FULL TEXT

Volume:7 • Number:4 • December 2009

Leflunomide Derivative FK778 Inhibits Production of Antibodies in an Experimental Model of Alloreactive T-B Cell Interaction

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Objectives: **The contribution of humoral immune response in allograft and xenograft rejection has been clearly demonstrated in recent years. For this reason, inhibition of alloantibody production has become essential in managing transplanted patients. Here, we assessed the effects of the leflunomide derivative FK778 (FK778) in the control of antibody production resulting from semi-allogeneic cognate T-B–cell interactions.**

Materials and Methods: **BALB/c mice were tolerized at birth with semiallogeneic spleen cells from (BALB/c × C57BL/6) F1 mice, with or without overexpression of human bcl-2 transgene in B cells. These tolerized mice were treated with different dosages of FK778, either from birth, or from the third week of age, when autoantibody production was detected. The production of autoantibodies, used as markers of semiallogeneic cognate T-B–cell interactions, was evaluated at different time points during drug administration or after the interruption of treatment.**

Results: **FK778 treatment started at birth inhibited the production of semiallogeneic-driven antibodies in a dose-dependent manner. In addition, FK778 also reduced the levels of preformed circulating autoantibodies in adult mice, although the dosage required was 4 times higher than that used in neonates. However, the levels of IgG antibodies in these tolerized mice increased after FK778 withdrawal, indicating that FK778 failed to induce tolerance to semiallogeneic host CD4+ Th2 and/or donor B cells.**

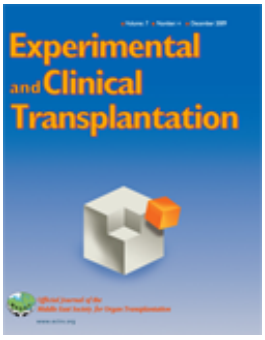
Conclusions: **Our results demonstrate the efficacy of FK778 in the control of antibody production resulting from semiallogeneic cognate T-B–cell interactions.**

Key words: Allograft rejection; Treatment, Transplant

In addition to the well-known contribution of T lymphocytes in allograft or xenograft rejection, multiple evidence indicates that B-cell–producing, alloreactive antibodies are actively involved in the pathogenesis of both acute and chronic allograft rejection (1-2) and xenograft rejection (3-4). The main antigenic targets of antibody-mediated rejection are major histocompatibility complex molecules (both class I and class II) (5) and the ABO group antigens (6). However, minor histocompatibility antigens or other autoantigens also can be targets of antibody-mediated rejection (7-9). Immunoadsortion by protein A has been considered as a tool to overcome hyperacute xenograft rejection by reducing circulating Ab (10). At present, the most common strategies for treatment of antibody-mediated rejection are based on the quick reduction of antibody titres using plasmapheresis in combination with aggressive immunosuppression (11). Although several immunosuppressor drugs (eg, mycophenolate mofetil or tacrolimus) can prevent antibody-mediated rejection (11), the discovery of new, immunosuppressive drugs that efficiently block alloantibody production in well-defined experimental models of allogeneic T-B–cell interactions is of paramount importance to define novel, and more-efficient therapies against antibody-mediated rejection.

FK778 (Astellas Pharma US, Inc., Deerfield, IL, USA), a synthetic malononitrilamide derived from the active metabolite of leflunomide (A77 1726), is an immunosuppressive drug that exerts its activity by inhibiting de novo pyrimidine synthesis by blocking the mitochondrial enzyme dihydro-orotate de-hydrogenase, and possibly, inhibition of tyrosine kinase activity (12-15). FK778 inhibits both T-cell and B-cell function (13, 16), and induces the generation of $CD4^+CD25^+$ regulatory T-cells in vitro (17).

In this study, we explore the efficacy of FK778 treatment in the prevention of antibody production resulting from an in vivo, cognate, semiallogeneic T-B–cell interaction. For this purpose, we used the experimental model of induction of neonatal tolerance to major histocompatibility complex alloantigens. In this model, the injection of semiallogeneic lymphoid cells differing in major histocompatibility complex class II molecules—but not in major histocompatibility complex class I or nonmajor histocompatibility complex alloantigens—into newborn parental mice induces immunologic tolerance to donor tissues, and the establishment of a donor B- and T-cell–chimerism (18, 19). However, host $CD4^+$ Th2 cells recognizing I-A or I-E major histocompatibility complex class II alloantigens induce the polyclonal activation of donor B-cells and the production of autoantibodies (autoAbs) (20, 21). An important feature of this model is that autoAbs production is self-limited, probably the consequence of a drop in donor B-cell chimerism (22). Indeed, the neonatal injection of semiallogeneic B-cells overexpressing a human bcl-2 transgene, promotes the maintenance of autoAbs production and the development of a lethal autoimmune syndrome (23). In this experimental model, using the production of IgG anti-DNA antibodies as a marker of the establishment of allogeneic T-B–cell interactions, we analyze the effect of FK778 in inhibiting the generation of allogeneic-driven autoAbs.



Volume : 7
Issue : 4
Pages : 218-224

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Acknowledgement: This work was supported by REDINREN RD06/0016 and PI070683 grants from the "Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo" (Spain) to MA, by grant No. SAF2005-00811 from the "Ministerio de Educación y Ciencia," and a grant from the "Fundación Ramón Areces" (Spain) to RM, by grants No. SAF2006-012520-C02-02 from the "Ministerio de Educación y Ciencia," and grant No. API07/08 from the "Fundación Marqués de Valdecilla" (Spain) to JM, and MAR-B is the recipient of a postdoctoral fellowship from the "Instituto Reina Sofía de la Fundación Renal Iñigo Alvarez de Toledo," Spain. IS is funded by the "Fundación Marqués de Valdecilla, API06/07" (Spain).
We thank Astellas Pharma US, Inc., (Deerfield, IL, USA) and Dr Stanley J. Korsmeyer, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, for their supply of FK778 and C57BL/6-hBcl-2 Tg mice, respectively; and Marcos Lopez-Hoyos for his insightful, critical reading of the paper.
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Materials and Methods

Mice and neonatal induction of tolerance to alloantigens.

BALB/c and C57BL/6 mice were acquired from Harlan Ibérica (Barcelona, Spain). C57BL/6-Ig-*human bcl-2* transgenic mice (C57BL/6-Ig-*human bcl-2* transgenic mice), overexpressing hBcl-2 in B cells (24), were generously provided by Dr Stanley J. Korsmeyer (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA). (BALB/c × C57BL/6) F1 mice were obtained in our animal facilities. The presence of hBcl-2 in F1 mice was assessed in peripheral blood mononuclear cells by flow cytometry using a specific monoclonal antibody against hBcl-2 (clone 6C8; Pharmingen, San Diego, CA, USA), as previously described (23).

Neonatal tolerance to H-2^b alloantigens was induced by an ip injection of 7×10^7 spleen cells from (BALB/c x C57BL/6) F_1-*hbcl-2* Tg mice or 10^8 spleen cells from (BALB/c × C57BL/6) F_1 mice into BALB/c mice within the first 24 hours of life, as described previously (23). These numbers of spleen cells were chosen to inject the same absolute number of B cells in both groups of mice, as determined by flow cytometry (23). Mice were bled from the retro-orbital plexus, and the resulting sera stored at -20°C until use. All studies were approved by the University of Cantabria Institutional Laboratory Animal Care and Use Committee.

Study protocol

Group 1: To explore and titrate, in vivo, the efficacy of FK778 to prevent antibody production resulting from allogeneic T-B–cell interactions, BALB/c mice were injected with 10^8 spleen cells from
(BALB/c × C57BL/6) F_1 mice and were treated ip with 5 mg/kg (group 1a, n=25) or 20 mg/kg (group 1b, n=7) of FK778 (Astellas Pharma US, Inc., Deerfield, IL, USA), diluted in 0.1% carboxymethylcellulose, 3 times per week, from day 1 of life until the third week of age.

Group 2: To explore the efficacy of FK778 in inhibiting the production of preformed allogeneic-driven antibodies, BALB/c mice injected at birth with 10^7 spleen cells from (BALB/c × C57BL/6) F_1-*hbcl-2*-Tg mice, overexpressing *hBcl-2* in B cells, were treated with FK778 ip 20 mg/kg (group 2a, n=7) or 80 mg/kg (group 2b, n=10) 3 times per week, for 6 weeks, from the third to the ninth week of age, when treatment was stopped.

Group 3 (n=16): In case of lack of effectiveness of the lower therapeutic regimen in group 2, we analyzed if this was related to the fact that the donor B cells used there overexpressed *hBcl-2*. With this aim, a group of mice were tolerized with 10^7 spleen cells from (BALB/c × C57BL/6) F_1-*hbcl-2*-Tg mice and received treatment with FK778 from birth up to the third week of age, with FK778 at a dose of 20 mg/kg.

Group 4 (n=10): To study whether the FK778 treatment could promote the induction of tolerance to alloantigens in vivo; thus, preventing the activation of allogeneic donor B-cell after FK778 withdrawal, BALB/c mice neonatally tolerized with spleen cells from (BALB/c × C57BL/6) F_1-*hbcl-2*-Tg mice were treated with 80 mg/Kg of FK778, 3 times a week, from the third week of age for 9 weeks, and then the treatment was stopped. After that, mice were followed serologically 3 weeks after the interruption of FK778 treatment.

Group 5: Tolerized controls. Group 5a (n=5): BALB/c mice injected with 10^8 spleen cells from (BALB/c × C57BL/6)F_1 mice ip solvent treated 3 times per week as tolerized controls. Group 5b (n=6): BALB/c mice injected with 10^7 spleen cells from (BALB/c × C57BL/6)F_1 *hbcl-2*-Tg mice ip solvent treated, 3 times per week, as tolerized controls.

Group 6 (n=6): BALB/c mice were ip solvent treated, 3 times per week, as nontolerized controls.

Serologic assays

Serum levels of total IgG and IgG anti-ssDNA autoAbs were determined by ELISA, as described elsewhere (23). Results were expressed in mg/mL in reference to a standard curve obtained with a mouse reference serum (ICN Biomedicals, Irvine, CA, USA), in the case of total IgG, or in titration units (U/mL) in reference to a standard curve obtained with a serum pool from a 6- to 8-month-old MRL.lpr/lpr mice, for IgG anti-ssDNA autoAbs.

Statistical analyses

Statistical analyses of differences between groups of mice were performed using the Wilcoxon 2-sample test. Probability values < .05 were considered significant.

Results

1. FK778 completely inhibits the generation of de novo antibodies secondary to allogeneic T-B–cell interactions.

In group 1b, FK778 at 20 mg/kg, totally inhibited the induction of IgG hypergammaglobulinemia and the production of IgG anti-ssDNA autoAbs in comparison to solvent treated tolerized controls (group 5a), which exhibited higher levels of circulating IgG (0.56 ± 0.04 mg/mL vs 5.29 ± 0.6 mg/mL) and IgG anti-ssDNA (10.7 ± 0.6 U/mL vs 180.2 ± 8.2 U/mL) autoAbs (*P* < .003 in both

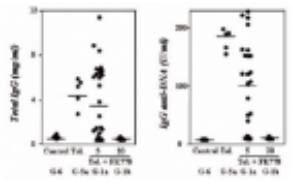

Figure 1. Effect of FK778 treatment in the production of de novo antibodies secondary to allogeneic T-B–cell interactions. BALB/c mice were tolerized at birth with spleen cells from (BALB/c × C57BL/6) F1 mice and treated from day 1 up to the thirdweek of agewith either 5mg/kg (group 1a) or 20mg/kg (group 1b) of FK778 3 times per week. Nontolerized (group 6) and tolerized BALB/c mice-treated with solvent (group 5a) were used as controls. Serum levels of total IgG (left) and IgG anti-ssDNA(right) after 3weeks of treatment are expressed in each individualmouse asmg/mL or U/mL, respectively

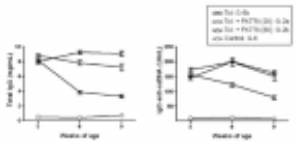

Figure 2. Effect of FK778 treatment in the production of preformed antibodies secondary to allogeneic T-B–cell interactions. BALB/c mice were tolerized at birth with spleen cells from (BALB/c × C57BL/6)F1-hbcl-2-Tg mice. From the third week of age, tolerized mice were treated with either 20 mg/kg (◊) (group 2a) or 80 mg/kg (Δ) (group 2b) of FK778 3 times perweek.Nontolerized (O) (group 6) and tolerized BALB/cmice treatedwith solvent (×) (group 5b)were used as controls. Themean ± SD of serumlevels of total IgG (left) and IgG anti-ssDNA(right) are expressed at different time points inmg/mL or U/mL.

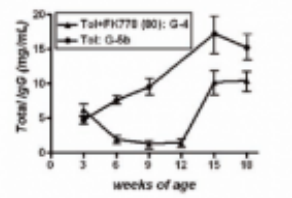

Figure 3. Effect of FK778 treatment in the induction of immunologic tolerance. BALB/c mice were tolerized at birth with spleen cells from (BALB/c × C57BL/6)F1-hbcl-2-Tgmice. Fromthe third week up to the 12th week of age, tolerized mice were treated with either 80 mg/Kg (▲) of FK778, 3 times per week (group 4), or solvent (●) (group 5b). The mean ± SD of serum levels of total IgGand are expressed inmg/mLat different time points during and after FK778 treatment.

cases) (Figure 1). In group 1a, FK778 at 5 mg/kg reduced significantly the levels of total IgG and antibodies after 3 weeks of treatment in only 44% of tolerized mice (11 mice from a total of 25 mice), compared to group 5a (4.82 ± 0.73 mg/mL vs 5.29 ± 0.64 mg/mL, *P* = NS) and IgG anti-ssDNA (101.6 ± 15.1 U/mL vs 180.2 ± 8.2 U/mL, *P* = .02) (Figure 1).

2. Higher dosages of FK778 are required to inhibit the production of preformed antibodies secondary to allogeneic T-B–cell interactions.

Mice from group 2 injected at birth with 7 × 10^7 spleen cells from (BALB/c × C57BL/6)F_1-*hbcl-2*-Tg mice exhibited, at the third week of age, in relation to solvent treated tolerized mice (group 5b), similar increased levels of circulating total IgG and IgG anti-ssDNA autoAbs (group 2a: 8.83 ± 0.27 mg/mL and 172.8 ± 17.7 U/mL; group 2b: 8.16 ± 0.72 mg/mL and 156.8 ± 18.7 U/mL; group 5b: 8.03 ± 0.27 mg/mL and 146.1 ± 16.5 U/mL) (P = NS in all cases) and, in relation to nontolerized controls (group 6), significantly increased levels of both circulating total IgG and IgG anti-ssDNA autoAbs (0.49 ± 0.02 mg/mL and 10.6 ± 0.7 U/mL) (P < .05) as expected (Figure 2). The initiation of FK778 treatment at this time point, at a dosage of 20 mg/kg 3 times per week (group 2a), the dosage used in neonatal mice to completely block de novo generation of autoAbs (Figure 1), was very inefficient in reducing serum levels of total IgG and IgG anti-ssDNA, after 6 weeks of treatment (9 weeks of age) compared with tolerized controls (group 5b) (7.2 ± 1.1 mg/mL vs 9.1 ± 0.8 mg/mL) (*P* = NS) and (151.0 ± 29.5 U/mL vs 162.6 ± 13.7 U/mL) (*P* = NS) (Figure 2). In fact, only 43% (3 of 7) of mice treated with 20 mg of FK778 from the third week of age showed a reduction in the titres of IgG anti-ssDNA (mean of 90.3 ± 32.9 U/mL), but none had reduced levels of circulating total IgG.

Treatment from birth up to the third week of age with FK778 at the dosage of 20 mg/kg, 3 times per week, completely interfered with de novo production of IgG anti-ssDNA autoAbs in mice tolerized with spleen cells from (BALB/c × C57BL/6)F_1-*hbcl-2*-Tg mice (group 3) compared to tolerized mice with (BALB/c × C57BL/6)F_1-*hbcl-2*-Tg spleen cells (group 5b): 7.1 ± 0.3 U/mL (n=16) vs 118.1 ± 73.7 U/mL (n=6) (*P* = .0009) and was similar to IgG anti-ssDNA autoAbs in nontolerized BALB/c mice (group 6): 8.2 ± 0.6 U/mL (n=6) (*P* = NS). However, a 4-fold increase in the dosage of FK778 (80 mg/kg of FK778, 3 times per week), administered from the third week of age into autoAb positive mice tolerized with (BALB/c × C57BL/6)F_1-*hbcl-2*-Tg spleen cells
(group 2b), could significantly reduce (compared to group 5b) the preformed anti-ssDNA autoAbs (80.7 ± 11.9 vs 162.6 ± 13.7 U/mL) (*P* = .001) and hyper-gamma-globulinemia in 100% of animals (3.32 ± 0.59 vs 9.1 ± 0.8 mg/mL) (P = .0008) (Figure 2).

3. Treatment with FK778 fails to promote immunologic tolerance of semiallogeneic host CD4+ Th2 and/or donor B cells

The interruption of FK778 treatment in group 4 was followed 3 weeks later by the appearance of IgG hypergammaglobulinemia at levels comparable to those observed in these animals before the treatment (group 5b) (10.2 ± 1.57 vs 17.19 ± 2.64 mg/mL) (P = NS) (Figure 3). Similarly, IgG hypergamma-globulinemia was observed 3 and 6 weeks after the interruption of drug administration in tolerized mice treated from birth up to the third week of age with 20 mg/kg of FK778 3 times per week (data not shown).

Discussion

The acceptance of allografts in animals or humans lacking T cells for a long time supports the misconception that these cells were the only, or at least, the major mediators, of allograft rejection. This idea largely underestimated the contribution of humoral immune responses in the pathogenesis of xeno and allograft rejection (1-4, 25-26). However, over the past few years, multiple evidence indicates that alloreactive antibodies play important roles in a significant proportion of both acute and chronic rejection episodes, particularly those involved in graft failure (1, 2, 25-27). For this reason, an adequate treatment to prevent xeno and allograft rejection should take in consideration not only the inhibition of T-cell alloresponses, but also the effective blockade of xeno and alloantibody production.

In the present study, we have evaluated in a well-established model of semiallogeneic-driven $CD4^+$ T-cell–mediated polyclonal activation of B cells, the effectiveness of FK778 in the prevention of antibody production. Our results demonstrate that FK778 largely blocks both de novo and the preformed production of semiallogeneic-driven autoAbs. However, the dosages required for the effective inhibition of preformed antibodies are significantly higher than those used for the inhibition of de novo produced antibodies. In addition, we demonstrate here that the in vivo treatment with FK778 fails to promote $CD4^+$ T- and B-cell tolerance.

We have used a previously well-documented model of semiallogeneic T-B–cell interaction secondary to the induction of neonatal tolerance to H-2 alloantigens (18-23). In this model, the injection of semiallogeneic (BALB/c × C57BL/6) F_1 H-$^{b/d}$ spleen cells, either with or without overexpression of hBcl-2 in B cells, into BALB/c H-2^d neonates, promotes the induction of tolerance of host $CD4^+$ Th1 and $CD8^+$ T cells, that is manifested by, among other findings, the persistence of donor B-cell chimerism. However, these donor B cells expressing the H-2^b alloantigen are polyclonally activated by non-tolerized H-2^b-specific BALB/c host $CD4^+$ Th2 cells to produce a wide repertoire of IgG1 and IgE antibodies, including multiple autoAbs (20). Several

characteristics make this experimental model suitable to test under stringent conditions the efficacy of new immunosuppressive drugs in the control of alloantibody production; a) the nature of the alloantigen, the H-2^b alloantigen, involved in this cognate T-B–cell interaction is well established (18, 19, 21), b) the number of precursors engaged in this interaction is even higher than in transplanted patients (especially if it is taken in consideration that all donor B cells may be activated in this model [28]), c) using different mouse donor sources (F_1 mice with or without overexpression of hBcl-2 in B cells) (23), it allows analysis of the effect of the drug in situations where alloreactive host $CD4^+$ T cells have not been previously exposed to the antigen (naive T cells), or when they have been activated, and memory T cells have been generated (see below), and d) autoAbs can be employed as serologic markers of this semiallogeneic interaction (28).

FK778 completely inhibits the generation of de novo antibodies secondary to allogeneic T-B–cell interactions; however, the protective effect of FK778 on autoAbs production was dose-dependent, because the dosage of 5 mg/kg of FK778, 3 times per week, was able to reduce only, either partially or totally, the levels of IgG anti-ssDNA and total IgG antibodies after 3 weeks of treatment in about 40% to 50% of tolerized mice (Figure 1), whereas 20 mg/kg of FK778, 3 times per week, put an end to autoAbs production.

An important finding of our study is that the dosage of FK778 required to reduce the levels of preformed allogeneic-driven autoAbs in tolerized adult mice treated from the third week of age is 4 times higher than the dosage used to block de novo production of such antibodies in mice treated from birth. In this case, we used a previously reported modification of the model of neonatal tolerance to alloantigens described above, consisting of the use of (BALB/c × C57BL/6) F_1-*hbcl-2*-Tg mice over-expressing hBcl-2 in B cells as the source of donor spleen cells injected at birth into BALB/c mice (23). In this model, and secondary to the enlarged survival of donor B cells that prolongs the persistence of donor B-cell chimerism, the increased levels of autoAbs are maintained chronically inducing a lethal autoimmune syndrome in these tolerized mice (23).

This difference in the FK778 dosages required is unrelated to the source of spleen cells used to induce neonatal tolerance to alloantigens, either (BALB/c × C57BL/6)F_1-*hbcl-2*-Tg or non-Tg F1 mice. In fact, the treatment from birth with FK778 at a dosage of 20 mg/kg, 3 times per week, is equally effective preventing the production of autoAbs in BALB/c mice tolerized with spleen cells from each F1 donor. Two mutually nonexclusive possibilities may explain this difference. First, although the dosage of immunosuppressant has been precisely adapted in every injection to the weight of individual animals, we cannot exclude the possibility of variations in the metabolism and/or body distribution of the drug between very young (mice from birth up to the third week of age) and more adult (from the third week of age on) mice; in this sense, a measurement of plasma levels of the drug could add additional information. Alternatively, a possible explanation for the different FK778 dosages required to inhibit antibody production in our experimental groups may be related to the presence in mice treated from the third week of age with semiallogeneic memory $CD4^+$ Th2 cells that are absent in neonatal mice. These memory $CD4^+$ Th2 cells could be more refractory to FK778 inhibition than the newly generated, semiallogeneic host $CD4^+$ Th2 cells in the neonates. The control of alloreactive memory T cells, which may be already present in nonpreviously, immunized, pre-transplanted patients, constitutes 1 of the major challenges for transplant immunotherapy (29, 30). In this regard, although successful attempts to control memory T-cell responses have been reported (31), human memory alloreactive $CD4^+$ T-cells stimulated in vitro seem to be refractory to the suppressive effects of steroids, deoxyspergualin, and sirolimus do not to the calcineurin inhibitor tacrolimus, or, to a lesser extent, cyclosporine (32).

It has been reported recently that in vitro FK778 induces the generation of regulatory T-cells from naive $CD4^+$CD25- T-cells (17). Because regulatory T-cells are very efficient at controlling the activation of B cells (33), this mechanism may explain the absence, or the reduction, of autoAbs in the different groups of tolerized mice treated with FK778. However, the reinduction of autoAbs production in these treated mice shortly after the interruption of the treatment in our in vivo model strongly argues against this possibility. Whatever the mechanism's involved in the split of tolerance observed in mice tolerized at birth with semiallogeneic spleen cells, we show here that FK778 is unable to promote tolerance in semiallogeneic host $CD4^+$ Th2 and donor B cells without apparently affecting the induction of tolerance of host $CD4^+$ Th1 and $CD8^+$ cells, evidenced by the persistence of B-cell chimerism for several weeks after FK778 withdrawal. In view of these results, we favor the idea that FK788 directly interferes with the activation of CD4+ T and B cells. In fact, A77 1726, the active metabolite of leflunomide from which FK778 is derived, has been shown to interfere with the phosphorylation of several tyrosine kinases involved in T-cell activation, to block the proliferation of T cells induced by several mitogens, and to inhibit the proliferation and antibody production of B cells in vitro (14, 16).

This study, however, has several limitations. One is that we cannot assure differences in metabolism and distribution of the drug in neonatal mice that could influence the response seen in our experiments. Secondly, unfortunately, during the development of the experiments, FK778 was withdrawn from the immunosuppression therapeutic arsenal in June 2006 owing to results of phase II trials that indicated unclear benefits over current treatment options. However, current studies suggest a role for FK778 in the prevention of chronic allograft nephropathy in a rat transplant model (34, 35), as well as decreasing the susceptibility of xenograft to acute or chronic rejection (36).

Together, our study indicates that FK778 is an efficient immunosuppressant for the control of antibody production resulting from allogeneic cognate T-B–cell interactions.

References:

1. Terasaki PI, Cai J. Humoral theory of transplantation: further evidence. Curr Opin Immunol. 2005;17(5):541-545.
2. Colvin RB, Smith RN. Antibody-mediated organ-allograft rejection. Nat Rev Immunol. 2005;5(10):807-817.
3. Kaufman CL, Gaines BA, Ildstad ST. Xenotransplantation. Annu Rev Immunol. 1995;13:339-367.
4. Platt JL, Lindman BJ, Geller RL, et al. The role of natural antibodies in the activation of xenogenic endothelial cells. Transplantation. 1991;52(6):1037-1043.
5. Erlich HA, Opelz G, Hansen J. HLA DNA typing and transplantation. Immunity. 2001;14 (4):347-356.
6. Vasilescu ER, Ho EK, Colovai AI, et al. Alloantibodies and the outcome of cadaver kidney allografts. Hum Immunol. 2006;67(8):597-604.
7. Derhaag JG, Duijvestijn AM, Damoiseaux JG, van Breda Vriesman PJ. Effects of antibody reactivity to major histocompatibility complex (MHC) and non-MHC alloantigens on graft endothelial cells in heart allograft rejection. Transplantation. 2000;69(9):1899-1906.
8. Dragun D, Müller DN, Bräsen JH, et al. Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection. N Engl J Med. 2005;352(6):558-569.
9. Austen WG Jr, Zhang M, Chan R, et al. Murine hindlimb reperfusion injury can be initiated by a self-reactive monoclonal IgM. Surgery. 2004;136(2):401-406.
10. Ramos A, Ruiz JC, de Francisco ALM, Gómez-Fleitas M, Arias M. Removal of xenoreactive antibodies by protein-A immunoadsorption: experience in 22 patients. Xenotransplantation. 2000;7(1):14-20.
11. Terasaki PI, Ozawa M. Predicting kidney graft failure by HLA antibodies: a prospective trial. Am J Transplant. 2004;4(3):438-443.
12. Rückemann K, Fairbanks LD, Carrey EA, et al. Leflunomide inhibits pyrimidine de novo synthesis in mitogen-stimulated T-lymphocytes from healthy humans. J Biol Chem. 1998;273(34):21682-21691.
13. Chong AS, Rezai K, Gebel HM, et al. Effects of leflunomide and other immunosuppressive agents on T cell proliferation in vitro. Transplantation. 1996;61(1):140-145.
14. Elder RT, Xu X, Williams JW, Gong H, Finnegan A, Chong AS. The immunosuppressive metabolite of leflunomide, A77 1726, affects murine T cells through two biochemical mechanisms. J Immunol. 1997 Jul 1;159(1):22-27.
15. Xu X, Williams JW, Bremer EG, Finnegan A, Chong AS. Inhibition of protein tyrosine phosphorylation in T cells by a novel immunosuppressive agent, leflunomide. J Biol Chem. 1995;270(21):12398-12403.
16. Siemasko K, Chong AS, Jäck HM, Gong H, Williams JW, Finnegan A. Inhibition of JAK3 and STAT6 tyrosine phosphorylation by the immunosuppressive drug leflunomide leads to a block in IgG1 production. J Immunol. 1998;160(4):1581-1588.
17. Kreijveld E, Koenen HJ, Hilbrands LB, van Hooff HJ, Joosten I. The immunosuppressive drug FK778 induces regulatory activity in stimulated human CD4+ CD25- T cells. Blood. 2007;109(1):244-252.
18. Schurmans S, Brighouse G, Kramer G, et al. Transient T and B cell activation after neonatal induction of tolerance to MHC class II or Mls alloantigens. J Immunol. 1991;146 (7):2152-2160.
19. Ramos A, Merino R, Merino J. Differences in non-MHC alloantigens promote tissue rejection but fail to mediate allogeneic co-operation and autoimmunity in mice neonatally injected with semi-allogeneic F1 B cells. Immunology. 1994;82(2):287-293.
20. Schurmans S, Heusser CH, Qin HY, Merino J, Brighouse G, Lambert PH. In vivo effects of anti-IL-4 monoclonal antibody on neonatal induction of tolerance and on an associated autoimmune syndrome. J Immunol. 1990;145(8):2465-2473.
21. Gonzalez AL, Conde C, Revilla C, Ramos A, Renedo B, Merino J. Autoimmune syndrome after induction of neonatal tolerance to I-E antigens. Eur J Immunol. 1993; 23: 2353-2357.
22. de la Hera M, de la Hera A, Ramos A, et al. Self-limited autoimmune disease related to transient donor B cell activation in mice neonatally injected with semi-allogeneic F1 cells. Int Immunol. 1992;4:67-74.
23. López-Hoyos M, Carrió R, Merino R, et al. Constitutive expression of bcl-2 in B cells causes a lethal form of lupuslike autoimmune disease after induction of neonatal tolerance to H-2b alloantigens. J Exp Med. 1996;183:2523-2531.
24. McDonnell TJ, Deane N, Platt FM, et al. bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell. 1989;57 (1):79-88.
25. Sandrin MS, Vaughan HA, Dabkowski PL, McKenzie IF. Anti-pig IgM antibodies in human serum react predominantly with Gal(alpha 1-3)Gal epitopes. Proc Natl Acad Sci USA. 1993;90(23):11391-11395.
26. Galili U. Interaction of the natural anti-Gal antibody with alpha-galactosyl epitopes: a major obstacle for xenotransplantation in humans. Immunol Today. 1993;14(10):480-