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mTOR Inhibition Suppresses Posttransplant Alloantibody Production through Direct Inhibition of Alloprimed B cells and Sparing of CD8⁺ antibody-suppressing T cells

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Abstract

Background—De novo alloantibodies (DSA) contribute to antibody-mediated rejection and poor long-term graft survival. Since the development of DSA is associated with early graft loss of cell transplants and reduced long-term survival of solid organ transplants, we hypothesized that conventional immunosuppressives, calcineurin inhibitors (CNI) and mammalian target of rapamycin inhibitors (mTORi), may not be as effective for suppression of humoral alloimmunity as for cell-mediated immunity.

Methods—Wild-type or CD8-depleted mice were transplanted with allogeneic hepatocytes. Recipients were treated with mTORi and/or CNI and serially monitored for alloantibody and graft survival. The direct effect of mTORi and CNI on alloprimed B cell function was investigated in Rag1^{-/-} mice adoptively transferred with alloprimed IgG1⁺ B cells. The efficacy of mTORi and/or CNI to suppress CD8-mediated cytotoxicity of IgG1⁺ B cells was evaluated in in vitro and in vivo cytotoxicity assays.

Results—mTORi, but not CNI, reduced alloantibody production in transplant recipients, directly suppressed alloantibody production by alloprimed IgG1⁺ B cells and delayed graft rejection in both low and high alloantibody-producers. Combination treatment with mTORi and CNI resulted in loss of the inhibitory effect observed for mTORi monotherapy in part due to CNI-suppression of CD8⁺ T cells which downregulate alloantibody production (CD8⁺ T_{Ab-suppressing} cells).

Conclusions—Our data supports that mTORi is a potent inhibitor of humoral immunity through suppression of alloprimed B cells and preservation of CD8⁺ T_{Ab-suppressing} cells. In contrast, alloantibody is readily detected in CNI-treated recipients because CNI does not suppress alloprimed B cells and interferes with downregulatory CD8⁺ T_{Ab-suppressing} cells.

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Introduction

Antibody-mediated rejection (AMR), caused by preformed or de novo donor-specific alloantibodies (DSA), is an important cause of graft rejection¹⁻³ and DSA is associated with reduced long-term allograft survival⁴. De novo DSA are particularly detrimental to cellular transplants, which have relatively smaller parenchymal cell mass and increased exposure to circulating antibodies⁵. Development of humoral alloimmunity after islet⁶⁻⁸ and hepatocyte transplant⁹ is associated with deterioration of graft function and is a barrier to long-term graft survival. Current therapies available for treatment of AMR include removal of deleterious alloantibodies, targeting IgG⁺ cells, cellular depletion, or a combination of these strategies^{10,11}. However, these therapies, initiated after the development of AMR, have produced unpredictable and often suboptimal results^{10,12}. Optimal maintenance immunosuppressive strategies to prevent posttransplant alloantibody production would mitigate the acute and long-term consequences of AMR.

In vitro data support the suppressive effects of mammalian target of rapamycin inhibitors (mTORi) on both murine and human B cell proliferation and maturation into antibody secreting cells (ASCs)¹³⁻¹⁶. When mTORi and calcineurin inhibitors (CNi) were compared, proliferation of LPS-stimulated mouse B cells in vitro, was suppressed following mTORi (but not CNi) treatment¹⁷. In contrast, other studies suggest CNi under select conditions inhibits B cell responses^{17,18}. Despite the fact that in vitro studies have shown efficacy of mTORi, and in some circumstances CNi, for suppression of human B cells, the clinical literature demonstrates a considerable number of recipients treated with these immunosuppressives continue to develop alloantibodies¹⁹⁻²². Surprisingly there is a relative paucity of published studies investigating the in vivo effects of these immunosuppressives on the humoral response after transplant.

Our group is the first to report that a population of CD8⁺ T cells, which we will refer to as CD8⁺ antibody-suppressing T (CD8⁺ T_{Ab-supp}) cells, negatively regulate humoral responses by killing allospecific IgG1⁺ B cells through the use of both Fas-FasL interactions and perforin²³. These studies were published in a well-validated model of hepatocyte transplant, characterized by a specific, Th2 driven IgG1-dominant pathway of alloantibody production²⁴⁻²⁹ which not only causes cell transplant rejection but is also known to result in graft rejection in vascularized cardiac transplant mouse models^{30,31}. Thus this CD8-dependent regulatory pathway applies to posttransplant alloantibody production after both cell and vascularized organ transplants.

The current studies were undertaken to address the relative efficacy of mTORi and CNi for suppression of in vivo humoral alloimmunity. We further determined whether combination CNi and mTORi produced additive or synergistic effects on humoral alloimmunity, and the effects on CD8⁺ T_{Ab-supp} cell and alloprimed B cell function.

Materials and Methods

Experimental animals

FVB/N (H-2^d MHC haplotype; Taconic, Hudson, NY) mice were used as allogeneic donors and C57BL/6, CD8 KO, and Rag1 KO (all H-2^b; Jackson Labs, Bar Harbor, ME) mouse strains were used as transplant and adoptive transfer (AT) recipients (6–10 weeks of age). Transgenic FVB/N mice expressing human alpha-1 antitrypsin (hA1AT) served as the source of “donor” hepatocytes, as previously described²⁴. All experiments were performed in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee of The Ohio State University (Protocol 2008A0068-R2).

Hepatocyte isolation, purification, and transplantation

Hepatocyte isolation, purification, and transplantation were performed, as reported²⁴. Graft survival was determined by detection of secreted hA1AT in serial recipient serum samples by ELISA^{24,28}. The reporter protein hA1AT does not elicit an immune response and syngeneic, hA1AT-expressing hepatocytes survive long-term²⁴.

Immunosuppressive treatments

Recipient mice were treated with in vivo doses of mTOR inhibitor (Rapamycin, Rapamune[®]) and/or CNi (FK506, Tacrolimus) (R-5000 and F-4900, respectively, LC laboratories, Woburn, MA) via intraperitoneal (i.p.) injection at concentrations of 0.001-5.0 mg/kg dissolved in PBS with 5% DMSO. In vitro assays utilized mTORi or CNi at concentrations of 1, 10, and 50 nM in 4-hour incubations with cells.

CD8⁺ T cell depletion

Recipients were depleted of circulating CD8⁺ T cells by i.p. injection of 100 mg of mAb (clone 53.6.72; days -2, -1), as described³². Depletion was confirmed through flow cytometric analysis of recipient peripheral blood lymphocytes.

CD8⁺ T cell isolation

CD8⁺ T cells were isolated from mouse splenocytes by negative selection columns as per manufacturer recommendations (MCD8C-1000, R&D Systems, Minneapolis, MN; purity routinely >90%). For AT, 10×10⁶ CD8⁺ T cells per mouse were suspended in serum free media.

B cell isolation

B cells were purified from splenocytes using anti-mouse B220⁺ magnetic beads (130-049-501) to isolate from untransplanted mice (naïve B cells) or anti-mouse IgG⁺ magnetic beads (130-048-401) to isolate from transplanted mice (alloprimed B cells) following the manufacturer's instructions (Miltenyi Biotec, Auburn, CA; purity routinely >95%).

Donor-reactive antibody

To quantify alloantibody titer, we analyzed recipient serum using published methods³³.

Alloprimed B cell reconstitution in Rag1 KO recipients

Naïve Rag1 KO mice underwent AT of 10×10^6 alloprimed IgG1⁺ B cells (isolated on day 5 posttransplant from high alloantibody-producing CD8 KO recipients). After AT on day 0, Rag1 KO received FVB/N splenocytes (20×10^6 cells; i.p.) as a source of alloantigen. On the day following AT, mice were treated with IL-4 (0.625 µg, 14-8041-80, eBioscience, San Diego, CA) and anti-CD40 (200 µg, BE0016-2, Bio × Cell, West Lebanon, NH).

***In vitro* CD8/B cell cytotoxicity assay**

Measurement of *in vitro* cytolytic elimination of alloprimed IgG1⁺ B cells was performed as previously published²³. The level of B cell-induced apoptosis was determined by propidium iodide uptake (LIVE/DEAD cell-mediated cytotoxicity kit, L-7010, ThermoFisher Scientific) and analyzed by flow cytometry, as previously published²³.

***In vivo* CD8/B cell cytotoxicity assay**

Measurement of *in vivo* cytolytic elimination of alloprimed IgG1⁺ B cells was performed as previously published²³.

Statistical analysis

General linear models were fit for each continuous outcome and contrasts used to compare relevant groups to test the primary hypothesis/hypotheses in each experiment. Model assumptions were assessed and violations to the normality assumption were addressed by transforming data to the natural log scale. Log rank tests were used to compare time to graft rejection. For experiments where all groups were compared to a control, Dunnett's method was used to adjust for multiple comparisons, otherwise, Holm's step down procedure was used to maintain the overall type 1 error rate at 5%. All analyses were conducted using SAS statistical Software Version 9.3 (SAS Institute, Inc., Cary, NC). To demonstrate the distribution of the data, alloantibody titer and *in vitro* and *in vivo* cytotoxicity results are listed as the mean plus or minus the standard error; graft survival results are listed as the median survival time (MST).

Results

Superior efficacy of mTORi compared to CNi for suppression of posttransplant alloantibody production and prolongation of allograft survival

C57BL/6 mice (wild-type; WT; H-2^b) were transplanted with allogeneic FVB/N hepatocytes (H-2^d) on day 0. On days 0-14 posttransplant mice were treated with a vehicle control (5% DMSO), mTORi (1 mg/kg/d), or CNi (1 mg/kg/d). Alloantibody titer was determined from recipient serum on day 14 posttransplant as mice exhibit peak antibody production at this time³². Recipients treated with CNi did not exhibit reduced alloantibody (titer=94±21) compared with DMSO-treated recipients (titer=113±31). mTORi-treated recipients had significantly reduced alloantibody (titer=15±3) compared to DMSO-treated ($p < 0.001$) and CNi-treated recipients ($p = 0.001$) (Figure 1A). Both mTORi-treated (MST=24.5 days, $p = 0.005$) and CNi-treated recipients (MST=26 days, $p = 0.006$) had prolonged allograft survival compared with DMSO-treated controls (MST=12 days) (Figure 1B).

In previous studies we found that CD8-depletion of wild-type recipients results in high alloantibody titer and graft rejection occurs primarily by an antibody-mediated (rather than cell-mediated) mechanism³⁴. To investigate the efficacy of mTORi and CNi for suppression of alloantibody production and prevention of AMR, we treated CD8-depleted recipients with mTORi and CNi. C57BL/6 mice were CD8-depleted, transplanted with FVB/N hepatocytes, and treated with the DMSO control, mTORi (1 mg/kg/d), or CNi (1 mg/kg/d) on days 0-14 posttransplant. Alloantibody titer was determined in recipient serum on day 14. High alloantibody titer occurred in DMSO-treated (titer=263±55) and CNi-treated recipients (titer=220±49). In contrast, mTORi treatment significantly inhibited alloantibody production (titer=28±8) compared to DMSO-treated ($p<0.001$) and CNi-treated recipients ($p<0.001$) (Figure 1C). This inhibition of alloantibody was associated with significant prolongation of graft survival in mTORi-treated recipients (MST=25.5 days) compared to controls (MST=14 days, $p=0.02$). Rejection in mTORi-treated recipients occurred approximately 2 weeks after cessation of mTORi therapy and 3 weeks after cessation of anti-CD8 mAb treatment. In these conditions, which focus on AMR without the contribution of CD8-mediated rejection, CNi-treated recipients (MST=12 days) had no survival advantage compared with controls (Figure 1D). Alloantibody production was inversely related to mTORi dosage (Supplemental Figure 1A) and high doses led to prolonged graft survival (Supplemental Figure 1B). Inhibition of alloantibody production and enhanced survival was not observed with high doses of CNi.

mTORi (but not CNi) treatment suppresses in vivo alloantibody production by alloprimed IgG1⁺ B cells

We developed a new model using immunoincompetent Rag1^{-/-} mice to directly investigate the effect of mTORi on alloprimed B cell effector function (alloantibody production) *in vivo*. IgG1⁺ B cells were isolated from CD8 KO hepatocyte transplant recipients on day 5 posttransplant and AT into naïve Rag1 KO mice. These mice received FVB/N splenocytes on day 0 as a source of alloantigen. On day 1 post-AT αCD40 mAb and IL-4 were injected i.p. as a surrogate for endogenous CD4⁺ T cell “help”. Transferred B cells produce high levels of alloantibody, which peaks on day 7 relative to AT, as can be observed in the DMSO-treated controls (titer=500±100) (Figure 2). Control Rag1 KO mice which received AT of naïve B cells (without allosplenocytes as an antigen source) did not produce alloantibody (data not shown). Cohorts of the mice reconstituted with IgG1⁺ B cells were treated with mTORi or CNi on days 1-7 relative to AT. CNi treatment did not suppress alloprimed B cell alloantibody production in reconstituted Rag1 KO mice (titer=500±100) compared with DMSO-treated controls. However, mTORi-treated mice had significantly reduced levels of alloantibody (titer=70±12) compared to control ($p<0.001$) and CNi-treated mice ($p<0.001$).

mTORi-mediated suppression of in vivo alloantibody production is negated when combined with CNi treatment

Given that many clinical protocols use combinations of therapies including mTORi and CNi, we investigated whether combination therapy might show additive or synergistic effects on alloantibody production or prolongation of allograft survival. C57BL/6 recipients underwent hepatocyte transplant on day 0, and were treated with the DMSO control, mTORi (0.25

mg/kg/d), CNi (1 mg/kg/d), combined mTORi (0.25 mg/kg/d) and CNi (1 mg/kg/d) on days 0-14 posttransplant. Additional recipients were CD8-depleted and treated with DMSO control, mTORi (1 mg/kg/d), CNi (1 mg/kg/d), combined mTORi (1 mg/kg/d) and CNi (1 mg/kg/d). Alloantibody titer in recipient serum was quantified on day 14 posttransplant. Unexpectedly, we found dual treatment with mTORi and CNi together did not result in additive or synergistic effects on alloantibody production but instead resulted in a loss of the inhibitory effects of mTORi monotherapy in both low and high-alloantibody producing recipients. Combination-treated WT recipients had higher levels of alloantibody (titer=75±11) compared to recipients treated with mTORi alone (titer=25±5, $p=0.005$) (Figure 3A). Combination-treated CD8-depleted recipients also had higher levels of alloantibody (titer=133±33) compared to recipients treated with mTORi alone (titer=28±8, $p=0.003$). (Figure 3A). Furthermore, whereas mTORi prolonged graft survival in WT recipients (MST=24.5), graft survival in combination-treated WT recipients (MST=12.5 days) was equivalent to DMSO-treated controls (MST=12 days, $p=ns$) (Figure 3B). On the other hand, graft survival was prolonged to a similar extent in CD8-depleted (high alloantibody producers) recipients treated with mTORi alone (MST=25.5) or with combined mTORi and CNi (MST=35.5 days, $p=ns$) compared to DMSO-treated recipients (MST=14 days, $p=0.02$, $p=0.002$ respectively) (Figure 3C). These unexpected results of immunosuppressants on in vivo alloantibody production prompted us to consider the effects of conventional mTORi and CNi immunosuppressants on other pathways, which regulate humoral alloimmunity.

CNi (but not mTORi) inhibits CD8-mediated killing of alloprimed B cells in vitro

We have previously reported a novel mechanism by which recipient CD8⁺ T_{Ab-supp} cells mediate *in vivo* cytotoxic killing of alloprimed B cells and downregulate posttransplant alloantibody production²³. If CNi treatment interfered with this downregulatory mechanism, this might explain why combination therapy adversely affected mTORi monotherapy in WT recipients. We utilized an in vitro cytotoxicity assay to determine if CNi treatment inhibits CD8-mediated killing of alloantibody-producing IgG1⁺ B cells. Co-culturing alloprimed CD8⁺ T cells with alloprimed IgG1⁺ B cells resulted in significant B cell apoptosis (11.4±1.2%). CNi-treatment resulted in significant suppression of CD8-mediated killing of B cells (3.5±0.8%) compared with control ($p=0.01$) and mTORi-treated (11.1±2.2%, $p=0.02$) cocultures. mTORi treatment had no effect on cytotoxicity compared with control cocultures. (Figures 4A & 4B) Neither mTORi nor CNi at any doses used (1, 10, 50 nM) was found to be directly cytotoxic to IgG1⁺/B220⁺ B cells or alloprimed/naïve CD8⁺ T cells (data not shown).²³ Taken together, these findings indicate that mTORi and CNi not only demonstrate differential effects on posttransplant alloantibody production, but they demonstrate distinct effects on at least 1 inhibitory immune pathway which downregulates humoral alloimmunity. Consequently, when these agents are combined, unexpected and deleterious consequences on humoral alloimmunity occur.

CNi (but not mTORi) inhibits CD8-mediated killing of alloprimed B cells in vivo

To investigate whether CNi inhibits CD8-mediated killing of alloantibody producing IgG1⁺ B cells in vivo, we utilized an in vivo cytotoxicity assay as previously described²³. A cohort of WT mice was transplanted with FVB/N hepatocytes and treated with the DMSO control,

mTORi (1 mg/kg/d), CNi (1 mg/kg/d), combined mTORi (1 mg/kg/d) and CNi (1 mg/kg/d) on days 1-7 posttransplant. In vivo killing of alloprimed B cells (IgG1⁺ B cells) was determined on day 7 posttransplant. Similar to the in vitro results, CNi-treatment interfered with CD8-mediated cytotoxic clearance of alloprimed B cells (27.5±5.4%) compared to DMSO-treated (61.5±5.3%, p=0.009) and mTORi-treated (65.2±7.4%, p=0.005) recipients. In vivo CD8-mediated B cell killing remained intact in mTORi-treated recipients. CD8-mediated B cell killing in combination-treated recipients was similar to CNi monotherapy treated recipients (31.3±6.1%). (Figures 5A & 5B) These findings support the interpretation that mTORi and CNi exert markedly different effects on de novo posttransplant alloantibody production, on alloprimed B cell effector function and CD8⁺ T cell antibody-regulating pathways.

Discussion

Given the importance of AMR in causing acute graft dysfunction¹¹ and the negative correlation of DSA with long-term allograft survival for both cell and organ transplants^{7-9,35-38}, it is of great interest to devise optimal maintenance immunosuppression to suppress the emergence of DSA posttransplant. The clinical literature includes publications which report that no current immunosuppressive regimens effectively prevent the development of DSA^{10,11}, while others infer relative efficacy of CNi by reporting that conversion of kidney transplant recipients from CNi to mTORi-based therapy is associated with a negative impact on DSA production and graft survival^{19,39-41}. These clinical data are difficult to interpret in aggregate and generally flawed due to the retrospective nature of the studies, the variability in treatment regimens (doses, timing, combination therapies, use of induction agents), and variability in reporting isotype, specificity and timing of DSA measurement. Investigation in animal models permits investigators to focus on specific immune pathways with rigorous controls. In the current studies, our aim was to investigate the efficacy of commonly used immunosuppressive agents mTORi and CNi upon posttransplant alloantibody production and allograft survival.

Results from these studies demonstrate for the first time that mTORi is a powerful suppressant of alloantibody production in part through inhibition of alloprimed B cell effector function. We found that mTORi's lack of interference with CD8⁺ T_{Ab-supp} cells is an added advantage, which contributes to reduced alloantibody titers in mTORi-treated recipients. Human and murine studies published by other groups suggest mTORi suppresses humoral alloimmunity through inhibition of CD40-mediated B cell activation, proliferation and antibody production^{13-15,17,42}. In vitro studies also show that mTORi suppress human B cell maturation and isotype switching in both IL-2-dependent and IL-2-independent humoral responses^{14,17,18}. Collectively, these published human studies and our current experimental studies in mice support the efficacy of mTORi for suppression of humoral alloimmunity.

The effects of CNi-treatment on human B cells are less clear. CNi has been shown to inhibit B cell responses to anti-IgM, low dose IgM and IL-4, but not to *Staphylococcus aureus* and CD40L stimulation^{14,17}. Heidt et al additionally showed that, in contrast to mTORi, CNi failed to inhibit B cell antibody production following coculture with pre-activated T cells⁴³. Interestingly a recent study shows that CNi inhibits naïve IL-2 and anti-CD40 stimulated

human B cell proliferation and differentiation but fails to inhibit total human B cell responses under similar conditions¹⁸. Thus the existing in vitro data supports CNi-mediated suppression of human B cells only under specific conditions.

The experimental model used in these studies focuses on Th2-driven IgG1 alloantibody production. There is some correlation between mouse and human IgG isotypes' biological functions and antigen-binding specificity. For example, murine IgG1 binds mast cells similar to human IgG4 and murine IgG2a and IgG2b isotypes fix complement and bind protein antigens similar to human IgG1 and IgG3⁴⁴. However, a better link between mouse and human alloantibody isotypes may be to consider the cytokines which stimulate their production. Our studies show that mTORi inhibits IL-4-dependent murine IgG1 alloantibody production^{32,45} which mediates graft rejection in both hepatocyte and vascularized cardiac transplant mouse models^{30,31,34}. In contrast to murine studies, where IL-4 drives primarily IgG1 and IgE⁴⁶, the human response to IL-4 is more complex. Kotowicz et al found that low doses of IL-4 drive IgG1 (and also IgG2 and IgG3 but not IgG4) production^{47,48}, while only high doses of IL-4 drive IgG4 production⁴⁸. Human IgG1 alloantibody is of particular importance due to reports correlating IgG1 with kidney allograft rejection and reduced allograft function⁴⁹. In addition, other reports suggest IL-4 drives human IgG4 and IgE production⁵⁰⁻⁵³. Extrapolation from the current rodent studies to humans would predict mTORi-mediated inhibition of multiple IL-4-driven alloantibody isotypes.

CNi are powerful immunosuppressives that inhibit the protein phosphatase calcineurin thereby blocking T cell IL-2 production, thus inhibiting T cell activation and downstream effector functions^{54,55}. Treatment with CNi after renal and other solid organ allograft transplantation is associated with reduced episodes of acute rejection and enhanced allograft survival. Consequently, CNi-based therapies are widely used for maintenance immunosuppression after transplantation^{56,57}. CNi-based immunotherapies are used following clinical cell transplantation including hepatocyte transplantation but have been associated with limited success for sustained hepatocellular allograft survival^{9,58-60}. While early studies have not sufficiently explored alloantibody responses following clinical hepatocyte transplant, recently Jorns et al reported the development of de novo donor-specific HLA antibody in 2 patients who underwent hepatocyte transplantation. The onset of humoral alloimmunity and graft loss occurred in the context of lowering and eventual discontinuation of immunosuppression in 1 patient and despite the use of maintenance immunosuppression with CNi and low dose steroids in the second case⁹. The current experimental studies which demonstrate the lack of efficacy of CNi for suppression of alloantibody production provide insight into clinical studies which demonstrate detection of DSA in CNi-treated recipients.

The current studies clearly show that treatment with CNi does not effectively suppress humoral alloimmunity after transplantation. In our previous studies we discovered an important regulatory pathway which decreases alloantibody production; we reported that CD8⁺ T cells kill IgG1⁺ B cells in an allospecific manner and that when CD8⁺ T cells are depleted significant augmentation of alloantibody levels occurs²³. In the current studies, we found that CNi suppresses CD8⁺ T_{Ab-supp} cell-mediated killing of IgG1⁺ B cells in vitro and in vivo. These studies do not exclude other potential causes for B cell death, however the

CD8-mediated cytotoxic killing of antibody-producing IgG1⁺ B cells is a dominant pathway, as recipients depleted of CD8⁺ T cells have a marked increase in alloprimed B cell survival and higher alloantibody production following hepatocyte²³, skin, or islet transplant (unpublished observations). To our knowledge this report provides first evidence for the failure of CNi to suppress alloprimed B cell production of alloantibody *in vivo* and for CNi-mediated suppression of CD8⁺ T cells with downregulatory functions (CD8⁺ T_{Ab-sup}) on humoral alloimmunity. However, these antibody-promoting effects of CNi therapy are counterbalanced by CNi-mediated immunosuppression of CD4⁺ T cells, which facilitate antibody production^{18,43}. This would account for minimal reduction of alloantibody production in CNi monotherapy and adverse effect on alloantibody production when CNi is combined with mTORi. However, this does not exclude other potential explanations for the unexpected effects of combination therapy with CNi and mTORi. For example, dose-dependent CNi-mediated antagonism of mTORi effects has been reported for mouse T cells⁶¹ and for mouse and human alloprimed B cells^{14,17}. The competitive inhibition between mTORi and CNi has been attributed to saturation of FK506-binding protein 12⁶². There is some evidence for this antagonism in our data. Since we observed that mTORi-mediated suppression of alloantibody production is reversed in both WT and CD8-depleted recipients following combination therapy, the antagonistic effects of CNi may occur independent of CD8⁺ T cells. While this CNi-mediated antagonism on alloantibody production (higher alloantibody titers) was associated with shortened graft survival in combination-treated WT recipients, this was not the case in combination-treated CD8-depleted recipients which had prolonged graft survival despite the higher alloantibody titers. The latter results may reflect the effect of higher doses of mTORi (1.0 mg/kg/d) used in the CD8-depleted recipients compared to the wild-type recipients (0.25 mg/kg/d) upon macrophages, which contribute to antibody-dependent cell-mediated cytotoxicity (ADCC)⁴⁵. mTORi, but not CNi, dampens macrophage-mediated ADCC in a dose-dependent fashion (unpublished observations). Thus, we speculate that in combination with CNi, the low dose of mTORi (0.25 mg/kg/d) did not suppress macrophage-mediated ADCC and graft rejection (MST=12.5 days) while the higher mTORi dose (1.0 mg/kg/d) did and led to prolonged hepatocyte survival (MST=35.5 days) despite the presence of alloantibody.

mTORi is known to inhibit T cell proliferation and cytokine production⁴³, which is the basis for use in transplantation to prevent cellular rejection, however accumulating evidence shows that T cell subsets demonstrate differential susceptibility to inhibition by mTORi. Mouse and human studies show that while mTORi mediates suppression of conventional alloreactive CD4⁺ T cells it does not suppress CD4⁺ T_{reg} cells⁶³. Our results extend these findings to CD8⁺ T cell populations and show that mTORi selectively spares CD8⁺ T_{Ab-sup} cell function but not CD8⁺ T cells mediating rejection. These results demonstrate a differential effect of mTORi and CNi on different CD8⁺ T cell subsets under the same circumstances. Our results have similarity to those published by Ferrer et al. in which treatment with mTORi did not suppress the number and effector function of CD8⁺ T cells which recognize OVA-peptide conjugated to *Listeria monocytogenes* while it did reduce the number and effector function of graft-specific CD8⁺ T cells which recognize OVA expressed on grafted skin⁶⁴. Alternatively, mTORi may have differential effects on T cells based on their utilization of the mTOR signaling pathway. The mTOR signaling pathway is a central

regulator of T cell development, and promotes Th1, Th2, and Th17 cells while inhibiting T_{reg} cells⁶⁵. Indeed Zeiser et al found that T_{reg} mTOR insensitivity is due to minimal usage of the mTOR-signaling pathway⁶³.

Collectively, these studies support the efficacy of mTORi for suppression of humoral alloimmunity in part through direct effects on alloprimed B cell function and by sparing of CD8⁺ T_{Ab-supp} cells. These studies also highlight the need to monitor not only the production of DSA but to include assessment of alloantibody isotypes and quantity. Our dose response studies emphasize the importance of considering the quantitative exposure to immunosuppressants for interpretation of mTORi and CNi effects on T and B cell responses in future clinical cell and solid organ transplant studies. Since our results conflict with some studies in humans some caution is warranted regarding direct application of these findings to humans. Nevertheless, our studies provide a strong rationale for future translational studies to determine how and under what conditions these apply to humoral immunity in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations

AMR	Antibody-mediated rejection
AT	Adoptive transfer
CNi	calcineurin inhibitor
DSA	donor-specific antibody
hA1AT	human alpha-1 antitrypsin
mTORi	mammalian target of rapamycin inhibitor

Figure 1. Superior efficacy of mTORi compared to CNi for suppression of posttransplant alloantibody production and prolongation of allograft survival

A) C57BL/6 (H-2^b, wild-type; WT) mice were transplanted with allogeneic FVB/N (H-2^q) hepatocytes. On days 0-14 posttransplant cohorts of recipients were treated with 1 mg/kg/d mTORi or CNi or a 5% DMSO-vehicle control. Alloantibody was quantified by analyzing recipient serum by antibody titer on day 14 posttransplant. mTORi-treated recipients had significantly reduced alloantibody (n=4, titer=15±3) compared with DMSO-treated recipients (n=4, titer=113±31, *p<0.001) and CNi-treated recipients (n=4, titer=94±21, †p=0.001). CNi-treated and control recipients had comparable alloantibody titers. **B)** Recipient graft survival was assessed through serum hA1AT levels. Treatment with either mTORi (MST=24.5 days, *p=0.005) or CNi (MST=26 days, †p=0.006) resulted in a significant prolongation of hepatocyte survival compared with control recipients (MST=12 days). **C)** CD8-depleted (days -2, -1) recipients (high-alloantibody producers) were similarly transplanted with allogeneic hepatocytes and treated with mTORi, CNi, or a DMSO-vehicle control on days 0-14 posttransplant. mTORi-treated recipients had significantly reduced levels of alloantibodies (n=4, titer=28±8) compared to the DMSO-treated (n=4, titer=263±55, *p<0.001) and CNi-treated recipients (n=5, titer=220±49, †p<0.001). In contrast, CNi-treated and control recipients had comparable alloantibody titers. **D)** mTORi (MST=25.5 days) prolonged hepatocyte survival in high alloantibody producers compared to

DMSO-treated recipients (MST=14 days, * $p=0.02$). CNi-treated (MST=12 days) and control recipients had similar graft survival. Data is representative of triplicate experiments.

Figure 2. mTORi (but not CNI) treatment suppresses *in vivo* alloantibody production by alloprimed IgG1⁺ B cells

Rag1 KO mice were adoptively transferred (AT) with alloprimed IgG1⁺ B cells (10×10^6 cells i.v.) and injected with FVB/N splenocytes as an antigen source (20×10^6 cells, i.p.). As a control, a cohort of mice received AT of only naive B cells and exhibited low levels of alloantibody (data not shown). On day 1 relative to AT, mice were given anti-CD40 mAb (200 mg) and IL-4 (0.625 mg) to mimic CD4⁺ T cell “help” normally absent in these mice. Cohorts of mice were treated with DMSO-vehicle control, mTORi (1 mg/kg/d), or CNI (1 mg/kg/d) on days 1-7. Alloantibody was quantified by analyzing recipient serum by antibody titer on day 7 posttransplant. AT of primed B cells resulted in high levels of alloantibody production in Rag1 KO mice ($n=4$, 500 ± 100). Treatment with mTORi resulted in significant inhibition of *in vivo* alloantibody production by alloprimed B cells ($n=5$, titer= 70 ± 12) compared to DMSO-treated (* $p < 0.001$) and CNI-treated mice ($n=6$, titer= 500 ± 100 , † $p < 0.001$). CNI-treated recipients exhibited no reduction in alloantibody compared with control mice. Data was combined from duplicate experiments.

Figure 3. mTORi-mediated suppression of in vivo alloantibody production is negated when combined with CNi treatment

WT mice received hepatocyte transplants from FVB/N donors. Recipients were treated on days 0-14 with mTORi (0.25 mg/kg/d), CNi (1 mg/kg/d), a combination of both mTORi and CNi, or DMSO vehicle control. Some recipients were CD8-depleted (d-2,-1) and treated with DMSO control, mTORi (1 mg/kg/d), CNi (1 mg/kg/d), combined mTORi (1 mg/kg/d) and CNi (1 mg/kg/d). **A**) Alloantibody was quantified by analyzing recipient serum alloantibody titer on day 14 posttransplant. In both WT and CD8-depleted recipients CNi-treatment did not result in a reduction of alloantibody (WT: n=4, 94±21; CD8-depleted: n=5, 220±49) compared with the control (WT: n=4, titer=113±31; CD8-depleted: n=4, titer=263±55). In both WT and CD8-depleted recipients mTORi-treated recipients had significantly reduced alloantibody (WT: n=4, titer=25±5; CD8-depleted: n=4, titer=28±8) compared to the control (p=0.002, p=0.0001 respectively). Unexpectedly, combination treatment with both mTORi and CNi resulted in significantly higher levels of alloantibody in both WT (n=6, titer=75±11) and CD8-depleted recipients (n=3, titer=133) compared to recipients treated with mTORi alone (*p=0.005, †p=0.003 for WT and CD8-depleted groups respectively). **B**) Recipient graft survival was assessed during treatment with CNi and/or mTORi and for an additional two weeks following cessation of immunosuppressive therapy. While mTORi treatment significantly prolonged graft survival (MST=24.5 days, *p=0.005) in WT recipients, combination treatment with mTORi and CNi did not (MST=12.5 days, p=ns) when compared with DMSO-treated control recipients (MST=12 days). **C**) In contrast, in CD8-depleted recipients both treatment with mTORi alone (MST=25.5), or combination treatment with mTORi and CNi (MST=35.5 days) significantly prolonged hepatocyte survival compared to DMSO-treated controls (MST=14 days, *p=0.02, †p=0.002 respectively). Data was combined from triplicate experiments.

Figure 4. CNI (but not mTORi) inhibits CD8-mediated killing of alloprimed B cells *in vitro*

WT and CD8 KO mice were transplanted with FVB/N hepatocytes. Naive and alloprimed CD8⁺T cells and B cells were isolated (day 7 posttransplant for alloprimed cells). CD8⁺ T cells and B cells were co-cultured at a 10:1 ratio for 4h with mTORi or CNI (10 nM) or 5% DMSO vehicle control. Prior to co-culture, B cells were stained with CFSE. Propidium iodide staining was used to determine apoptosis within CFSE-stained B cells (percent PI positive by flow cytometry). **A**) Bulk alloprimed CD8⁺T cells (“aCD8”) caused significant apoptosis of alloprimed (IgG1⁺) B cells (n=3, 11.4±1.2%) compared to naive CD8⁺ T cells (“nCD8”, n=3, 3.5±0.2%). mTORi treatment did not inhibit CD8-mediated apoptosis of primed B cells (n=3, 11.1±2.2%). However, CNI inhibits CD8-mediated cytotoxicity to B cells (n=3, 3.5±0.8%) compared to DMSO-treated (*p=0.012) and mTORi-treated co-cultures (†p=0.02). Data is representative of triplicate experiments. **B**) Representative flow cytometry plots are shown. To analyze for apoptotic B cells in the co-cultures, cells were first gated on lymphocytes and then subsequently on the CFSE⁺ labeled-B cells. Uptake of propidium iodide (PI) by CFSE⁺ labeled B cells was analyzed.

Figure 5. CNi (but not mTORi) inhibits CD8-mediated killing of alloprimed B cells in vivo
 WT transplant recipients were treated with mTORi (1 mg/kg/d), CNi (1 mg/kg/d), combined mTORi (1 mg/kg/d) and CNi (1 mg/kg/d), or 5% DMSO vehicle control on days 1-7 posttransplant. In vivo cytotoxicity of B cells was determined by transferring equal numbers of CFSE-labeled alloprimed IgG1⁺ (CFSE^{hi}) and naïve (CFSE^{lo}) B cells into WT mice 7 days after transplant. The spleen of recipient mice was collected 18 hours later and evaluated for recovery of both naïve and alloprimed IgG1⁺ B cells to calculate the specific lysis of alloprimed IgG1⁺ B cells. **A**) WT transplant recipient mice treated with DMSO vehicle control (n=4, 61.5±5.3%) or mTORi (n=5, 65.2±7.4%) exhibited relatively high cytotoxicity, while those receiving CNi (n=5, 27.5±5.4%) exhibited significantly less *in vivo* cytotoxicity of alloprimed IgG1⁺ B cells compared to DMSO-treated (*p=0.009) and mTORi-treated recipients ([‡]p=0.005). Combined mTORi (1 mg/kg/d) and CNi (1 mg/kg/d) treatment (n=3, 31.3±6.1%) had similar in vivo cytotoxicity of alloprimed IgG1⁺ B cells compared to CNi-treated recipients. Data was combined from duplicate experiments. **B**) Representative flow cytometry plots are shown. To analyze the proportion of CFSE-labeled alloprimed (CFSE^{hi}) and naïve (CFSE^{lo}) B cells remaining 18 hours after adoptive transfer, splenocytes from recipient mice were analyzed by first gating on lymphocytes and then subsequently on the CFSE⁺ labeled-B cells.