SUPPRESSIVE TREG CELL ACTIVITY IS POTENTIATED BY GLYCOGEN SYNTHASE KINASE 3β INHIBITION

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Running head: Increased Treg Suppression and GSK-3β Inhibition

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The mechanism by which regulatory T cells (Tregs) suppress the immune response is not well defined. A recent study has shown that β-catenin prolongs Treg survival. Since β-catenin is regulated by GSK-3β directed phosphorylation, we focused on GSK-3β and the role it plays in Treg function. Inhibition of GSK-3β leads to increased suppression activity by Tregs. Inhibitor-treated Tregs exhibited prolonged FoxP3 expression and increased levels of β-catenin and of the anti-apoptotic protein, Bcl-XL. Systemic administration of GSK-3β inhibitor results in prolonged islet survival in an allotransplant mouse model. Our data suggest GSK-3β could be a useful target in developing strategies designed to increase the stability and function of Tregs for inducing allotransplant tolerance or treating autoimmune conditions.

Regulatory T cells (Tregs) are charged with the important task of maintaining homeostatic T cell reactivity and are thought to prevent autoimmunity by restraining self-reactive T cells that escape thymic deletion (1,2). Moreover, Treg cells have been shown to down-regulate all T cell driven immunity (3,4), which is of increasing interest in the transplant community. However, expansion of Tregs has proved challenging due to their anergic potential. While some studies have shown induction of CD4+CD25+ Treg cells from CD4+CD25- cells with various exogenous cytokines or peptides (5-7), their functional profile may not be the same as that of naturally occurring CD4+CD25+ Treg cells (8). This difference has given impetus to those searching for a method to improve CD4+CD25+ Treg cell stability and activity.
Recent work shows that β-catenin may play a central role in Treg cell stabilization. Ding and colleagues have shown that ectopic expression of a mutated form of β-catenin that lacks the classic Ser33/Ser37/Thr41/Ser45 N-terminal amino acids and is therefore nondegradable, was able to enhance Treg survival in vitro (9). β-catenin is best known for its role in Wnt signaling, a pathway which governs patterning and cell fate determination during embryogenesis (10,11). β-catenin is regulated by the serine/threonine kinase, glycogen synthase kinase-3 or GSK-3β. Phosphorylation of β-catenin by GSK-3β targets β-catenin for ubiquitination and degradation, terminating the signal (11). In contrast, inhibiting GSK-3β activity with Li+ or other GSK-3β inhibitors or by expressing a dominant inhibitory mutant of GSK-3β, leads to a significant increase in β-catenin levels (12). When cellular levels of β-catenin rise, β-catenin interacts with transcription factors of the HMG box family, LEF-1 and Tcf, enters the nucleus, and regulates induction of genes believed to be involved in the proliferative response(13). It is this downstream activation of cellular growth that has made β-catenin especially interesting to immunologists searching for immunosuppressive targets. Recently, manipulation of the Wnt/β-catenin pathway has been shown to alter T cell expansion and their cytokine profile (14-17). This association between β-catenin and T cell proliferation has prompted strong query into β-catenin’s action in Tregs.

By utilizing the GSK-3β inhibitor, SB216763, we examined what role GSK-3β plays in Treg activity. We also suggest a possible mechanism by which SB216763 may act on increasing Treg suppressive function. Treg cells treated with SB216763 were found to maintain elevated levels of FoxP3 protein and the anti-apoptotic protein Bel-XL as compared to the untreated controls. Given the possible overt relationship between SB216763 and prevention of apoptosis in Treg cells, we also examined what effects DEVD-CHO, a caspase-3 inhibitor, had on Treg function. Caspase-3 inhibitor, when used alone, resulted in an increase in Treg suppression activity. When used in combination with GSK-3β inhibitor, we observed no further augmentation in this suppressive effect, suggesting that the two inhibitors may be acting on the same or parallel pathways. Additionally, when SB216763 is given systemically in an islet allotransplant mouse model, islet allograft survival is observed. Nevertheless, SB216763 and drugs similar in function provide obvious laboratory and clinical advantages in defining the role of Treg cell activity in models for autoimmunity and transplantation.

**Experimental Procedures**

*Animals*- C57BL/6 (designated B6 for clarity) (H-2b), BALB/c (H-2d) and C3H (H-2k) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). All mice were kept in a pathogen-free, filter top isolator cages. All animals were cared for according to methods prescribed by the American Association for the Accreditation of Laboratory Animal Care. All protocols were approved by the Institutional Committee for Research Animal Care.

*CD4+CD25- Effector T cell and*
**CD4+CD25+ Treg cell Isolation** - Single cell suspensions were prepared from B6 and BALB/c mice using standard methods. After a brief erythrocyte lysis using Red Blood Cell Lysing Buffer (Sigma-Aldrich), the cells were resuspended in degassed PBS with 0.5% BSA and 2mM EDTA. Separation of CD4+25- and CD4+CD25+ cells was accomplished with magnetic labeling using a MACs CD4+CD25+ Treg Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4+CD25- cells were negatively selected with the flow through fraction, while the CD4+CD25+ cells were positively selected. The purity of CD4+CD25+ fractions was confirmed by Fluorescence Activated Cell Sorting (FACS) analysis since anti-CD25 PE antibody was used for purification. Final purity of the cell population was 90-95% (data not shown).

**In Vitro Suppression Assay** - CD4+CD25+ Tregs were co-cultured with CD4+CD25- T cells stimulated with anti-CD3/CD28 Dynabeads (Invitrogen) with 5 µM SB216763 (Sigma-Aldrich) or with 0.05% DMSO (vehicle control). CD4+CD25+ Treg cells were added in increasing ratios to CD4+CD25- T cells for dose response measurements. Proliferation was measured in triplicates by the incorporation of tritiated thymidine over the last 18-20 h of the coculture. All cells were cultured in complete RPMI medium.

**Western Blot Analysis** - Column-purified CD4+CD25+ cells were plated at 400,000 cells/well in a 96 well plate and incubated in the absence or presence of 10 µM of SB216763 (SIGMA-Aldrich). Cells were counted and lysed in a volume of 1X SDS loading buffer (BIORAD), proportionate to the number of cells, at 42 and 66 hours. To evaluate Bcl-2 levels, proportionate samples were loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, electrophoresed, and transferred onto Immobilon-P membrane (Millipore). Western blotting was performed as described previously (18) by using anti-Bcl-2 antibodies (1:1,000; Cell Signaling). As a loading control, the immunoblot was stripped and reprobed with anti-β-actin antibody (1:1,000; Cell Signaling).

**Mouse Islet Isolation and Transplantation** - In this in vivo model, B6 mice were used as recipients, and C3H mice were used as islet donors. Prior to islet transplantation, B6 mice were rendered diabetic by intraperitoneal (IP) injections of 200mg/kg Streptozotocin (STZ) (Sigma-Aldrich). Cells were then washed and permeabilized with 0.2% Igepal CA-630 (Sigma-Aldrich) and stained intracellularly with 0.5 µg antibody for 30 min at room temperature with FITC-conjugated anti-mouse Foxp3 (FJK-16s) (eBioscience), anti-mouse GSK-3β (pTyr 216) (Cell Signaling Technologies), anti-mouse β-catenin (pSer33/37/Thr41) (27C10) (Cell Signaling Technologies), anti-mouse β-catenin (H-102) (Santa Cruz Biotechnology) or anti-mouse Bcl-XL (Santa Cruz Biotechnology). Secondary goat anti-rabbit Alexa-488-conjugated antibody was purchased from Invitrogen.
Diabetes was defined as blood glucose levels >200 mg/dl for at least two consecutive days. Islets from C3H mice were isolated by the standard technique of collagenase digestion and Ficoll purification. Following isolation, 500 fresh islets were transplanted under the kidney capsule of diabetic B6 mice treated with saline containing 1% DMSO in the control group or with daily IP injections of SB216763 (100 µM) in the experimental group. Euglycemia was defined as a non-fasting blood glucose level <200 mg/dl. Rejection was diagnosed when animals became hyperglycemic again with BG>200 mg/dl for at least 2 consecutive days. For Treg depletion studies, mice were injected with PC61 anti-CD25 (BioXCell, Lebanon, NH) 250µg IP day -8 and day -3 prior to islet transplantation. On the day of transplant, peripheral blood was analyzed with GK1.5 anti-CD4 and 7D4 anti-CD25 (eBioscience) to demonstrate Treg depletion.

Statistical analyses- Data were presented as means ± s.e.m. Where indicated, we determined the statistical significance between two groups by the logrank test (Mantel-Cox test). Values of P <0.05 were considered statistically significant.

RESULTS

Naïve CD4+CD25+FoxP3+ Treg cells exhibit elevated GSK-3β activity and increased β-catenin phosphorylation in vitro. Activated GSK-3β is characterized by phosphorylation of tyrosine-216 (Tyr216). In Figures 1A and 1B, we FACS analyzed isolated, column-purified, CD25-PE labeled B6 splenic, naïve CD4+CD25+ cells for the presence of phospho-Tyr216 and showed that CD4+CD25+hi cells exhibited the greatest staining with phospho-Tyr216 antibody (Figure 1A.C, 1A.F and 1A.I). GSK-3β phosphorylates β-catenin on serine 33/37 and threonine 41, resulting in the degradation of β-catenin. Using antibodies specific to phospho-β-catenin (Ser33/37,Thr41), we show that CD4+CD25+hi cells also displayed the greatest phospho-β-catenin (Ser33/37,Thr41) staining, as revealed by FACS analysis (Figure 1A.B). We obtained similar FACS analysis results with similarly purified CD4+CD25+ cells from B6 naïve lymph nodes (Figures 1A.E) and from BALB/c naïve spleen (Figures 1A.H). These results are important because they show that naïve CD4+CD25+hi cells, which also have the greatest FoxP3 levels, possess the highest GSK-3β activity, as indicated by having the highest degree of phospho-β-catenin (Ser33/37,Thr41) (60-70%) labeling, when compared to CD4+CD25+med and CD4+CD25+lo cells. In addition, analysis of CD4+CD25- FoxP3- cells revealed a lower GSK-3β activity when compared to CD4+CD25+hi cells as ascertained by phospho-β-catenin (Ser33/37,Thr41) labeling (Figure 1B).

Inhibition of GSK3β with SB216763 increased suppressive activity of CD4+CD25+FoxP3+ cells. To assess the effect of GSK-3β inhibition on the functional properties of Treg cells, we performed an in vitro suppression assay in the absence or presence of SB216763, a specific GSK-3β inhibitor (Figure 2). We isolated CD4+CD25- effector T cells and CD4+CD25+ Treg cells using magnetic separation. CD4+CD25- effector T cells
were incubated in the presence or absence of anti-CD3/CD28 microbeads with increasing amounts of CD4+CD25+ Treg cells, with or without SB216763. Proliferation was assessed by H\(^3\)-thymidine uptake. Notably, CD4+CD25- T effectors cells incubated with an equal number of anti-CD3/CD28 microbeads and in the absence of Treg cells showed a 25% increase in H\(^3\)-thymidine uptake with the addition of SB216763 when compared to vehicle treated control. This is not unprecedented since Oheteki and colleagues have previously demonstrated that inhibition of T cells with lithium, an inhibitor of GSK-3β, resulted in increased T cell proliferation (19). More importantly, there was a marked difference in suppression of CD4+CD25- T cells cultured with CD4+CD25+ Tregs with and without SB216763. At a 1:1 ratio of CD4+CD25- T cells and CD4+CD25+ Treg cells, the suppression was 65% as measured by H\(^3\)-thymidine uptake. While this was not unexpected, we observed an increase in suppression by CD4+CD25+ Treg cells in cultures supplemented with SB216763. Moreover, this enhanced suppression was titratable with increasing numbers of SB216763 treated CD4+CD25+ Tregs yielding better suppression than control CD4+CD25+ Tregs. Namely, 95% suppression was seen when CD4+CD25- T cells were combined in 1:8 ratio with CD4+CD25+ Tregs and SB216763, while CD4+CD25- without the GSK-3β inhibitor yielded 78% inhibition.

Inhibition of GSK3β with SB216763 results in the stabilization of β-catenin in CD4+ CD25+hi FoxP3+ cells. Ding and colleagues have shown that the introduction of a stable mutant form of β-catenin leads to the increased survival of Tregs (9). GSK-3β has been shown to phosphorylate β-catenin, targeting it for degradation via ubiquitination. Therefore, we analyzed what effect inhibition of GSK-3β by SB216763 would have on β-catenin levels in our experimental system. We co-cultured CD4+CD25- effector T cells and CD4+CD25+Tregs with anti-CD3/CD28 microbeads in the presence or absence of SB216763. Using FACS analysis we assessed untreated and SB216763-treated cultures for β-catenin while gating on the CD4+CD25+hi FoxP3+ Treg cells (Figure 3). In this experimental design β-catenin is elevated in Treg cells during all time points. CD4+CD25- cells also show increased β-catenin levels (data not shown), which would explain why they exhibit greater proliferation in the presence of SB216763 in the absence of Tregs (Figure 2). This is unsurprising, given that β-catenin is implicated in cellular proliferation with its heightened presence due to GSK-3β inhibition. Interestingly, while there are increased β-catenin levels in treated cells, we also observe β-catenin protein stabilization in untreated CD4+CD25+hiFoxP3+ cells. This stabilization of β-catenin was observed in CD4+CD25+hiFoxP3+ cells only when they were co-cultured with CD4+CD25- cells (data not shown).

CD4+CD25+ FoxP3+ Treg Cells Exhibit Less FoxP3 Protein Turnover in the Presence of SB216763. We have noticed that CD4+CD25+hiFoxP3+ cells, when grown in vitro, exhibit rapid loss of FoxP3 protein (data not shown). Given that inhibition of GSK-3β enhances Treg cell activity, we wanted to assess whether SB216763 treatment influenced FoxP3 turnover.
CD4+CD25- T cells and CD4+CD25+ Treg cells were isolated using magnetic separation and co-cultured in a 1:1 ratio with anti-CD3/CD28 microbeads with and without SB216763. The collective grouping of T effector and Treg cells was fixed and stained with anti-CD4 perCyP and Foxp3 FITC in intervals of 0, 24, 48 and 72 hours (Figure 4). CD4+CD25- T cells alone incubated with anti-CD3/CD28 microbeads with and without SB216763 served as controls for this experiment. Using FACS analysis and gating on FoxP3+ cells, we found that CD4+CD25+ Treg cells co-cultured with CD4+CD25- T cells and treated with SB216763 maintain their FoxP3+ signature. Foxp3 expression in untreated CD4+CD25+ Treg cells was detected in 39.7% of cells at 24 hours, 51.6% at 48 hours and 18.3% at 72 hours. Contrastingly, SB216763 treated CD4+CD25+ Tregs displayed a slower pace of loss of FoxP3 protein. These cells had a FoxP3 signal of 54.6% at 24 hours, 32.5% at 48 hours and 31.2% at 72 hours. This finding suggests that treatment with SB216763 slows FoxP3 cell turnover in Tregs in vitro. When CD4+CD25- effector T cells that had proliferated in the presence of anti-CD3/CD28 microbeads were examined, they were found to display minimal to no Foxp3 expression (data not shown).

Increase in the levels of the anti-apoptotic protein, Bcl-XL and Bcl-2, in Tregs following GSK-3β inhibition. Previous studies have shown that inhibition of GSK-3β results in increased cell survival by the stabilization of β-catenin (9). Our data suggest that SB216763 treatment results in increased Bcl-XL protein expression in Tregs. Bcl-XL is an anti-apoptotic protein that works to counteract cellular death through inhibitory binding of apoptotic mediators (9). Interestingly, we demonstrate heightened Bcl-XL protein levels in CD4+CD25+ Treg cells when they are co-cultured with CD4+CD25- T cells and with anti-CD3/CD28 microbeads. FACS analysis on gated CD4+CD25+hi cells demonstrates that untreated cells exhibit a 19% increase in Bcl-XL signal while SB216763-treated cells exhibit a 31% increase in Bcl-XL within 24 hours (Figure 5A). By 48 hours, untreated cells return to their original Bcl-XL levels while treated cells maintained 13% higher levels of Bcl-XL protein even after 72 hours. Western blot analysis of purified CD4+CD25- treated in vitro with SB216763 showed that Bcl-2 levels are maintained at least up to 66 hours following GSK-3β inhibition versus untreated cells (Figure 5B). The increase in Bcl-XL and Bcl-2 protein levels correlated with increased cell survival of CD4+CD25+ cells, where SB216763-treated cells exhibited 20% more cells at 66 hours versus controls (data not shown). Collectively these findings suggest that the GSK-3β inhibitor, SB216763 may work through BcL-XL and Bcl-2 to protect CD4+CD25+Tregs from apoptosis in vitro.

Caspase-3 Inhibition with CPP32/Apopain Results in Increased Suppression Activity in Tregs. GSK-3β has been shown to play a role in apoptosis, in part by destabilizing Bcl-2 (20,21). Since we observe a stabilization of the anti-apoptotic proteins Bcl-XL and Bcl-2 following GSK-3β inhibition, we next examined whether SB216763 inhibition acted concordantly with inhibition of apoptosis. To explore this, we made use of a caspase 3-inhibitor, DEVD-CHO. In Figure 5C, we show that addition of 100 µM of DEVD-CHO
resulted in a 30% increase in suppressive activity at a 1:1 ratio of CD4+CD25- T cells to CD4+CD25+ Treg cells, in the presence of anti-CD3/CD28 microbeads. There is no additive effect when cells are co-treated with SB216763 and DEVD-CHO. These results suggest that DEVD-CHO and SB216763 may act through similar and/or parallel pathways to lessen Treg cell apoptosis and enhance suppressive activity.

**Prolonged Survival of Islet Allotransplants Following SB216763 Treatment.** In an attempt to extrapolate our in vitro work to an in vivo system, we used an allograft islet transplantation system to assess any potential effects on islet allograft survival in the presence of GSK-3β inhibition. Systemic inhibition of GSK-3β has been used in animal models ranging from inflammation, to Alzheimer's disease, to type II diabetes (22-27) but the effect of inhibiting GSK-3β in animal models for organ transplantation has not yet been reported. In an islet transplant model where C3H islets were transplanted into B6 mice, we show that there is at least a modest prolongation of allograft islet survival in mice that were treated daily with 100 μM SB216763 beginning one day post transplantation (Figure 6A). We next depleted Tregs using anti-CD25 antibody (PC61) and assessed if the SB216763 effect was negated. The two-dose PC61 treatment of mice prior to transplantation resulted in an 87-90% depletion of CD4+FoxP3+ cells in PBL, as determined by FACS analysis (data not shown). In Figure 6B, we show that prolongation of allograft islet survival is not seen if Tregs are depleted.

**DISCUSSION**

A recent study has shown that introduction of a stable form of β-catenin results in increased Treg cell survivability. This paralleled the need for fewer modified Treg cells in the alleviation of inflammatory bowel disease in a mouse model (9). In this study, we analyzed what upstream components of β-catenin regulation may play a role in Treg function. We focused on the serine/threonine kinase, GSK-3β, which phosphorylates β-catenin, marking it for ubiquitination, resulting in its degradation. Our results show that the inhibition of GSK-3β potentiates Treg suppressive activity, and that it does so by increasing Treg survivability, consistent with observations by Ding and colleagues.

To address the issue of GSK-3β’s role in Treg function, we isolated CD4+CD25+ cells and by FACS analysis showed that the highest portion of activated GSK-3β, as characterized by phosphorylation of tyrosine-216, was observed in CD4+CD25+hi cells. These cells also exhibited the greatest level of phospho-β-catenin (Ser33/37, Thr41), indicative of greater GSK-3β activity. Inhibition of GSK-3β using SB216763, a specific GSK-3β inhibitor, resulted in increased Treg activity as exhibited by at least 20% greater suppression in cells treated with SB216763. This enhanced suppression was not a result of direct inhibition of CD4+CD25- proliferation by SB216763 since treatment of CD4+CD25- alone with inhibitor resulted in increased cellular proliferation. This is consistent with previous observations by Oheteki and colleagues who demonstrated that inhibition of T cells with lithium, an inhibitor of GSK-3β, resulted in increased T cell proliferation and IL-2 production (19). But this is in contrast
with what is observed by Ding and colleagues where they found that ectopic expression of stable β-catenin in CD4+CD25- effector cells results in anergy. This could be simply due to the fact that mutant β-catenin in CD4+CD25- is competing with the endogenous wild type form, either by competing for binding to transcriptional factors and/or regulatory molecules, such as GSK-3β complex, resulting in the alteration in proliferation of T effector cells.

Using the Miltenyi kit for the isolation of CD4+CD25+ cells gives the added advantage that the cells are pre-stained with anti-CD25 PE antibody and therefore phenotypic changes can be assessed in mixed cultures with unlabeled CD4+CD25- cells by following the labeled cells. Analysis of β-catenin protein showed that there is an increase in total β-catenin protein in CD4+CD25+hiFoxP3+ cells treated with GSK-3β inhibitor versus untreated cells. But while there is an increased level of β-catenin protein in treated cells, there is β-catenin protein stabilization occurring also in untreated CD4+CD25+hiFoxP3+ cells. This was only observed in CD4+CD25+hiFoxP3+ cells co-cultured with CD4+CD25- cells. This could be attributable to the fact that β-catenin stabilization may be a molecular event that occurs in Tregs during the process of suppression, and that what Ding and colleagues as well as our laboratory have shown, is an enhancement of this process, either through the introduction of non-hydrolyzable β-catenin or through the inhibition of GSK-3β, respectively. It is possible that co-staining for FoxP3 and beta catenin could serve as identifying markers for assessing activated Tregs in vivo.

We also looked at the status of FoxP3 expression in cells treated with or without SB216763 and found that treated CD4+CD25+hiFoxP3+ cells exhibited prolonged FoxP3 expression as compared to untreated cells. A possible explanation for this is that active GSK-3β has been shown to phosphorylate the transcriptional factor NFAT, resulting in its exit from the nucleus, negatively regulating its NFAT activity (28). NFAT is important in the transcriptional activation of FoxP3 expression (29). Therefore, inhibition of GSK-3β may result in the stabilization of NFAT activity, resulting in increased FoxP3 expression. In addition, it is well documented that TGF-β treatment results in increased FoxP3 expression (5,30-33), in part by activating the Smad3 transcriptional factor (29), and it also can result in the inhibition of GSK-3β (34-38).

Ding and colleagues have shown that messenger RNA levels of the anti-apoptotic protein, BCL-XL, are increased in Treg cells that ectopically express stable β-catenin, as assessed by RT-PCR (9). Using FACS analysis and gating on FoxP3+ cells, we found that BclXL protein levels were increased in SB216763-treated CD4+CD25+hiFoxP3+ cells versus untreated cells. This is in agreement with Ding and colleagues and suggests that increased Treg suppressive function following GSK-3β inhibition may be due in part to increased Treg survival. To further validate that inhibition of apoptosis increases Treg function, we treated cells with the caspase inhibitor, DEVD-CHO, and observed about a 30% increase in the suppressive activity of Tregs. When cells are treated with DEVD-CHO together with SB216763, we observe no additive effect,
suggesting that GSK-3β inhibition may affect similar or parallel pathways to those inhibited by DEVD-CHO.

Since GSK-3β inhibition potentiates the suppressive activity of Tregs in vitro, we have begun to study the effect of inhibition of GSK-3β in vivo using an islet transplant model where C3H islets are transplanted into B6 mice. Moreover, while we cannot draw definitive inferences on the effect of Treg cells in SB216763 treated animals, it is notable that euglycemia is prolonged in grafted animals in the treated group. Interestingly, while explanted islet grafts from both the control and experimental groups displayed lymphocytic infiltration suggestive of rejection (data not shown), the time to rejection was significantly different in animals treated with SB216763. While we cannot exclude a myriad of variables involved, it is striking to note that islet grafts survived twice as long with GSK-3β inhibition than in the control. Depletion of Tregs by PC61 resulted in the elimination of the SB216763 effect on prolonging allograft islet survival. While the imprecise nature of these experiments cannot be understated, this disparity coupled with our in vitro work does raise the possibility that enhanced Treg cells, due to GSK-3β inhibition, contribute in promoting tolerance. Preliminary data suggest that naïve mice treated daily for 7 days with 100 μM SB216763 show a decrease in the number of FoxP3 positive cells in the spleen with an increase in the number of FoxP3 positive cells in the peripheral blood, without a change in the overall number of FoxP3+ cells in treated versus untreated naïve mice (data not shown).

In summary, we have shown that inhibition of GSK-3β results in increased suppression activity by Treg cells. Analysis of SB216763-treated Treg cells shows that there is a stabilization of β-catenin and that this in part may account for increased functionality of Tregs. This is consistent with the findings of Ding and colleagues when they ectopically expressed a non-hydrolyzable mutant form of β-catenin in Tregs. We further show that GSK-3β inhibition is accompanied by increased stabilization of FoxP3 protein in Treg cells as well as an increase in BclXL protein levels. There was no additive effect when cells were co-treated with SB216763 and DEVD-CHO, suggesting that the inhibitors are acting on similar and/or parallel pathways. When SB216763 is given systemically to recipients of transplanted islets, these allospecific cells enjoyed prolonged survival. Knowledge of how GSK-3β affects FoxP3 stability and function, and what role this plays in increasing Treg suppressive activity is timely, especially in light of the recent study by Zhou and colleagues showing that the loss of FoxP3 expression in Treg cells results in the generation of “exFoxP3” T cells that have a potent, pathogenic memory T cell phenotype (39). Therefore, understanding the important molecular mechanism(s) underlying this form of immune regulation could aid in the development of therapeutics for managing allotransplantation rejection and autoimmunity.
REFERENCES


**FOOTNOTES**

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The abbreviations used are: GSK-3β, glycogen synthase kinase 3 beta; DEVD-CHO, N-acetyl-L-alpha-aspartyl-L-alpha-glutamyl-N-(2-carboxy-1-formylethyl)-L-valinamide; DMSO, dimethyl sulfoxide
FIGURE LEGENDS

Fig. 1. A. FACS analysis of purified CD4+CD25+ cells for FoxP3, active GSK-3β and phosphorylated β-catenin. Single cell suspensions of isolated CD4+CD25+ cells from B6 and BALB/c spleen and lymph nodes demonstrate co-staining of FoxP3+, GSK-3β (pTyr216) and phosphorylated β -catenin (pSer33/37/Thr41). This finding demonstrates that CD4+CD25+hi FoxP3+ cells exhibit high GSK-3β activity. Moreover, the signal overlaps with CD4+CD25+hi stained for β -catenin (pSer33/37/Thr41) suggesting that CD4+CD25+hi FoxP3+ cells possess elevated GSK-3β activity, reflected in increased β-catenin phosphorylation. (A-C) B6 spleen; (D-F) B6 lymph nodes (LNs); (G-I) BALB/c spleen. Data are representative of at least three independent experiments, gating on the purified, lymphocyte population. B. Histogram analyses of purified CD4+CD25- and CD4+CD25+ cells for phosphorylated β -catenin (pSer33/37/Thr41). This finding demonstrates that CD4+CD25+hi cells exhibit higher GSK-3β activity than CD4+CD25-FoxP3- cells. (A) B6 spleen; (B) B6 lymph nodes (LNs); (C) BALB/c spleen. (Shaded area), CD4+CD25+hi cells plus Alexa488-labeled secondary antibody alone; (---, dashed line), CD4+CD25-FoxP3- cells plus Alexa488-labeled secondary antibody alone; (—, solid line), CD4+CD25+hi cells plus anti-phosphorylated β-catenin (pSer33/37/Thr41) antibody; (….., dotted line), CD4+CD25-FoxP3- cells plus anti-phosphorylated β-catenin (pSer33/37/Thr41) antibody. Data are representative of at least three independent experiments, gating on the purified, lymphocyte population.

Fig. 2. Effect of GSK-3β inhibition, using SB216763, on Treg Suppression. Performing a classical suppression assay, CD4+CD25- T cells and CD4+CD25+ Treg cells were isolated using magnetic separation and co-cultured in a 1:1 ratio with anti-CD3/CD28 microbeads with and without SB216763. Cells were pulsed with tritiated thymidine after 18-20 hours and proliferation was measured. Using this assay we show that SB216763 potentiates Treg function in vitro, as judged by increased suppression of Teff CD4+CD25- proliferation when Teff CD4+CD25-cells were incubated at a 1:1 ratio with CD4+CD25+ Treg cells in 5 µM SB216763 (86% inhibition vs 65% without the drug [DMSO vehicle control]). Moreover, cells incubated in 1:8 ratio respectively with Tregs again in 5 µM SB216763 demonstrate 95% inhibition vs 78% without the drug, suggesting that GSK-3β inhibition can significantly enhance Treg cell suppression. Data are represented as means ± s.e.m. of triplicate samples. All data are representative of at least two independently performed experiments.

Fig. 3. FACS analysis of total β-catenin protein levels in the presence or absence of SB216763. To assess β-catenin levels in active Treg cells we elected to mimic a suppression assay without radioactive isotopes. A time course was performed to trend the β-catenin intensity in Tregs in intervals of 0, 24, 48 and 72 hours. Given that GSK-3β targets β-catenin for degradation, the effect of GSK-3β inhibition on β-catenin was measured using flow cytometry with targeting FITC antibodies. Unsurprisingly, an increase in total β-catenin protein was observed in FoxP3+Treg cells co-cultured with
CD4+CD25- cells and treated with SB216763. All data are representative of at least two independently performed experiments, gating on CD4+CD25+hi FoxP3+ cells.

**Fig. 4.** Assessment of FoxP3 protein levels in the presence or absence of SB216763. Again a co-culture of isolated Tregs and Teff cells in a 1:1 ratio with anti-CD3/CD28 microbeads with and without 5 µM SB216763 was performed. The collective grouping of T effector and Treg cells was fixed and stained with Foxp3 FITC in intervals of 0, 24, 48 and 72 hours. Using FACs analysis we clearly show that CD4+CD25+ Treg cells co-cultured with CD4+CD25- T cells and treated with SB216763 maintain their Foxp3+ signature. This striking finding suggests that SB216763 slows Foxp3 cell turnover *in vitro*. All data are representative of at least two independently performed experiments, gating on CD4+CD25+hi FoxP3+ cells.

**Fig. 5.** A. FACS analysis of the anti-apoptotic protein, Bcl-XL, following SB216763 treatment. Our co-cultured group of cells treated with SB216763 demonstrates a higher intensity of Bcl-XL after 24 hours, than untreated cells. Moreover, the kinetics Bcl-XL loss is significantly slower temporally in the treated group. Collectively these findings suggest that the GSK-3β inhibitor, SB216763, may work through Bcl-XL to protect Tregs from apoptosis *in vitro*. All data are representative of at least two independently performed experiments, gating on CD4+CD25+hi FoxP3+ cells. B. Western blot analysis of CD4+CD25+ cells treated *in vitro* with SB216763. Cells treated with SB216763 maintain increased Bcl-2 levels up to 66 hours versus untreated cells. The Western blot was stripped and reprobed with anti-β-actin antibody as a loading control. C. Effect of caspase 3 inhibition, using DEVD-CHO, on Treg Suppression. Performing a classical suppression assay, the use of 100 µM of the DEVD-CHO, a caspase-3 inhibitor, resulted in a 20% increase in suppression activity. When caspase-3 inhibitor was used with GSK-3β inhibitor, we observed no additive affect, suggesting that the two inhibitors may be acting on the same or parallel pathways. Data are represented as means ± s.e.m. of triplicate samples. All data are representative of at least two independently performed experiments.

**Fig. 6.** Increased survival of allograft islet transplantation following SB216763 treatment. A. Diabetogenic B6 recipients of C3H islets were injected daily with 100 µM SB216763 post-operatively. Interestingly, these islets had a 2-fold slower rejection time than untreated B6 recipients as demonstrated by prolonged euglycemia. \( P = 0.0056 \) (Logrank test). B. Diabetogenic B6 recipients of C3H islets, depleted of CD25+ cells prior to transplantation, were injected daily with 100 µM SB216763 post-operatively. Depletion of Tregs results in the elimination of SB216763 effect. This demonstration of GSK-3β inhibition in an *in vivo* model hints at the powerful possibilities of potential Treg cell therapy in transplantation. \( P = 0.1616 \) (Logrank test).
Figure 1A

CD25

FoxP3  phospho-β-catenin (pSer33/37/Thr41)  phospho-GSK3β (pTyr216)
phospho-\(\beta\)-catenin
(pSer33/37/Thr41)
Figure 5b

GSK-3β inh.  

<table>
<thead>
<tr>
<th></th>
<th>42 hrs</th>
<th>66 hrs</th>
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- Bcl-2

- β-actin
Figure 5c

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Figure 6

A

Log rank test: \( P=0.0050^{(**)} \)

B

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\( \text{PC61} n=6 \)

\( \text{PC61 + GSKi} n=6 \)
Suppressive Treg cell activity is potentiated by glycogen synthase kinase 3β inhibition
Jay Graham, Michael Fray, Stephanie de Haseth, Kang Mi Lee, Moh-Moh Lian, Catharine M. Chase, Joren C. Madsen, James Markmann, Gilles Benichou, Robert B. Colvin, A. Benedict Cosimi, Shaoping Deng, James Kim and Alessandro Alessandrini

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