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Dendritic cell maturation occurs through the inhibition of GSK-3 β

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Abstract

Dendritic cell (DC) maturation results in changes in antigen processing and presentation, governing the fate of adaptive immunity. Understanding the intracellular signaling pathways governing DC maturation is therefore critical. In this study, we observed that the kinase, GSK-3 β , is present in its active form in resting immature DCs isolated from the spleen and bone marrow of mice. Induction of DC maturation using GM-CSF, IL-4 and TNF α resulted in GSK-3 β inhibition, as reflected by increased phosphorylation of Serine 9 on the kinase, and concomitant stabilization of its substrate, beta-catenin. Treatment of immature DCs with a GSK-3 β inhibitor increased cell surface expression of CD80, CD86 and CD40 on DCs, enhancing their ability to present antigen and activating IL-2 secretion by T cells. GSK-3 β inhibition also parallels dendritic cell maturation *in vivo*. Our results show that GSK-3 β signaling controls DC maturation and suggest that this kinase could be manipulated to modulate adaptive immunity.

1. Introduction

Dendritic cells (DCs) go through various stages during their maturation, a process primarily influenced by exposure to antigens and cytokines. At an *immature state*, DCs express low levels of MHC class II and CD80 and CD86 on their surface. These cells are highly

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Conflicts of Interest.

The authors have no financial conflict of interest.

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endocytic, taking up antigens and processing them. As DCs reach an intermediate state in maturation, the number of MHC class II molecules expressed on the plasma membrane increases, as do levels of the co-stimulatory molecules, CD80 and CD86. These DCs migrate to lymphatic vessels and eventually infiltrate the lymph nodes. Exposure to granulocyte macrophage-colony-stimulating factor (GM-CSF) further matures DCs, a process associated with even greater expression of MHC class II and co stimulatory molecules. This primes DCs for terminal differentiation into mature DCs which occurs upon exposure to inflammatory agents, such as TNF-alpha and LPS [1]. DCs attaining this *mature state*, express high levels of peptide-loaded, membrane MHC class II molecules and co-stimulatory molecules; and they secrete cytokines, such as IL-1, IL-12 and gamma-IFN, that stimulate T cell functions. Mature DCs are potent antigen presenting cells (APCs) required for the initiation of effector T cell responses to foreign antigens. In contrast, there is accumulating evidence suggesting that immature DCs induce and maintain T cell tolerance to self-antigens, presumably through the activation of regulatory T cells (T regs) [2; 3]. Based upon these dual properties of DCs, it has been proposed that these cells could be used to manipulate T cell responses in vivo. Some evidence has been provided suggesting that injection of donor immature dendritic cells into transplant recipients could achieve allotransplant tolerance [4; 5]. However, successful development of tolerance protocols using immature DCs has been difficult due to the fact that immature DCs rapidly mature upon injection, which stimulates inflammatory conditions [6]. This phenomenon is associated with the risk of sensitizing rather than tolerizing the host. Therefore, in order to design reliable tolerance protocols using immature DCs in transplantation, it is crucial to arrest permanently DCs at an early stage of their development, a task that requires an in depth understanding of the molecules and signals governing DC maturation.

While phenotypic markers identifying different stages of dendritic cell development are well characterized, little is known about the signaling pathways involved in their maturation of DCs. Granulocyte macrophage-colony-stimulating factor (GM-CSF), an essential cytokine in DC maturation, activates the phosphoinositide 3-OH (PI3K)/AKT pathway [7]. AKT kinase activation plays a key role in the survival of DCs whose maturation has been induced by LPS [8]. At the same time, AKT is known to inhibit another kinase, GSK-3 β by phosphorylating it on Serine-9 [9; 10; 11; 12; 13]. It is noteworthy that GSK-3 β regulates the growth factor-dependent transcription of c-Jun N-terminal kinase [14] and NF-AT by increasing nuclear export of NF-ATc [15]. JNK activation and NF-AT induced transcription control the DC maturation process by regulating the expression of cytokines and co-stimulatory molecules [16; 17]. Collectively, these observations suggest that GSK-3 β may play a critical role in DC maturation, a hypothesis that we investigated in this study.

In this study, we show that immature DCs display high levels of GSK-3 β activity. Exposure of immature DCs to a specific GSK-3 β inhibitor consistently resulted in the maturation of these cells as evidenced by increased surface expression of CD80 and CD86. Likewise, physiological maturation of DCs induced by GM-CSF, is associated with GSK-3 β inhibition, as assessed by increased phosphorylation on serine 9. We also show that GSK-3 β inhibition in DCs enhanced their ability to activate T cell function. Finally, using C57BL/6.Foxp3^{DTR} mice (B6.Foxp3^{DTR}) [18], we observed concomitant inhibition of GSK-3 β and

dendritic cell maturation *in vivo*. These results demonstrate the role of GSK-3 β activity in the maturation and antigen presenting functions of DCs. The implications of this finding for the design of tolerogenic DCs are discussed.

2. Materials and Methods

2.1 Mice

Male C57BL/6(H-2b) and male BALB/c (H-2d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.Foxp3^{DTR} mice, which have a knock-in of the Diphtheria Toxin Receptor (DTR) coding sequence into the 3' untranslated region of *Foxp3* as well as GFP, were kindly provided by A. Rudensky [18]. The mice were housed in a pathogen-free environment with food and water ad libitum in our facility at Massachusetts General Hospital and were used at 8–12 wk of age.

2.2 Dendritic Cell Isolation and Culture

Bone marrow and spleens were obtained from male C57BL/6/J and male BALB/c mice. Dendritic cells were isolated using CD11c positive selection magnetic cell beads and separation columns from Miltenyi Biotec (Auburn, CA, USA) as per the manufacturer's directions. CD11c positive cells were isolated to 90–95% purity, as determined by FACS analysis using CD11c⁺ or I-A^b staining (data not shown). After selection, the CD11c⁺ cells were plated at 3×10^5 cells per well in 96 well plates. The control plates were cultured in 100 μ l per well of RPMI 1640 (Mediatech Inc.) supplemented with 1% gentamicin, 1% nonessential amino acids, 1% L-glutamine, 2% HEPES, 10% FCS, and 50 μ M beta-mercaptoethanol (complete media). The experimental plates were cultured in 100 μ l per well of complete media containing GM-CSF (Sigma-Aldrich) at 2 ng/ml, 20 ng/ml or 200 ng/ml, plus 20 ng/ml of IL4. The cells were incubated at 37°C and 5% CO₂. On the fifth day, cells were again fed. Control plates received another 100 μ l of complete media and the experimental plates received complete media plus either 2 ng/ml, 20 ng/ml or 200 ng/ml GM-CSF, 20 ng/ml IL4 (final concentration), and 40 ng/ml of TNF- α (final concentration). After 2 days, the cells were fixed and labeled. Experiments involving the use of kinase inhibitors and their effects on splenic dendritic cell maturation were performed as follows. After CD11c⁺ cells were purified, they were plated at 3×10^5 cells per well in 96 well plates. Dendritic cells were incubated for 2 days in the absence or presence of specific kinase inhibitors (1 μ M U0126, a MEK1 inhibitor [SIGMA]; or 5 μ M SB216763, a GSK-3 β inhibitor [SIGMA]; or 10 μ M Akt inhibitor [Calbiochem]) in complete RPMI media. After 2 days, the cells were washed, fixed and FACS analyzed for cell surface expression of the maturation markers, CD80 and/or CD86.

2.3 Diphtheria Toxin Treatment B6.Foxp3^{DTR} Mice

B6.Foxp3^{DTR} mice were then randomly selected to receive either 50 μ g/kg of DT in 200 μ l of water or vehicle i.p. on two consecutive days. Diphtheria toxin (DT) (Sigma) was reconstituted according to the manufacturer's protocol. DT stocks were frozen, thawed once and diluted to 5 μ g/ml in water. Two groups were compared: B6.Foxp3^{DTR} given DT and B6.Foxp3^{DTR} given vehicle. Six days after the last DT treatment, the mice were sacrificed, the spleen removed and CD11c⁺ cells were purified as described above.

2.4 Labeling and FACS Analysis

Fluorescence-labeled antibodies were purchased from BD Biosciences (CD4, CD8, B220, I-A^b), eBioscience (CD80, CD86, and CD40), Miltenyi Biotec (mPDCA-1) and Invitrogen (Alexa 488-labeled, anti-rabbit antibody). GSK-3 β ser9 labeling antibody was purchased from Sigma-Aldrich. After seven days in culture, the cells were washed, incubated with antibodies at 4°C for 30 minutes and washed again. The cells were then fixed by incubation in 2% paraformaldehyde for 10 minutes at room temperature. Cells were permeabilized by incubation in a solution of PBS plus 2% BSA and 2% IGEPAL (Sigma-Aldrich) at room temperature for 30 minutes. Following another wash, the cells were co-labeled with GSK-3 β labeling antibodies and incubated for 30 minutes at room temperature. The cells were washed again, incubated with Alexa 488-labeled, anti-rabbit antibody for 30 minutes, and then washed a final time and resuspended in PBS + 0.1% NaN₃. FACS data collection was performed on a FACScan (Becton Dickinson) using CellQuest Pro software. 10,000 cells were collected per sample. Analysis was performed using FlowJo software.

2.5 Pretreatment of purified BMDCs with SB216763, a specific GSK-3 β inhibitor

BMDCs were isolated as described above and 2×10^6 cells were pretreated with vehicle (DMSO) and 2×10^6 cells were pretreated with 5 μ M SB216763 for 4 days in 1 ml each of RPMI 1640 (Mediatech Inc.) supplemented with 1% gentamicin, 1% nonessential amino acids, 1% L-glutamine, 2% HEPES, 10% FCS, and 50 μ M beta-mercaptoethanol (complete media) using 12-well Falcon plates. After 4 days, the cells were washed three times and then resuspended in complete media and counted.

2.6 ELISPOT

Sterile plates 96-well plates (MultiScreen HTS IP Sterile Plates, Millipore, Billerica, MA, USA) were incubated with 70% ethanol for 2 minutes, then washed four times using sterile PBS. Capture antibodies (IL-2 and IFN- γ , BD Biosciences) in sterile phosphate-buffered saline (PBS) were then incubated on the plates overnight at 4° C. The next day the plates were washed four times with sterile PBS. Cells were added to the wells in the indicated concentrations. The plates were placed in a 37° C, 5% CO₂ incubator. The number of purified T cells used per well was 125,000 and the number of DMSO-treated or SB216763-treated BMDCs was 35,000. After 48 hours, the plates were washed and secondary antibodies conjugated with biotin (BD Biosciences) were added to the plate at concentrations of 2 μ g/ml suspended in PBS/0.25% Tween/1% BSA. After two hours at room temperature, the plates were washed again. A solution of horseradish peroxidase streptavidin (SAV-HRP, BD Biosciences) diluted 1/2000 in PBS/0.25% Tween/1% BSA was added to the wells and the plates were incubated for one hour in the dark at room temperature. After a final wash, an AEC solution (3-Amino-9-ethylcarbazole, SIGMA, solution prepared as per eBioscience's ELISPOT protocol) was added and the plates were developed for approximately 15 minutes in the dark before the reaction was stopped by H₂O. The plates were dried and the spots were counted using an ELISPOT reader (CTL-ImmunoSpot, Cellular Technology Ltd., Shaker Heights, OH, USA). T cells were purified from spleen using Pan T Cell Isolation Kits (Miltenyi Biotec Inc., Auburn, CA, USA) as per the manufacturer's instructions, resulting in untouched, purified T cells.

2.7 Phagocytosis Assay

Phagocytosis by dendritic cells was assessed by the uptake of FITC-dextran (70 kDa; SIGMA). Briefly, 200,000 DCs were incubated for 30 minutes in the absence or presence of specific kinase inhibitors (1 μ M U0126, a MEK1 inhibitor; or 5 μ M SB216763, a GSK-3 β inhibitor; both were purchased from SIGMA) in complete RPMI media. The DCs were then incubated for an additional 30 minutes at 37°C in the presence of 1 mg/ml FITC-dextran, with or without inhibitors. The reaction was stopped by the addition of ice cold PBS. Cells were analyzed using a FACScan flow cytometer (Becton Dickinson). As a control for non-specific FITC-dextran binding to the surface of cells, some cells were incubated on ice throughout the assay and these values were subtracted from the experimental values.

2.8 Statistical analysis

Statistical analysis was performed using a two-tailed Student t test and data are expressed as means $\pm$ SD.

3. Results

3.1 Treatment of bone marrow- and spleen-derived CD11c⁺ dendritic cells with maturation factors leads to the inhibition of GSK-3 β activity

In resting cells, GSK-3 β is usually found in an activated state. Phosphorylation of GSK-3 β on serine 9 (p-GSK-3 β [Ser9]) correlates with increased inhibition of its activity. Here, we compared GSK-3 β activity in resting and activated dendritic cells by measuring the levels of intracellular p-GSK-3 β [Ser9] using flow cytometry. In Figure 1, we show that exposure of DCs to GM-CSF and LPS for 7 days induced the maturation of bone marrow-derived and spleen-derived DCs and resulted in a 3.0- to 3.5-fold increase in the levels of p-GSK-3 β [Ser9]. Maturation of bone marrow derived DCs treated with GM-CSF, IL-4 and TNF-alpha, resulted in a 3- to 4-fold increase in the levels of p-GSK-3 β [Ser9] (Figure 2A). Treatment of DCs with increasing amounts of GM-CSF (2 ng/ml, 20 ng/ml, 200 ng/ml) while maintaining the same concentrations of IL-4 and TNF-alpha also significantly showed a dose-dependent increase in the phosphorylation of GSK-3 β on serine 9 (Figure 2A). The overall percentage increase of p-GSK-3 β [Ser9] in a sampling of several experiments, using 200 ng/ml of GM-CSF, is 12.3% $\pm$ 3.7 (S.D.) for untreated cells versus 63.4% $\pm$ 15.3 (S.D.) for cells treated with 200 ng/ml GM-CSF. Low levels of p-GSK-3 β [Ser9] were always detected in resting immature DCs (Figure 2B), suggesting that one of the distinguishing characteristics between immature and mature DCs may be the number of GSK-3 β molecules that are phosphorylated on Serine 9, referred to as p-GSK-3 β [Ser9]^{lo} (immature DCs) and p-GSK-3 β [Ser9]^{hi} (activated/mature DCs).

3.2 Treatment of BMDCs with GM-CSF results in the stabilization of beta-catenin

β -catenin, which is regulated by GSK-3 β , is best known for its role in Wnt signaling, a pathway which governs patterning and cell fate determination during embryogenesis [19; 20]. Recently, β -catenin together with the adenomatous polyposis coli (APC) gene product has been implicated in tumorigenesis (reviewed in [21]). Phosphorylation of β -catenin by GSK-3 β targets β -catenin for ubiquitination and degradation, thereby terminating the signal [20]. In contrast, inhibition of GSK-3 β activity leads to β -catenin stabilization [22]. Here, we

compared the expression of beta-catenin protein in immature and mature DCs. Figure 2C shows that treatment of DCs with 20ng/ml GM-CSF results in at least a 3-fold increase in the percentage of cells expressing β -catenin when compared to untreated DCs. The increased stabilization of β -catenin in GM-CSF-treated BMDCs confirms that GSK-3 β is inhibited as DCs undergo maturation.

3.3 Co-expression of CD80 (B7-1) and CD86 (B7-2) receptors and p-GSK-3 β [Ser9] in mature DCs

We next investigated whether the population of BMDCs that show an increase in CD80 and CD86 expression also exhibit an increase in the inhibition of GSK-3 β . In Figure 3A, we show that BMDCs, treated with 20 ng/ml of GM-CSF, exhibited an increase surface expression of CD80 and CD86 and that the majority of these cells exhibited co-staining with phospho- GSK-3 β [Ser9]^{hi}. We observed similar results when 200 ng/ml GM-CSF was used to treat BMDCs (data not shown). Addition of SB216763, a specific GSK-3 β inhibitor, with GM-CSF did not result in an additive increase in CD80 and CD86 surface expression, suggesting that GM-CSF addition may act by inhibiting GSK-3 β in the process of DC maturation (data not shown). It is important to note that treatment of DCs with a suboptimal dose of GM-CSF (2 ng/ml) induced the surface expression of CD80 but not CD86 and p-GSK-3 β [Ser9] (Figure 3B). This suggests that under these conditions, DCs go through an intermediate stage of maturation characterized by a CD80⁺ CD86⁻ p-GSK-3 β [Ser9]^{lo} phenotype. It is noteworthy that DCs treated with similar low levels of GM-CSF have been shown to induce T cell tolerance [23; 24]. Whether co-staining of CD80 with p-GSK-3 β [Ser9]^{lo} could be used to assess the tolerogenicity of DCs, needs further investigation.

3.4 p-GSK-3 β [Ser9] expression in CD4⁺CD11c⁺ and CD8⁺CD11c⁺ and B220⁺CD11c⁺ BMDC subsets

The population of CD11c⁺ DCs is comprised of different subsets displaying different markers and functional properties. Since the observations summarized in Figure 1 and 2 indicate that not all maturing DCs express increased p-GSK-3 β [Ser9], it is apparent that GSK-3 β inhibition is associated with the maturation of select DC subsets. This suggestion is confirmed in Figure 4A, where we observe that under conditions when 200 ng/ml of GM-CSF is used in the maturation of DCs, both CD4⁺CD11c⁺ and CD8⁺CD11c⁺ cells co-stain with p-GSK-3 β [pSer9]^{hi}. The majority of B220⁺CD11c⁺ plasmacytoid DCs were p-GSK-3 β [Ser9]^{lo}. After treatment with 200 ng/ml GM-CSF, we observed a 5-fold decrease in B220 expression (Figure 4B) and a 4-fold increase in PDCA-1 (plasmacytoid dendritic cell antigen -1) expression in cells that also co-stain with p-GSK-3 β [Ser9]^{hi} (Figure 4C). A similar reduction in B220 staining has been previously observed by Gilliet and colleagues with plasmacytoid DCs treated with GM-CSF [25]. While we observed an increase in p-GSK-3 β [Ser9] in all the subsets that we analyzed, there is still a large subset of cells that are p-GSK-3 β [pSer9]^{hi} that do not co-stain with CD4, CD8 or PDCA-1. We believe that these represent CD4⁻CD8⁻CD11c⁺ cells.

3.5 DC treatment with a GSK-3 β inhibitor, SB216763, is sufficient to induce the surface expression of CD80, CD86 and CD40 but not MHC class II

We next investigated whether inhibition of GSK-3 β activity is sufficient on its own to induce the maturation of DCs. To address this, CD11c⁺ DCs were treated for four days with a specific GSK-3 β inhibitor, SB216763 (5 μ M). As shown in Figure 5A, this treatment resulted in an increase of the surface expression of CD80 and CD86 on spleen DCs. We obtained similar results with BMDCs (data not shown). Therefore, in the absence of GM-CSF, IL-4 and TNF-alpha cytokines, GSK-3 β inhibition is sufficient to trigger surface expression of CD80 and CD86 in both splenic and bone marrow-derived DCs. Recently, Lam and colleagues have shown that leptin induces CD40 expression in DCs via activation of AKT [26]. Since AKT inhibits GSK-3 β , we investigated if exposure of DCs to the SB216763 GSK-3 β inhibitor influences CD40 expression on DCs. In Figure 5B, we observed a modest increase in the number of cells expressing CD40 following the addition of the GSK-3 β specific inhibitor. We also observe co-staining of CD40 and p-GSK-3 β [Ser9] after treatment of DCs with GM-CSF (Fig. 5C). Interestingly, treatment of DCs with SB216763 did not result in the increased surface expression of MHC class II molecules (Figure 5D). Treatment of DCs with inhibitors of the kinases AKT and MEK1 did not result in increased surface expression of CD80 (Figures 5E and 5F), suggesting that this observation, in the absence of cytokine induced maturation, is at least specific to inhibition of GSK-3 β . These data suggest that while inhibition of GSK-3 β in the absence of maturation-inducing cytokines, can result in the increased surface of expression of CD80 and CD86, it does not result in the complete maturation of DCs, since MHC II surface levels are unaffected.

3.6 DCs pretreated with SB216763 can increase IL-2 secretion of alloantigen-activated T cells

We have shown that pretreating BMDCs with SB216763 resulted in increased surface expression of CD80 and CD86. We next tested the effect of pretreating BMDCs on cytokine production when these DCs were incubated with purified T cells. In Figure 6A we show that there was a 4-fold increase in IL-2 production when purified T cells from BALB/c skin graft primed B6 mice were incubated with treated vs. untreated BALB/c DCs. There was no significant difference in IFN-gamma production between the two groups of DCs (Figure 6B). None of the wells with naïve T cell/DC combinations displayed any cytokine production, which may indicate that BMDCs, treated or untreated with inhibitor, are not fully mature to induce a response in naïve T effector cells, and that the IL-2 and IFN-gamma produced was a result the direct pathway interaction between memory (alloantigen experienced) T cells and the DCs. The increased expression of IL-2 can be explained by the previously shown significant upregulation in the CD80 and CD80 costimulatory molecules on SB216763 treated DCs, as it is these molecules that implicated in inducing IL-2 secretion [27]. On the other hand, the effect of GSK-3 β inhibition has a modest affect on CD40 expression on DCs, and correspondingly does not have as much impact on CD40/CD40L induced IFN-gamma secretion [28], though levels are already high to begin with (Figure 6B).

3.7 GSK-3 β inhibition does not affect phagocytosis by immature DCs

A major characteristic of immature DCs is their ability to perform phagocytosis. We tested if inhibition of GSK-3 β affected FITC-dextran phagocytosis in CD11c⁺ cells. DCs treated with 5 μ M SB216763, a concentration that was determined to completely inhibit GSK-3 β , showed no reduction in FITC-dextran incorporation by DCs (Fig 7). The MEK/ERK pathway is known for its involvement in phagocytosis by DCs [29; 30], an observation which prompted us to test if inhibition of this pathway can affect phagocytosis in CD11c⁺ cells and use it as a positive control. When cells were treated with 1 μ M U0126, a MEK1 specific inhibitor, we observed a 57 % reduction in FITC-dextran phagocytosis (Figure 7). The lack of an effect on phagocytosis following GSK-3 β inhibition with SB216763 in DCs further shows that while these cells express increased surface expression of CD80 and CD86 in response to this inhibitor, they are not fully mature.

3.8 GSK-3 β inhibition parallels dendritic cell maturation in vivo

Kim and colleagues have previously shown that *in vivo* depletion of regulatory T cells using the B6.Foxp3^{DTR} mouse model results in the expansion and accelerated maturation of dendritic cells in the spleen [18]. Depletion of regulatory T cells results in the maturation of dendritic cells, as monitored by increased expression of MHC-II (I-Ab) on isolated splenic DCs (Figure 8A). In Figure 8B, we show a concomitant increase in phospho-GSK-3 β (Ser9) costaining with CD86^{med} and CD86^{hi} (7-fold increase) or I-A^{bmed} and I-A^{bhi} (20% increase) cells. Interestingly, we observed a linear relationship between phospho-GSK-3 β (Ser9) and CD86 or I-A^b costaining in isolated spleen dendritic cells from both DT treated and untreated mice (Figure 8).

4. Discussion

DCs play a critical role in adaptive immunity by processing and presenting antigens to T cells. It is now firmly established that the ability of DCs to serve as APCs depends upon their degree of maturation. Some evidence has been provided suggesting that antigen presentation by mature DCs is immunogenic while antigen presentation by immature DCs tends to induce T cell tolerance presumably via activation of regulatory T cells [2; 3]. Therefore, in order to manipulate the immune response using DCs, it is critical to control DC maturation, a task that requires a better understanding of the molecular events governing this process.

In this study, we showed that DC maturation induced by GM-CSF is associated with an inhibition of GSK-3 β as evidenced by an increase of its phosphorylated counterpart, p-GSK-3 β [Ser9]. In addition, our study is the first to show that inhibition of GSK-3 β also parallels dendritic cell maturation *in vivo*. Furthermore, we observed that exposure of DCs with a GSK-3 β inhibitor was sufficient on its own to induce DC maturation characterized by the expression of CD80, CD86 and CD40 surface molecules. Recent work from Rodionova et al., who used human CD14⁺ monocytes, and Jiang et al, who used murine DCs, have also observed that addition of GSK-3 β specific inhibitors resulted in the increased expression of CD80 and CD86 [31; 32]. This is in contrast to what was observed by Ono and colleagues where they observed a decrease in CD86 and CD40 expression following GSK-3 β inhibition

[33]. This may be due in part to differences in DC purification methods between the groups. But while we observe increased surface expression of CD80, CD86 and a modest increase of the number of cells expressing surface CD40 just by the addition of SB216763, it did not result in increased surface expression of MHC-II or in the inhibition of phagocytosis. This implies that GSK-3 β inhibition is essential but not sufficient to induce full DC maturation. The functional consequence of the increase of these co-stimulatory molecules is evident by the ability of SB216763 pre-treated BMDCs to increase IL-2 production in purified T cells exposed to their antigen (Figure 6A). But while SB216763 treated BMDCs can increase IL-2 production, there is no difference in the level of IFN- γ produced by purified T cells when incubated with treated or untreated BMDCs (Figure 6b). This is in agreement with our finding that BMDCs, treated or untreated with SB216763, express roughly the same levels of surface CD40, a result presumably that accounts for the similar levels of IFN- γ observed, since the production of the cytokine is a CD40/CD40L driven event, as previously described [28]. On the other hand, IL-2 production by T cells requires interaction with CD80/CD86 co-stimulatory molecules [27]. And in fact we observe a four-fold increase in the number of T cells secreting IL-2 following incubation with SB216763-pretreated BMDCs, that we know exhibit greater surface expression of CD80 and CD86. It is also important to note that we observed a marked increase in intracellular expression of CD40 by DCs following GM-CSF treatment (data not shown), though there was a very modest increase in the number of cells expressing CD40 on the surface. The only other study that has documented cytosolic expression of CD40 was from Lin-Lee and colleagues using neoplastic human B lymphocytes [34]. Analysis of untreated CD11c⁺ cells also show that all the cells express endogenous CD40 and perhaps the translocation of endogenous CD40 to the plasma membrane may play a role in the process of DC maturation. Possible modification of this step, in other words, the inhibition of CD40 translocation to the cell surface, could be important in determining the immunogenicity versus tolerogenicity of DCs.

Analysis of CD4⁺CD11c⁺ and CD8⁺CD11c⁺ cells show that under maturation conditions, both these cell types co-stain with p-GSK-3 β [pSer9]^{hi}. Untreated, immature plasmacytoid dendritic cell population shows that the majority of these cells are p-GSK-3 β [Ser9]^{lo}B220⁺PDCA1⁻. Following treatment with 200 ng/ml GM-CSF, we observed at least a 5-fold decrease in B220⁺ staining when compared to B220-staining in untreated cells. The reduction in B220⁺ staining is consistent with a previous study by Gilliet and colleagues who reported that 100 ng/ml of GM-CSF could block the development of pDCs (25). Though B220 surface expression was reduced, PDCA-1 (plasmacytoid dendritic cell antigen -1) expression increases and co-stains with p-GSK-3 β [Ser9]^{hi} cells. This suggests that there are at least two stages of pDC maturation, governed by exposure to high concentrations of GM-CSF: one that is represented by p-GSK-3 β [Ser9]^{lo}B220⁺PDCA1⁻ cells and the other represented by p-GSK-3 β [Ser9]^{hi}B220^{lo}PDCA1⁺. The functions of these two sets of cells need further characterization.

5. Conclusions

In this study, we show that GSK-3 β is inhibited during the maturation of DCs, as seen by the increase in the levels of p-GSK-3 β [Ser9]^{hi} DCs (Model in Figure 9). These cells express

both CD80 and CD86 when treated with 20 ng/ml or 200 ng/ml GM-CSF. Treatment of DCs with 2 ng/ml GM-CSF, a concentration that has been shown to render DCs tolerogenic, results in increased expression of just CD80, which co-stains with p-GSK-3 β [Ser9]^{lo} DCs. This suggests that the level of p-GSK-3 β [Ser9] might be used as a hallmark of tolerogenic versus immunogenic DCs. We are currently introducing the constitutively active form of GSK-3 β , GSK-3 β [S9A], into DCs in order to test if this treatment will arrest them in an immature stage. This strategy, if successful, would allow us to generate tolerogenic DCs that can be used to prevent allotransplant rejection and for the treatment of immune-mediated pathologies, including autoimmune diseases and allergies.

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Abbreviations used in this paper

GSK-3β	glycogen synthase kinase 3 beta
DC	dendritic cells
BM	bone marrow
pDC	plasmacytoid dendritic cells
APC	antigen presenting cells

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Figure 1. *In vitro* GM-CSF and LPS induced maturation of DCs results in the inhibition of GSK-3 β

A and B), CD11c⁺ DCs, isolated from spleen or bone marrow of B6 mice, were treated *in vitro* with 20 ng/ml GM-CSF for 5 days then for 2 days with 20ng/ml GM-CSF and 5 μ g/ml LPS. Cells were fixed, permeabilized, stained with anti-phospho-GSK-3 β (Ser9) antibodies, and analyzed by flow cytometry. The histograms are representative stainings of endogenous phospho-GSK-3 β (Ser9) on gated CD11c⁺ cells. Data represent triplicate samples. All data are representative of at least two independently performed experiments.

Figure 2A

Figure 2B

Figure 2C

Figure 2. *In vitro* GM-CSF, IL-4 and TNF- α induced maturation of bone marrow DCs results in the inhibition of GSK-3 β

A), CD11c⁺ DCs, isolated from bone marrow of B6 mice, were treated *in vitro* with increasing dose concentrations of GM-CSF and 20 ng/ml IL-4 for 5 days then for 2 days with the appropriate dose of GM-CSF, 20 ng/ml IL-4 and 40 ng/ml TNF-alpha. Cells were fixed, permeabilized, stained with anti-phospho-GSK-3β(Ser9) antibodies, and analyzed by flow cytometry. The dot blots are representative stainings of endogenous phospho-GSK-3β(Ser9) on gated CD11c⁺ cells. B), histogram representation of the results from 2A), including isotype control staining. = isotype control; = untreated DCs; = GM-CSF, IL-4 and TNF-alpha treated DCs. C), representative staining for total β-catenin protein in untreated and treated CD11c⁺ bone marrow DCs. Data represent triplicate samples. All data are representative of at least two independently performed experiments.

Figure 3A

Figure 3B

Figure 3. Co-staining of CD80 and CD86 with phospho-GSK-3 β (Ser9)

A) representative dot blot of untreated and 20 ng/ml treated CD11c⁺ cells co-stained with anti-CD80 or CD86 and anti-phospho-GSK-3 β (Ser9). B), representative dot blot of untreated and 2ng/ml treated CD11c⁺ cells co-stained with anti-CD80 or CD86 and anti-phospho-GSK-3 β (Ser9). Data represent triplicate samples. All data are representative of at least two independently performed experiments.

Figure 4A

Figure 4B

Figure 4C

Figure 4. Co-staining of CD4, CD8, B220 and PDCA-1 with phospho-GSK-3 β (Ser9)

A) representative dot blot of untreated and 200 ng/ml treated CD11c⁺ cells co-stained with anti-CD4 or CD8 and anti-phospho-GSK-3 β (Ser9). B), representative dot blot of untreated and 200 ng/ml treated CD11c⁺ cells co-stained with anti-B220 and anti-phospho-GSK-3 β (Ser9). C), representative dot blot of untreated and 200 ng/ml treated CD11c⁺ cells co-stained with anti-PDCA-1 and anti-phospho-GSK-3 β (Ser9). Data represent triplicate samples. All data are representative of at least two independently performed experiments.

Figure 5A

Figure 5B

Figure 5C

Figure 5D

Figure 5E

CD80 -FITC

Figure 5F

CD80 -FITC

Figure 5. Treatment of CD11c⁺ cells with SB216763 results in the increased surface expression of CD80, CD86 and CD40

A) Splenic DCs were treated for 2 days with 5 μ M of the specific GSK-3 β inhibitor, SB216763. Cells were fixed, stained with either anti-CD80 or anti-CD86 antibodies, and analyzed by flow cytometry. The histograms are representative stainings of the surface expression of CD80 and CD86 on gated CD11c⁺ cells. B), CD11c⁺ DCs were treated for 2 days with 5 μ M of the specific GSK-3 β inhibitor, SB216763. Cells were fixed, stained with either anti-CD40, and analyzed by flow cytometry. The histograms are representative stainings of the surface expression of CD40 on gated CD11c⁺ cells. C), representative dot blot of untreated and 20 ng/ml treated CD11c⁺ cells co-stained with anti-CD40 and anti-phospho-GSK-3 β (Ser9). The histograms are representative stainings of CD40 and anti-phospho-GSK-3 β (Ser9) on gated CD11c⁺ cells. D) Splenic DCs were treated for 2 days with 5 μ M of the specific GSK-3 β inhibitor, SB216763. Cells were fixed, stained with anti-I-A^b antibodies, and analyzed by flow cytometry. The histograms are representative stainings of the surface expression of I-A^b on gated CD11c⁺ cells. E) Splenic DCs were treated for 2 days with 10 μ M of a specific Akt inhibitor. Cells were fixed, stained with either anti-CD80 antibodies, and analyzed by flow cytometry. The histograms are representative stainings of the surface expression of CD80 on gated CD11c⁺ cells. F) Splenic DCs were treated for 2 days with 1 μ M of the specific MEK1/2 inhibitor, U0126. Cells were fixed, stained with either anti-CD80 antibodies, and analyzed by flow cytometry. The histograms are representative stainings of the surface expression of CD80 on gated CD11c⁺ cells. Shades histograms, untreated DCs; line histogram, kinase inhibitor-treated DCs. Data represent

triplicate samples. All data are representative of at least two independently performed experiments.

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Figure 6. SB216763 pre-treated BMDCs induce IL-2 production in purified T cells

A) IL-2 ELISPOT; B) IFN- γ ELISPOT. “Naïve Mouse” refers T cells purified from naïve B6. “Primed Mouse” refers to T cells purified from B6 mouse that had undergone a skin transplant (BALB/c to B6) 30 days prior to the experiment (and had rejected in about 10 days after transplantation); 1 and 3 = Purified T cells from B6 incubated with purified DMSO pre-treated CD11c⁺ BM cells from BALB/c; 2 and 4 = Purified T cells from B6 incubated with purified SB216763 (GSK-3 β inhibitor) pre-treated CD11c⁺ BM cells from BALB/c. Data are represented as means $\pm$ S.D. of triplicate samples. All data are representative of at least two independently performed experiments.

Figure 7. Inhibition of GSK-3 β does not affect phagocytosis in CD11c⁺ cells

Isolated CD11c⁺ cells were incubated for 30 minutes at 37 C with 1 mg/ml FITC-dextran (70 kDa) in the absence or presence of the kinase inhibitors, SB216763 (5 μ M, GSK-3 β inhibitor) or U0126 (1 μ M, MEK1 inhibitor). Cells incubated on ice in the presence of FITC-Dextran were controls for non-specific binding and those values were subtracted from the experimental values. Values are representative as means $\pm$ S.D. of triplicate samples.

A. **B.**

Figure 8. Dendritic cell maturation parallels GSK-3 β inhibition *in vivo*

A) Representative histogram of splenic dendritic cells undergoing maturation *in vivo* following depletion of regulatory T cells. Purified CD11c+ cells were stained with anti-I-Ab antibody and FACS analyzed. Dashed line, isotype control; shaded area, CD11c+ cells from untreated mice; solid line, CD11c+ cells from DT treated mice. All data are representative of at least two independently performed experiments. B) Representative dot blots of CD11c+ cells purified from the spleens of untreated or DT-treated B6.Foxp3^{DTR} mice. Cells were co-stained with anti-CD86 or I-Ab and anti-phospho-GSK-3 β (Ser9). All data are representative of at least two independently performed experiments.

Figure 9. Schematic model of GSK-3β and the state of dendritic cell maturation

Immature dendritic cells exhibit a low level of inactive GSK-3β, as exhibited by the phosphorylation on Serine 9 (pSer9), which may be a result of a low level of AKT activity. Upon exposure to maturation factors, such as GM-CSF, AKT gets fully activated, resulting in the increased number of GSK-3β molecules that are inactivated. This in turn allows for the increased surface expression of co-stimulatory molecules, such as CD80, CD86 and CD40.