 or CD25 (CedarLane).

2.7. Conjugate formation assay

CTL binding to target cells was determined by conjugate formation assay as described (Hoskin et al., 1996). Data are presented as the mean percentage of conjugated T cells ($\pm$SD).

2.8. Colorimetric granzyme B assay

Granzyme B activity in the cytosolic fraction of MHC-unrestricted CTL induced with anti-CD3 mAb was measured as described (Fitzpatrick et al., 1996) using the granzyme B-specific synthetic substrate Boc–Ala–Ala–Asp thiobenzyl ester (Enzyme Systems Products, Dublin, CA). Data are expressed as mean units of esterolytic activity/10^6 anti-CD3-activated T cells in quadruplicate samples ($\pm$SD).

2.9. Statistical analysis

Data analysis was performed using the Instat statistics program (GraphPad Software, San Diego, CA). Statistical significance of data was tested using the *t* statistic evaluation and Student's *t* distribution. *P* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Lymphoproliferation and CTL induction in the presence of A77 1726

We tested the effect of A77 1726 on murine (C57BL/6) T cell proliferation and CTL induction at 48 h of culture in response to in vitro stimulation with anti-CD3 mAb. T cell proliferation was measured by [^{3}H]TdR incorporation and cytotoxicity was measured against P815 mastocytoma target cells in a Cr^{51}-release assay. P815 cells are highly sensitive to nonspecific CTL induced with anti-CD3 mAb (Kaiser and Hoskin, 1992). We have previously determined that peak [^{3}H]TdR incorporation and cytolytic activity occurs 48 h following in vitro T cell activation with anti-CD3 mAb (Hoskin et al., 1996). As depicted in Fig. 1A, A77 1726 exhibited a dose-dependent inhibitory effect on DNA synthesis in anti-CD3-activated T cell cultures with an approximate IC_{50} of 5 μM. In contrast to this decrease in DNA synthesis at all concentrations tested, Fig. 1B shows that a low concentration of A77 1726 (0.5 μM) potentiated the generation of cytotoxic cells in anti-CD3-activated T cell cultures. Higher concentrations of A77 1726 inhibited the development of cytotoxic cells in these cultures ($IC_{50} \sim 10$ μM). The inhibitory effect of A77 1726 on T cell proliferation and CTL induction at higher concentrations was not due to nonselective toxicity because T cell viability at 48 h of culture, as assessed by trypan blue dye exclusion, was unaffected by the drug.

3.2. IFN-γ and IL-2 synthesis in the presence of A77 1726

IFN-γ and IL-2 are important cytokines in the development of CTL (Maraskovsky et al., 1989). Although A77 1726 has been reported to have little or no effect on IL-2 production by mouse T cells at the concentrations used in our experiments (Cherwinski et al., 1995), the effect of A77 1726 on IFN-γ synthesis has not yet been investigated. We, therefore, employed ELISA to determine whether A77 1726 affected IFN-γ synthesis in cultures of anti-CD3-activated T cells. Following 48 h of culture, we observed that T cell cultures exposed to 20 μM A77 1726 consistently contained reduced levels of IFNγ in comparison to control cultures (Table 1). In contrast, IFN-γ levels in T cell cultures performed in the presence of 0.5 μM A77 1726 were slightly above control levels, although the increase was not always statistically significant. We were unable to demonstrate any

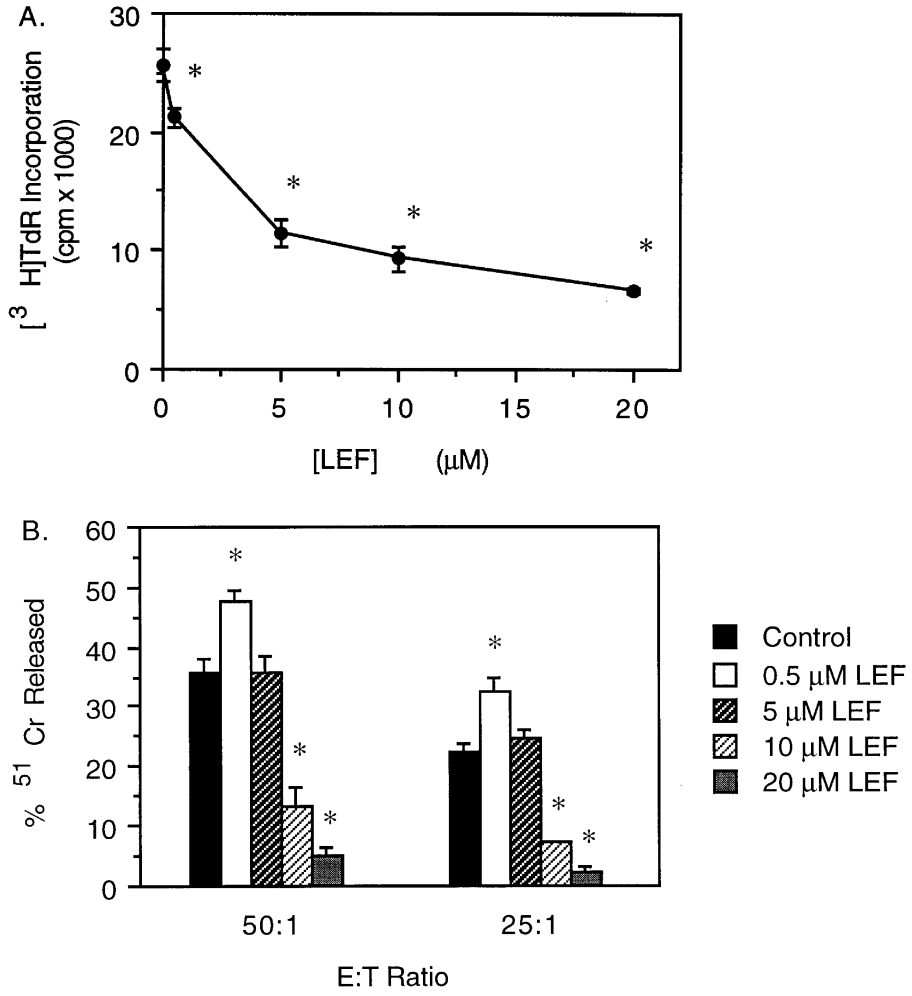

Fig. 1. Effect of A77 1726 on (A) T cell proliferation and (B) CTL development induced with anti-CD3 mAb. T cells were stimulated with anti-CD3 mAb in the absence or presence of the indicated concentrations of A77 1726. Following 48 h of culture, T cell proliferation was measured by [^{3}H]TdR incorporation. Results are expressed as mean cpm ($\pm$SD) from quadruplicate wells. Cytolytic activity against P815 target cells at the indicated E:T ratios was measured in a ^{51}Cr-release assay. Data are expressed as mean % ^{51}Cr released ($\pm$SD) by P815 target cells in triplicate wells. Asterisk indicates a statistically significant difference in comparison with the control response, as determined by Student's *t*-test. The data show one of three experiments performed with similar results.

significant inhibitory effect of A77 1726 on IL-2 synthesis in anti-CD3-activated T cell cultures (data not shown).

3.3. Effect of A77 1726 on granzyme B expression

Since granzyme B is crucial for the granule-dependent cytolysis of allogeneic target cells by cytotoxic lymphocytes (Heusel et al., 1994) and granzyme B expression is upregulated in response

Table 1
Effect of A77 1726 on IFN-γ production and granzyme B activity in anti-CD3-activated T cell cultures

Experiment	Culture treatment[a]	IFN-γ in culture supernatants (pg/ml)	Granzyme B enzymatic activity (Units/10^6 cells)
1	Medium	$1{,}034 \pm 44$	8.1 ± 1.1
	A77 1726, 0.5 μM	$1{,}189 \pm 32^{*}$	$11.0 \pm 0.9^{*}$
	A77 1726, 10 μM	ND	$5.0 \pm 0.8^{**}$
	A77 1726, 20 μM	$449 \pm 35^{***}$	ND
2	Medium	$2{,}238 \pm 76$	7.6 ± 1.0
	A77 1726, 0.5 μM	$2{,}314 \pm 49$	8.5 ± 0.9
	A77 1726, 10 μM	ND	$2.7 \pm 0.7^{***}$
	A77 1726, 20 μM	$1{,}548 \pm 30^{***}$	ND

[a] T cells were stimulated with anti-CD3 mAb alone or in the presence of 0.5, 10, or 20 μM A77 1726. Following 48 h of culture, IFN-γ levels in culture supernatants were measured by ELISA. At the same time, lysates were prepared from the T cell cultures and granzyme B enzymatic activity was measured as described (Fitzpatrick et al., 1996). ND = not determined.

$^{*}p < 0.01$; $^{**}p < 0.005$; $^{***}p < 0.001$ compared to the medium control and determined by Student's *t*-test.

to IFN-γ (Fitzpatrick et al., 1996), we next used a colorimetric granzyme B assay to determine the effect of A77 1726 on granzyme B protein levels in anti-CD3-activated CTL at 48 h of culture. Results from two representative experiments are shown in Table 1. On a per cell basis, granzyme B enzymatic activity was significantly decreased in lysates of CTL induced in the presence of 10 μM A77 1726, in comparison to control CTL lysates. In contrast, exposure to 0.5 μM A77 1726 resulted in a slight, albeit not always significant, increase in granzyme B enzymatic activity of CTL.

3.4. Effect of A77 1726 on CD8 and CD25 expression

T cells activated with anti-CD3 mAb in the absence or presence of 0.5 μM or 20 μM A77 1726 were analyzed by flow cytometry to determine whether altered expression of the α chain (CD25) of the high affinity IL-2 receptor or a change in the percentage of CD8-bearing T lymphocytes might contribute to the effects of A77 1726 on cytotoxicity. A77 1726 did not alter the percentage of $CD25^+$ T cells or the mean channel fluorescence of $CD25^+$ T cells, which is an indicator of cell-surface molecule density (data not shown). Although the percentage of $CD8^+$ T cells was similarly unaffected by either concentration of A77 1726, exposure to the highest concentration of the drug resulted in a marked decrease (36 ± 2% reduction) in mean channel fluorescence, indicating that these T cells expressed less cell-surface CD8 on a per cell basis.

3.5. Conjugate formation in the presence of A77 1726

We also tested whether CTL induced in the presence of A77 1726 were able to conjugate normally to P815 target cells. Control CTL and CTL which were generated in the presence of either 0.5 or 20 μM A77 1726 adhered equally well to P815 cells. In one experiment (typical of three separate experiments) 33.9 ± 4.5% of T cells from control cultures bound to P815 cells

whereas 30.9 ± 5.4% and 29.3 ± 2.9% of anti-CD3-activated T cells treated with 0.5 or 20 μM A77 1726, respectively, conjugated with P815 cells.

4. Discussion

At higher concentrations (IC_{50} of 50 μM) A77 1726 has been shown to inhibit early tyrosine phosphorylation events in IL-2 signal transduction involving Jak1 and Jak3 kinases, while at lower concentrations (IC_{50} of 2 μM) A77 1726 targets pyrimidine biosynthesis in T lymphocytes (Elder et al., 1997). Here we show that A77 1726 inhibits mouse T cell proliferation with an IC_{50} of ~5 μM, which approximates the IC_{50} for A77 1726 acting at the level of pyrimidine synthesis. Our data are in line with a previous report that A77 1726 interferes with mouse T cell cycle progression by inhibiting pyrimidine synthesis, since uridine and cytidine antagonize the antiproliferative effect of the drug (Chong et al., 1996). In contrast, we observed that the IC_{50} of A77 1726 for inhibition of CTL induction was ~10 μM, suggesting that CTL development may be more sensitive to A77 1726-mediated inhibition of protein tyrosine kinases involved in IL-2 signal transduction. Impaired CTL induction in the presence of 20 μM A77 1726 could not be attributed to diminished IL-2 synthesis or reduced expression of the high affinity IL-2 receptor. These findings are consistent with the well established principle of IL-2 autoinduction of CD25 expression by T cells (Depper et al., 1985), and are in agreement with earlier reports that A77 1726, at the concentrations used in our experiments, fails to significantly affect IL-2 synthesis or CD25 expression by T lymphocytes (Chong et al., 1993; Cherwinski et al., 1995). CTL induced in the presence of 20 μM A77 1726 also conjugated normally with P815 target cells. This finding is consistent with the observation that A77 1726 fails to alter LFA-1 expression by anti-CD3-activated T cells, as determined by flow cytometric analysis (Richard and Hoskin, unpublished). Taken together, these data suggest that A77 1726 does not affect the expression or function of the adhesion molecule LFA-1 which is involved in recognition of P815 target cells by anti-CD3-activated CTL with nonspecific cytotoxic activity (Stewart and Hoskin, 1997a).

The inhibitory effect of 20 μM A77 1726 on CTL development was associated with diminished IFN-γ synthesis in anti-CD3-activated T cell cultures. The inhibitory effect of A77 1726 on protein tyrosine kinases associated with the high affinity IL-2 receptor might account for the observed reduction in IFN-γ synthesis since T lymphocyte production of IFN-γ is known to be induced by IL-2 (Vilcek et al., 1985). Because IFN-γ upregulates granzyme B gene expression in mouse CTL (Fitzpatrick et al., 1996), reduced IFN-γ synthesis in anti-CD3-activated T cell cultures exposed to 20 μM A77 1726 is likely to account, at least in part, for the inhibitory effect of A77 1726 on CTL induction and granzyme B synthesis. However, decreased levels of cell-surface CD8 on CTL exposed to 20 μM A77 1726 might also contribute to reduced cytotoxicity since CD8 has been implicated in the nonspecific cytolytic activity of CTL induced with anti-CD3 mAb (Stewart and Hoskin, 1997b).

We were surprised to find that a low concentration (0.5 μM) of A77 1726, while still slightly inhibitory to mouse T cell proliferation, had an enhancing effect on CTL induction. A slight, but not always significant, increase in IFN-γ and granzyme B synthesis was also observed in anti-CD3-activated T cell cultures performed in the presence of 0.5 μM of A77 1726. However, these slight increases in IFN-γ and granzyme B synthesis are unlikely to account for the potentiating effect of

low dose A77 1726 on CTL development. The mechanism by which a low dose of A77 1726 might upregulate the cytotoxic activity of CTL remains unclear at the present time. It is interesting, though, that the in vitro inhibition of recombinant human dihydroorotate dehydrogenase by A77 1726 has an apparent IC_{50} of 0.3 μM (Davis et al., 1996). The enhancing effect of low dose A77 1726 on CTL development may therefore involve alterations in the pyrimidine biosynthesis pathway of T lymphocytes. In any case, our data suggest that it might be prudent to carefully monitor serum levels of the active metabolite of leflunomide in any future clinical trials of the drug in organ transplant recipients. We predict that high doses of leflunomide will inhibit alloantigen-induced T cell proliferation and prevent the induction of allograft-specific CTL. However, it is conceivable that if serum levels of the active metabolite were to fall below a critical threshold level as a result of decreased metabolism of the prodrug or patient noncompliance, the generation of allograft-reactive CTL might actually be enhanced.

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References

Bartlett, R. R., Dimitrijevic, M., Mattar, T., Zielinski, T., Germann, T., Rüde, E., Thoenes, G. H., Küchle, C. C. A., Schorlemmer, H. -U., Bremer, E., Finnegan, A., & Schleyerbach, R. (1991). Leflunomide (HWA 486), a novel immunomodulating compound for the treatment of autoimmune disorders and reactions leading to transplantation rejection. *Agents Actions*, *32*, 11–21.

Cherwinski, H. M., McCarley, D., Schatzman, R., Devens, B., & Ransom, J. T. (1995). The immunosuppressant leflunomide inhibits lymphocyte progression through cell cycle by a novel mechanism. *J. Pharmacol. Exp. Ther.*, *275*, 1043–1049.

Chong, A. S. -F., Finnegan, A., Jiang, X. L., Gebel, H., Sankary, H. N., Foster, P., & Williams, J. W. (1993). Leflunomide, a novel immunosuppressive agent. The mechanism of inhibition of T cell proliferation. *Transplantation*, *55*, 1361–1366.

Chong, A. S. -F., Rezai, K., Gebel, H. M., Finnegan, A., Foster, P., Xu, X. L., & Williams, J. W. (1996). Effects of leflunomide and other immunosuppressive agents on T cell proliferation in vitro. *Transplantation*, *61*, 140–145.

Davis, J. P., Cain, G. A., Pitts, W. J., Magolda, R. L., & Copeland, R. A. (1996). The immunosuppressive metabolite of leflunomide is a potent inhibitor of human dihydroorotate dehydrogenase. *Biochemistry*, *35*, 1270–1273.

Depper, J. M., Leonard, W. J., Drogula, G., Kronke, M., Waldmann, T. A., & Greene, W. C. (1985). Interleukin 2 augments transcription of the IL-2 receptor gene. *Proc. Natl. Acad. Sci. USA*, *82*, 4230–4234.

Elder, R. T., Xu, X., Williams, J. W., Gong, H., Finnegan, A., & Chong, A. S. -F. (1997). The immunosuppressive metabolite of leflunomide, A77 1726, affects murine T cells through two biochemical mechanisms. *J. Immunol.*, *159*, 22–27.

Fitzpatrick, L., Makrigiannis, A. P., Kaiser, M., & Hoskin, D. W. (1996). Anti-CD3-activated killer T cells: Interferon-

γ and interleukin-10 cross-regulate granzyme B expression and the induction of major histocompatiblity complex-unrestricted cytotoxicity. *J. Interferon. Cyto. Res.*, *16*, 537–546.

Heusel, J. W., Wesselschmidt, R. L., Shresta, S., Russell, J. H., & Ley, T. J. (1994). Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell*, *76*, 977–987.

Hoskin, D. W., Phu, T., & Makrigiannis, A. (1996). Pentoxifylline inhibits granzyme B and perforin expression following T-lymphocyte activation by anti-CD3 antibody. *Int. J. Immunopharmac.*, *18*, 623–631.

Hutchinson, I. (1991). Cellular mechanisms of allograft rejection. *Curr. Opin. Immunol.*, *3*, 722–728.

Kaiser, M., & Hoskin, D. W. (1992). Expression and utilization of chymotrypsin-like but not trypsin-like serine protease enzymes by nonspecific T killer cells activated by anti-CD3 monoclonal antibody. *Cell. Immunol.*, *141*, 84–98.

Legros-Maida, S., Soulie, A., Benvenuti, C., Wargnier, A., Vallee, N., Berthou, C., Guillet, J., Sasportes, M., & Sigaux, N. (1994). Granzyme B and perforin can be used as predictive markers of acute rejection in heart transplantation. *Eur. J. Immunol.*, *24*, 229–233.

Maraskovsky, E., Chen, W. -F., & Shortman, K. (1989). IL-2 and IFNγ are two necessary lymphokines in the development of cytolytic T cells. *J. Immunol.*, *143*, 1210–1214.

Podack, E. R. (1995). Functional significance of two cytolytic pathways of cytotoxic T lymphocytes. *J. Leukoc. Biol.*, *57*, 548–552.

Siemasko, K. F., Chong, A. S. -F., Williams, J. W., Bremer, E. G., & Finnegan, A. (1996). Regulation of B cell function by the immunosuppressive agent leflunomide. *Transplantation*, *61*, 635–642.

Stewart, B. H., & Hoskin, D. W. (1997a). Inhibition of protein tyrosine kinases or protein kinase C prevents nonspecific killer T lymphocyte-mediated tumoricidal activity. *Biochim. Biophys. Acta*, *1356*, 333–342.

Stewart, B. H., & Hoskin, D. W. (1997b). Regulatory role of CD8 in major-histocompatibility complex-unrestricted tumoricidal activity of mouse T cells activated with anti-CD3 monoclonal antibody. *Immunol. Invest.*, *26*, 601–614.

Swan, S. K., Crary, G. S., Guijarro, C., O'Donnell, M. P., Keane, W. F., & Kasiske, B. L. (1995). Immunosuppressive effects of leflunomide in experimental chronic vascular rejection. *Transplantation*, *60*, 887–890.

Vilcek, J., Henricksen-Destefano, D., Siegel, D., Klion, A., Robb, R. J., & Le, J. (1985). Regulation of IFN-γ induction in human peripheral blood cells by exogenous and endogenously produced interleukin 2. *J. Immunol.*, *135*, 1851–1856.

Williams, J. W., Xiano, F., Foster, P., Clardy, C., McChesney, L., Sankary, H., & Chong, A. S. -F. (1994). Leflunomide in experimental transplantation: Control of rejection and alloantibody production, reversal of acute rejection and interaction with cyclosporine. *Transplantation*, *57*, 1223–1231.