Mechanism of expansion of T-regulatory cells by GM-CSF Induced
Bone Marrow Derived Dendritic cells.

BY

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THESIS
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I dedicate this thesis to my parents Yadagiri and Renuka GOPISETTY for their never-ending support and dedication to my education.
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<td>EAT</td>
<td>Experimental autoimmune thyroiditis</td>
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<td>TSHR</td>
<td>Thyrotropin receptor</td>
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<td>mTg</td>
<td>Mouse thyroglobulin</td>
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<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
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<td>Flt3-L</td>
<td>FMS-like tyrosine kinase 3 ligand</td>
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<td>BMDCs</td>
<td>Bone marrow derived dendritic cells</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>DCs</td>
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<td>G-spDCs</td>
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<td>Control splenic DCs</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>TPO</td>
<td>Thyroid peroxidase</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
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<tr>
<td>CFA</td>
<td>Complete Freund's adjuvant</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>APC</td>
<td>Allo-phycocyanin</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>TMRM</td>
<td>Tetramethylrhodamine, methyl ester</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TW</td>
<td>Transwell</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>Foxp3</td>
<td>Forkhead box P3</td>
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<td>NOD</td>
<td>Non-obese Diabetic</td>
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SUMMARY

T regulatory cells (Tregs) are known to be the important cells required for the suppression of many autoimmune diseases and to maintain tolerance. Tregs have been well characterized and different conditions/factors that lead to their activation have been extensively studied over the years. Investigation of Treg biology is still an active field of research as the molecules/mechanisms that contribute to Treg homeostasis are not clearly understood. Our laboratory had previously demonstrated that GM-CSF treatment of mice with induced experimental autoimmune thyroiditis (EAT) could not only prevent but also reverse the ongoing disease. EAT is a mouse model of Hashimoto's thyroiditis, a T cell mediated chronic inflammatory organ specific autoimmune disease that can be induced in mice by mouse thyroglobulin (mTg) injections. Furthermore, this modality of treatment had been successfully used to treat various other autoimmune diseases in mice such as diabetes in Non obese Diabetes (NOD) mice and experimental autoimmune myasthenia gravis (EAMG). We had earlier shown relative to mTg immunized mice, mTg mice immunized mice treated with GM-CSF had increased number myeloid CD8α dendritic cells (DCs) that subsequently increased the number of Tregs and suppressed the T-effector response through IL-10 production.

Although the therapeutic efficacy of GM-CSF in different autoimmune diseases was mediated through its effects on DCs had been shown, the specific action of GM-CSF on DCs and the molecular interactions necessary for Treg activation had not been explored. Therefore, the present study was performed to investigate if GM-CSF primarily acts on bone marrow precursors or differentiated splenic DCs and to investigate the DC-Treg interactions that are required for Treg activation.
SUMMARY (continued)

In order to evaluate the effects of GM-CSF on bone marrow (BM) cells or on differentiated DCs, we first tested the efficacy of BM derived DCs or splenic DCs in activating the Treg response. Our basic experimental strategy involved co-culture of naive CD4+ T-cells with GM-CSF derived BMDCs and splenic DCs cultured in the presence or absence of GM-CSF and compared their efficacy to induce/expand Tregs \textit{in vitro}.

The first part of our study revealed that GM-CSF derived BMDCs had a superior capacity to selectively expand existing Treg population in the absence of any TCR stimulation \textit{in vitro} compared to the splenic DCs. Based on the dogma that any T-cell response would require TCR activation for expansion, this was a novel finding. However, we found that TCR played an indirect role in Treg expansion. TCR activation on CD25- T-cells by the endogenous antigen presented through MHC class-II on BMDCs was required for IL-2 production, which enabled the selective proliferation of Tregs. This was further substantiated when addition of exogenous IL-2, in the absence of TCR signalling was sufficient to expand Tregs in the presence of GM-CSF treated BMDCs from MHC class-II knockout mice. Although IL-2 was required, it was not sufficient for the expansion of Tregs by GM-CSF derived BMDCs and required contact dependent interactions between BMDCs and T cells. Further investigation, revealed that the critical molecule involved was OX40L, which was expressed on BMDCs and not on splenic DCs. OX40L is a TNF family member that was earlier thought to be important for T-effector and memory T-cell proliferation. Interestingly, we also found that BMDCs secreted TGF-β and along with TCR activation was able to induce Tregs.
SUMMARY (Continued)

These studies showed that BMDCs can expand natural Tregs in a contact dependent but TCR independent fashion in the presence of IL-2. Moreover, the BMDCs also could induce Tregs (convert non-Tregs into Tregs; i.e. convert CD4+CD25- cells into CD4+ CD25+ Tregs) in a TGF-β and TCR mediated signalling dependent manner. These studies conclusively showed two distinct mechanisms of action of BMDCs one caused Treg expansion while the other was involved in Treg induction.

Our experiments to determine if OX40L alone is sufficient for the in vitro expansion of Tregs by BMDCs revealed that although OX40L was required, it was not sufficient. Our further literature search and investigative studies enabled us to determine the role for a notch ligand Jagged1, along with OX40L in nTreg expansion. Interestingly only OX40L+Jagged1+ BMDCs were able to expand Tregs in vitro. Proliferating Tregs had activated notch receptor notch3, indicating its activation by notch ligand Jagged1 expressed on BMDCs. Furthermore, adoptive transfer of OX40L+Jagged1+ BMDCs into mTg immunized mice caused increase in the percentage of Tregs and, in protective cytokine, IL-4+IL-10+ T-cells compared to control mice that were immunized with mTg and mTg immunized and treated with OX40L+Jagged1- BMDCs. Collectively, our studies showed that (i) GM-CSF primarily acted on BM precursors, which helped increase the number of Tregs by two distinct mechanisms: One was they expanded nTregs selectively in a contact and IL-2 dependent, but TCR independent manner; and the other is they induced Tregs in a contact independent, but TGF-β and TCR signal dependent manner (ii) OX40L and Jagged1 expressed on BMDCs were required for the expansion of Tregs in vitro (iii) OX40L+Jagged1+ BMDCs alone could expand Tregs
SUMMARY (continued)

*in vitro and were able to suppress ongoing autoimmune thyroiditis in mice. Therefore the DCs mobilized upon treatment of mice with GM-CSF caused increase in Tregs and in IL-4+ IL-10+ T-cells, which likely suppressed the ongoing autoimmune thyroiditis. Our studies also shed some light on the molecules that may play critical roles in Treg homeostasis *in vivo.*
I. INTRODUCTION

T-cell activation is the first step in the initiation of a cascade of events in the adaptive immune response. A productive T-cell activation requires 'signal-2/co-stimulation' in addition to the T-cell receptor mediated (TCR) 'signal-1', which is provided by antigen presenting cells (APCs) such as dendritic cells (1). In addition to the above mentioned two-signals required for T-cell activation, there are other factors such as nature of the antigen, local cytokine milieu and additional co-stimulatory signals from APCs that further determine the quality and the nature of a specific T-cell response. The complexity involved in the T-cell immune response to various self and non-self antigens makes it immensely interesting as well as challenging, and provides a very exciting field of research to the immunologists. Furthermore, tweaking the T-cell response has been demonstrated to be beneficial in various autoimmune disease animal models.

Although the factors that trigger a specific kind of T-cell response to various infections are not completely understood, there are two well characterized types of T-helper cell responses induced by APCs upon antigen uptake and presentation to T cells: (1) Th1 type of T cell response, which is more effective against intracellular pathogens, is characterized by the production of IL-12/IFN-γ and plays a major role in delayed type hypersensitivity reactions including macrophage activation, and in many destructive autoimmune disorders such as EAE, Hashimoto’s thyroiditis and type-1 diabetes. (2) Th2 type of T cell response is characterized by the production of IL-4 and IL-5, and it mediates immune reactions involved in the activation of B-cell response against extracellular bacteria, parasites and toxins (2).
In normal conditions, there is immunological balance maintained between Th1/Th2 T-cell responses. Aberrant immune reactions that can lead to skewing toward a specific T-cell response could result in autoimmune conditions.

Advances in the field of immunology have led to the discovery of additional T-cell responses such as Th17 and T-regulatory (Treg) T-cell responses. While Th17 T cell response has been implicated in various autoimmune disease models and in the initiation of antimicrobial immunity (3), Treg cell response could limit the activation of harmful effector T-cell responses and enhance protective immunity (4-6). And lately, most of the efforts are directed towards eliciting Treg responses to suppress autoimmunity in animal models of autoimmune diseases. Therefore, the factors/molecules that induce or expand Tregs under autoimmune conditions are currently 'hot topics' of investigation in immunology.

As the predominant antigen presenting cells that can optimally initiate an adaptive immune response, DCs play a major role in determining the nature of T cell response. DCs not only have the capacity to activate either a Th1 or Th2 type of effector T-cell response, but also the Treg cell response (7). The nature of T cell response is dictated by the strength of the antigenic signal, the nature of the antigen, the type of cytokine secreted and the co-stimulation delivered by the DCs. And most importantly, it has been demonstrated that a specific T-cell response can be elicited by a specific subset of DCs. Lymphoid DCs (CD8α+ DCs) elicit Th1 type of response, while myeloid DCs (CD8α- DCs) promote Th2 response (8-10). Over the years there have been studies demonstrating that maturation status of the DCs also affect the T-cell response. Semi-mature DCs characterized by high levels of expression of co-stimulatory molecules and lower inflammatory cytokine production have been shown to be important for eliciting Treg cell response (11-14). Therefore, a combination of factors determines Treg cell response. More information on the modulation of DCs for Treg induction will be discussed in the later sections.
Experimental autoimmune thyroiditis (EAT) is a well established murine model of Hashimoto thyroiditis (HT), a common human autoimmune thyroid disease. HT is characterized by infiltration of thyroglobulin specific T-cells into the thyroid which eventually destroys the thyroid hormone producing thyroid tissue and results in clinical hypothyroidism (15). EAT can be induced by immunizing mice with mouse thyroglobulin (mTg) emulsified in complete Freund's adjuvant (CFA). The disease pathology in mice is similar to that noted in humans and is characterized by the infiltration of mTg specific CD4+ T-cells leading to the destruction of thyroid follicles. It is now apparent that Tregs play a profoundly important role in regulating mTg specific immune response. Our earlier studies showed that treating mice with GM-CSF can prevent the onset of, as well as reverse ongoing, EAT (16). These studies also showed that animals treated with GM-CSF had increased numbers of semi-matured CD8α- myeloid DCs and that antigen presentation by such DCs resulted in an expansion of Tregs. These Tregs produced higher levels of IL-10, which suppressed the autoimmune response. Moreover, either blockade of IL-10 receptor or neutralization of IL-10 using specific antibodies abrogated the protective effects of GM-CSF treatment against EAT. Subsequently, our laboratory demonstrated that GM-CSF treatment can also suppress ongoing experimental autoimmune myasthenia gravis (EAMG), and prevent the development of type-1 diabetes (T1D) (16-19).

More recent studies from laboratory showed that GM-CSF acted primarily on DCs and caused an expansion of CD8α- DCs (20). These DCs expressed very low to negligible levels of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, but expressed higher levels of TGF-β (i.e. tolerogenic phenotype), which was sustained even when exposed to inflammatory stimuli such as complete Freund’s adjuvant (CFA) or bacterial lipopolysaccharide (LPS). Adoptive transfer of ‘tolerogenic’ DCs from GM-CSF treated donor mice to recipient mice followed by immunization with mouse thyroglobulin (mTg) led to an expansion of Tregs and prevented
the development of EAT (20). Additionally, we demonstrated that the CD4^+CD25^+ Tregs could suppress EAT through increased production of IL-10 (14). Similarly, adoptive transfer of *in vivo* GM-CSF exposed DCs, and not the unexposed DCs, obtained from NOD SCID mice into NOD mice caused a significant delay in T1D onset (18). However the underlying mechanism of expansion of Tregs relative to pathogenic T effector cells (Teff) remained unknown.

Tregs are characterized by the expression of transcription factor, Foxp3. On the basis of their origin, two different types of Foxp3^+ T regulatory cells (Tregs) have been defined (21). Foxp3^+ natural Tregs (nTregs) are generated in the thymus through MHC class II dependent T cell receptor (TCR) interactions (21-23). In normal animals, depletion of nTregs alone can cause autoimmune diseases irrespective of the presence of other T-cell subsets or efficiency of naive T-cells to differentiate into Tregs (24). Deficiency of certain co-stimulatory molecules such as CTLA4, GITR on T-cells or other molecules on antigen presenting cells (APCs) or a cytokine (such as IL-2) can result in autoimmune condition by negatively affecting nTreg numbers or function. There is increasing evidence suggesting that in animal models, *in vivo* or *ex vivo* expanded nTregs not only can prevent autoimmune diseases but also suppress ongoing autoimmune disease (25, 26). The TCR repertoire of nTregs is broad and is skewed more toward the recognition of self peptide and MHC complex in the thymus and periphery. Although nTregs have been shown to proliferate *in vitro* and *in vivo* following strong antigenic stimulation and high dose of IL-2 (27-31), peripheral homeostasis of nTregs is still not clearly understood. Identification of molecules that regulate this process will provide us a far better understanding of Treg biology and ways to harness their potential to suppress autoimmune responses to treat autoimmune diseases. Other studies have shown that Foxp3^+ Tregs can also be generated in the periphery (32, 33). These peripherally generated Tregs are commonly termed adaptive Tregs (iTregs). Cytokine TGF-β
plays a key role in the conversion of TCR activated naïve T-cells to Foxp3\(^+\) adaptive Tregs (34, 35). The dendritic cells have been shown to generate adaptive Tregs in the presence of TGF-β through programmed death ligand-1 (PDL-1) co-signalling (36).

Like classic effector T-cells, Tregs also require 'signal-2' for their function (37). Although previously it had been demonstrated that absence of 'signal-2' induces unresponsiveness/anergy in T-cells (38), it is now known that co-stimulation does not merely provide a on/off switch but rather is a part of complex receptor ligand interactions (1). The most important co-stimulatory molecules implicated in Treg function are CD28 and CTLA4. Additionally a growing field of evidence shows that co-stimulatory molecules on T-cells previously known to be involved in positive regulation such as program cell death-1 (PD-1), CD40 Ligand (CD40L), 4-1BB (CD137) are now implicated in the regulation of Treg biology (39-42). The role of some molecules such as OX40 is still controversial. OX40L belongs to TNFR family and its role in Treg generation and homeostasis is not fully resolved. OX40L expressed on antigen presenting cells binds to OX40 expressed on T-cells. Such an interaction has been thought to provide co-stimulation and inhibit induction of Tregs or alter their suppressive function (43, 44). Conversely, other studies have shown that OX40 mediated signalling can promote generation, survival and expansion of Tregs (45-47). While, Ruby et al demonstrated that in an experimental autoimmune encephalomyelitis model, OX40 stimulation after disease induction exacerbated the disease, Griseri et al and Piconese et al (46, 47) have shown that under inflammatory conditions OX40 is required for Treg mediated suppression of colitis. However, these earlier studies also indicated that additional signalling may be required for the expansion of Tregs. Therefore, co-stimulation required for the Treg homeostasis and function may depend on the context of the immune response. Recently, it has been shown by Bleck et al (48) that up-regulation of OX40L and a Notch ligand Jagged1 on
human myeloid DCs enhances Th2 responses, suggesting that Notch signalling may represent the second co-stimulation required for nTreg expansion.

Co-stimulation by Notch ligands and their role in Treg function is a novel concept and its potential role in nTreg expansion is emerging. The Notch signalling has been implicated in the development and differentiation of DCs and T-cells (49-53), and is required for hematopoiesis. Notch receptors, encoded by genes Notch 1-4, and five surface-bound ligands encoded by Jagged1, 2, and Delta-like1, 3 and 4 (Dll1, Dll3 and Dll4) have been described. The type of cell and the specific ligand/receptor mediated signals regulate the outcome of Notch signalling (54, 55). Because hematopoietic progenitors expressing Jagged2 and antigen presenting cells (APCs) over-expressing Jagged1 can activate Tregs (56, 57), a role for Notch signalling in BMDC mediated Treg expansion has become apparent. Although Jagged family Notch ligands have been shown to enhance Tregs, specific role for a particular Notch mediated signalling in Treg expansion, in the presence or absence of canonical TCR mediated signalling, is yet to be defined.

Owing to the available literature on Treg biology and the previous work from our laboratory we wanted to investigate if the effect of GM-CSF is on differentiated splenic DCs or bone marrow derived DCs. Our current investigation revealed that \textit{ex vivo} treatment of SpDCs with GM-CSF followed by adoptive transfer failed either to increase the numbers of Tregs or confer protection, and suggested that the GM-CSF may be acting primarily on DC precursors in the bone marrow. Interestingly, BM precursor cells differentiated in the presence of GM-CSF (BMDCs) \textit{in vitro} were able to selectively expand nTregs in co-cultures while the spleen derived DCs (SpDCs) could not (58). Further, these BMDCs induced expansion of nTregs in a TCR independent but in a contact dependent manner.

Additionally, we have shown that GM-CSF primarily acts on BM precursors and its predominant tolerogenic effect is through the mobilization of BM precursors to
develop into tolerogenic DCs. Moreover, while these tolerogenic DCs could expand nTregs upon direct cell to cell contact in the absence of antigenic stimulation (independent of TCR stimulation), they could facilitate adaptive conversion of CD4⁺CD25⁺ T cells into Tregs through cytokine secretion only upon TCR activation. The predominant co-stimulatory molecules on BMDCs involved in the in vitro contact dependent expansion of nTregs and the particular subset of BMDCs capable of inducing/expanding Tregs in autoimmune thyroiditis have not yet been elucidated.

In the current study we show that in vitro expansion of nTregs by GM-CSF induced BMDCs is mediated by both OX40L and a Notch ligand Jagged1 in the absence of any antigenic stimulation. Blocking or knockdown of either ligand abrogated the proliferation of nTregs in vitro. Additionally, adoptive transfer of OX40L+Jagged1+ BMDCs, and not OX40L+Jagged1- BMDCs, was able to suppress thyroiditis. Moreover, the protection from thyroiditis correlated with enhanced production of suppressor cytokines such as IL-10. This observation is consistent with our finding that the GM-CSF confers protection by activating IL-10+CD4+Foxp3+Tregs. Therefore, our data provides the mechanistic basis for the action of GM-CSF in vivo in the sense that GM-CSF primarily acts on BM precursors and mobilizes them leading to increase in Tregs in the periphery. More specifically OX40L+Jagged1+ BMDCs can expand nTregs, which also activate IL-10 production in T-cells and suppress autoimmune thyroiditis.
II. Literature Review

A. Autoimmune thyroid diseases.

Classically, autoimmune diseases are characterized by the activity of autoreactive lymphocytes, which cause tissue or organ damage through the formation of autoantibodies or self reactive T-cells. Autoimmune diseases are broadly classified as organ specific or systemic (59). Organ specific autoimmunity involves chronic T-cell or antibody responses directed against antigens that are unique to a target tissue. By contrast, systemic autoimmunity is often the result of the breakdown of immunological tolerance to ubiquitous self-antigens resulting in immune complex formation that could be deposited throughout the body resulting in damage at several body sites (60).

Thyroid autoimmune diseases represent more than 30% of all organ-specific autoimmune diseases. Autoimmune thyroid diseases include two extremes of a clinical spectrum, Hashimoto’s thyroiditis (HT) and Grave's disease (GD). Dysfunction of the diseased thyroid gland varies from hypothyroidism due to glandular destruction in HT or blocking antibodies in primary myxedema, to hyperthyroidism in Graves' disease due to thyroid stimulating antibodies. Abnormalities in magnitude range from destruction of thyroid in HT, atrophy in primary myxedema to Goiter in Graves' disease. These different clinical features of autoimmune thyroid diseases are primarily a consequence of either lymphocytic infiltration into the thyroid gland and/or specificity and activity of various autoantibodies. Although T-cells are shown to be critical in the induction, development and maintenance of the disease, the events leading to the different diseases are unclear.

In both cases there is humoral response, with a major difference being in the target antigen specificity. The stimulating antibodies critical in GD are specific for the thyrotropin receptor (TSHR), but antibodies to thyroid peroxidase (TPO) and thyroglobulin are also found
in patient's sera, and they serve as diagnostic markers without being directly involved in the pathogenesis.

Experimental animal models of thyroiditis have helped confirm the autoimmune etiology of many thyroid diseases. There are animal models of human thyroid diseases with autoimmune etiology that develop spontaneously. In these animal models the autoimmune response is directed to Tg or TPO (61). Immunization of certain animals with Tg or TPO has been reported to induce thyroiditis (62). In contrast, experimental Graves' hyperthyroidism can be induced with thyrotropin receptor protein or cDNA that encodes the receptor (63).

**Experimental autoimmune thyroiditis (EAT):**

EAT, the murine model for Hashimoto’s thyroiditis, is a chronic organ-specific autoimmune disease which develops in genetically susceptible strains of mice following immunization with either autologous or xenogenic thyroglobulin (Tg) emulsified in adjuvants. The pathogenesis of EAT is genetically controlled, but how it contributes to disease pathogenesis has not been fully elucidated. Furthermore, EAT is also inducible in irradiated susceptible recipients by transfer of mouse thyroglobulin (mTg)-activated spleen cells from mTg primed donor mice (64). EAT and Hashimoto’s thyroiditis are characterized by circulating anti-Tg and anti-thyroid peroxidase (TPO) peroxidase auto-Abs, and infiltration of the thyroid glands by lymphoid cells including CD4+, CD8+ T-cells, and other mononuclear cells (65), (66). Tg is synthesized by the follicular epithelial cells and stored in the follicular lumen. This molecule consists of tyrosine residues, which are iodinated resulting in the production of T3 and T4 thyroid hormones. TPO is responsible for the iodination of thyroglobulin tyrosine residues, and is located in the apical portion of the follicular epithelial cells and in the cytoplasm of these cells.
In Tg induced EAT model, the antigen is presented to T-cells by classical antigen presenting cells (dendritic cells and macrophages). Antigen specific T cells get activated and release a variety of cytokines. Using monoclonal antibodies to enrich or to deplete specific T-cells subsets, it has been shown that CD4+ T-cells are required for the initiation of EAT, CD8+ T-cells operate at a later stage, presumably acting as cytokine effectors (67). Cytokines like IL-1β and IFN-γ influence the immune response (68). There were several modalities of treatment proposed for the down regulation of EAT: The involvement of thyroiditogenic Th1 cells in EAT has been shown by the beneficial effect of anti-IFNγ treatment, which reduced the severity of Tg induced EAT. Mice with EAT can be treated by injecting de-aggregated mTg (69). Thyroid inflammation, T cell proliferative response, and autoantibody production were minimal or undetectable in these mice. Similarly, our laboratory has demonstrated that GM-CSF treatment of mTg immunized mice can suppress EAT by activating Treg response (16).

**B. Immunological Tolerance:**

The balanced act of mammalian immune system protects the host from a wide variety of pathogens at the same time protecting the host from excessive immune reactions. T and B cells are an integral part of the adaptive immune system that mediates both protective and harmful immune responses principally because of their superior antigen specificity and long lasting memory. Aberrant immune responses trigger autoimmunity, allergy and harmful inflammation. One of the major challenges in immunology and medicine is to understand how immune responses to foreign antigens are regulated without harmful side effects to the host and unresponsiveness to self antigens (i.e. immunological tolerance) is maintained. "Tolerance" is a functional condition, in which either immune response to self antigens from somatic cells does not exist or there is an anti-self immune response that is not apparent
because of the absence of any tissue damage. Understanding the underlying mechanism of self tolerance is likely to help develop methods to either augment immune responses against tumour antigens and chronic microbial infections or limit excessive immune responses against allografts or self-antigens.

Immunological tolerance is maintained at two levels; one is central and the other is peripheral. In the neonatal period, central tolerance is maintained in the thymus by deletion of self-reactive T cells. Self antigens are presented by medullary dendritic cells in the thymus and T cells that possess higher affinity (i.e. above a threshold affinity) for the self antigen are actively deleted by negative selection (70). Thus, self reactive T-cells with lower affinity for self antigen may escape central deletion. Based on transgenic mouse models, it is now evident that self reactive T-cells that might have escaped due to lack of relevant self peptide presentation by thymic APCs are exposed to self antigens expressed at peripheral sites (71). Additionally, it has been demonstrated that thymic low level expression of self antigens is also expressed by peripheral somatic cells (72). These studies suggest that there is potential for immune suppression/regulation of self-reactive T cells in the periphery.

Control of self reactivity outside the thymus is referred to as "Peripheral tolerance". This can be broadly grouped into T cell intrinsic and T cell extrinsic mechanisms. T cell intrinsic mechanisms include clonal ignorance, anergy or activation induced cell death (clonal deletion). Lack of peripheral response to self-antigens may be due to "ignorance" of self reactive T-cells that possess below the threshold level TCR affinity for self antigens (73, 74). It is also possible that these self-antigens are not presented by DCs, and thus autoreactive T-cells are not activated; a process known as immunological ignorance (75). In some instances, ignorance is due to presentation of different epitopes of self antigens by non-APCs in the periphery (72). Active peripheral tolerance also exists and is maintained by deletion of self reactive T-cells in draining lymph nodes and uninflamed peripheral organs and tissues (76).
Under other circumstances, TCR signalling may lead to unresponsiveness or anergy, perhaps due to lack of co-signalling (77). However, T cell extrinsic mechanism involves regulation of self reactive T-cells by a specialized population of T-cells known as regulatory T-cells (Tregs). The Tregs constitute a major component of peripheral tolerance through which autoimmunity is prevented on an ongoing basis. Balance between effector and Tregs is not only important for quality and proper control of adaptive immune response but for establishing and breaching tolerance to self and non self antigens as well. Until recently, it was not possible to phenotypically differentiate Tregs from Teff cells. However, now a functional T regulatory cell lineage is established based on the expression of a transcription factor known as Foxp3 (78-82).

**Foxp3, a marker for Tregs, controls Treg development and function**

Foxp3, a member of the forkhead/winged-helix family of transcription factors is the master regulator of Treg development and function. Foxp3 is mutated in patients with immune deregulation, polyendocrinopathy, enteropathy and X-linked syndrome (IPEX), and in the spontaneous mouse mutant scurfy (83, 84). These findings further demonstrated the indispensable role of FoxP3 in immunoregulation. Mice harboring a loss of function of FoxP3 mutation suffer from multi-organ autoimmune diseases while reconstitution of FoxP3+ Tregs can inhibit autoimmunity suggesting that Tregs are crucial for maintaining immunological self tolerance (85, 86). The studies determining the role of Foxp3 in Treg development and function were prompted by immunological similarities between IPEX in humans and the development of autoimmunity/inflammation in animals devoid of Tregs (37, 78, 79). These studies also suggested that Foxp3 is expressed in CD4+CD25+ thymocytes but not in resting or activated other lymphocytes. Ectopic expression of FoxP3 in CD4+CD25- T-cells converted them to CD4+CD25+ T-cells and conferred the ability to suppress the proliferation of other T-cells.
of other T-cells, thereby inhibited the development of autoimmune diseases (79). The Foxp3 transduction in T-cells leads to elevated expression of Treg cell markers such as IL-2 receptor (CD25), cytotoxic T cell associated antigen-4 (CTLA4), glucocorticoid-induced TNF receptor family-related gene/protein (GITR). Conversely, FoxP3 expression can repress the production of IL-2, IL-4, and IFN-γ. The severe systemic inflammation in scurfy mice is attributed to the lack of fork head domain in Foxp3 which leads to reduced numbers of CD4+CD25+ T-cells, and the condition can be reversed by inoculating CD4+CD25+ T-cells from normal mice (78). Transgenic expression of Foxp3 in mice led to increased numbers of CD4+CD25+ T-cells and Foxp3 expression in CD4+CD25-T-cells and CD8+ T-cells which had suppressor capacity in vitro (80). Conversely, in bone marrow chimera experiments using wild type and Foxp3 deficient cells it was shown that mice receiving Foxp3 deficient bone marrow cells failed to develop Tregs while those receiving wild type cells developed Tregs. Recent studies have revealed that Foxp3 cells appear shortly after birth and their depletion leads to the development autoimmune/inflammatory conditions (39, 87). Therefore, Foxp3 expression in T-cells is critical for the development of Tregs. The fact that disruption of Foxp3 gene leads to the development of autoimmune/inflammatory condition and Foxp3 expression is sufficient to confer suppressive function in non-Tregs unequivocally demonstrate the critical role of Foxp3 in Treg development and function. Thus, currently Foxp3 is considered, the reliable marker for Tregs.

The most intriguing question about Foxp3+ Tregs is how does Foxp3 orchestrate cellular and molecular programs involved in Treg function? It has been shown that Foxp3 interacts with various transcription factors such as NFAT (Nuclear factor of activated T-cells), ALM2 (acute myeloid leukaemia 1), histone acetyl transferase (HAT), and possibly NF-kB (88). Significance of the above mentioned transcription factors have been studied by disrupting their interaction with Foxp3. For instance, it has been shown that amino acid
substitutions in Fopx3 domain that disrupt Foxp3-NFAT interaction impair the ability of Foxp3 to repress IL2, activate CTLA4 and CD25 (89). Similarly, it has been shown that disrupting binding between Foxp3/ALM1 in natural Tregs impairs suppressor activity of Tregs (90). Therefore, it seems that FoxP3 hijacks the effector T-cell transcription machinery and converts them into Tregs. In this regard, it had been shown that in mice genetically engineered to express substantially lower amounts of Foxp3 the natural Tregs not only lose suppressor activity but also spontaneously develop into effector T-cells (91). However, the questions as to how Foxp3 interacts with other transcription factors and controls the genes that mediate suppression remain unexplored.

C. Types of T-regulatory cells:

1. Natural T-regulatory cells

Two different types of Tregs have been defined on the basis of their origin (53). Auto-antigen specific natural Tregs (nTregs) are generated in the thymus through MHC class II dependent T cell receptor (TCR) interactions (22-24, 92). However recently various groups have shown that Foxp3+ Tregs can also be generated outside the thymus (93, 94) and they are referred to as adaptive or induced Tregs (iTregs).

For nearly 40 years it has been known that manipulation of the thymus and T-cells can cause autoimmune diseases in normal animals (95). Thymectomy in normal neonates around day 3 results in damage to various organs (thyroid, stomach, ovaries and testes) and the appearance of tissue specific antibodies in the circulation. On the other hand, adult thymectomy in rats followed by various rounds of sub-lethal irradiation produces autoimmune thyroiditis and type 1 diabetes. More importantly, adoptive transfer of normal T-cells, specifically CD4+ T-cells from untreated syngeneic mice, inhibits the development of autoimmunity (96, 97). These studies indicate that there are two types of T-cells in normal
animals: Pathogenic self-reactive T-cells and T-cells that suppress autoimmunity. Efforts to identify a specific marker for a population of T-cells that can prevent autoimmunity led to the identification of CD25 (IL-2 receptor α) (98). Adoptive transfer of CD4+ T-cells depleted of CD25+ T-cells into athymic nude mice caused autoimmune diseases where as co-transfer of CD25+ T-cells inhibited the development of autoimmunity (99). CD4+CD25+ Tregs that develop in the thymus constitute about 5-10% of the circulating T-cell population in healthy humans and mice. Treg depletion also augments immune response to non-self antigens. For instance, depletion of Tregs produces inflammatory bowel disease, which likely results from excessive immune response to commensal bacteria in the intestine (100). Thus Treg depletion not only causes autoimmunity but also augments immune response to non-self antigens. T-cells that are reactive to specific self-antigens such as glutamic acid decarboxylase in type-1 diabetes can be expanded in vitro when stimulated with respective self-antigen after depletion of Tregs (101).

The above studies allow us to draw certain generalizations regarding self-tolerance and immune regulation. First normal thymus produces both self reactive T-cells and mature Tregs. Second, normal immune system produces CD4+CD25+ T-cells that suppress immune response to self and non-self (microbes and allografts) antigens. Third, Deficiency of Treg is sufficient to elicit chronic T-cell mediated autoimmunity. Fourth, Activated CD4+ T-cells also express CD25, but it is transient and is of low magnitude compared to Tregs. Fifth, the gene product that clearly distinguishes between CD4+CD25+ T-regs and conventional, activated T-cells is Foxp3 (26, 80)

2. Induced T regulatory cells:

The existence of CD25- T-cells in IL-2 deficient mice that were surprisingly suppressive led to investigation which revealed that acquisition of CD25 expression in CD25-
T-cells is a requisite for functional Tregs (102). Therefore, CD25- T-cells from IL-2 deficient mice were able to convert to CD25+ Tregs in the presence of IL-2 and they also gained Treg function. More importantly, when the key role of Foxp3 was demonstrated it was also shown that non-Tregs can be converted to functional Tregs under certain conditions (22, 103). Early evidence of peripheral conversion of Foxp3- T-cells into Tregs came from the adoptive transfer experiments in which polyclonal CD25- T-cells were injected into lymphopenic mice devoid of nTregs. The donor population became CD25+.CTLA4+ GITR+ and acquired Foxp3 expression and suppressive capacity (102, 104). In the above experimental setting it is possible that Foxp3+ T-regs came from small population of pre existing CD25-Foxp3+ T-cells. This was not the case because in a study where generation of TCR transgenic Foxp3+ T-cells were studied, kinetic and other considerations indicated that they were derived from Foxp3- T-cells. Additionally in a study by Knoechel et al, 2005 it was shown that in a disease model of antigen specific lymphocyte deficient RAG2-/- mice, the mice developed acute Graft versus host like disease but recovered upon injection of antigen specific naive CD4+ T-cells that converted in to Foxp3+ T-cells, which was dependent on IL-2 but did not require thymus (32).

One of the possibilities for how a small population of antigen specific Tregs suppress effector T-cell response in vivo is that Tregs may induce suppressor phenotype in effector cells thereby expanding suppressor cells. "Infectious tolerance", a term originally coined by Gershon and Kondo is referred to as a mechanism where naive lymphocyte population is suppressed by cells with regulatory phenotype (105). Later on, this concept was expanded by Qin et al (106) and is now used to describe a process where tolerance state is induced from one cell population to another. Later on it was shown that TGF-β is a potent inducer of Foxp3 expression and Treg production of TGF-β is required for protection against autoimmune disease (34, 107, 108). Also in a SCID mouse model, protection from colitis has
been shown to be dependent on TGF-β and another suppressor cytokine, IL-10 (109). After in vitro demonstration of induction of Foxp3 by TGF-β in naive TCR activated T-cells, protocols have been developed to generate Tregs in vitro (34, 35). Thus naive T-cells marked as Foxp3- T-cells can develop in to Foxp3+ Tregs in vitro and in vivo.

3. Maintenance of Tregs:

The Treg marker CD25 is a component of the high affinity IL-2 receptor (IL-2R) and is required for Treg development and function. The fact that CD25 (IL2R) distinguishes a major subset of Tregs suggests an important role for IL-2 in Treg development, expansion, and function. Systemic autoimmunity seen in IL-2- and IL-2R deficient mice is reminiscent of that seen when Tregs are depleted by neonatal thymectomy or in Foxp3 deficient mice (110). Additionally, it was later shown that mice lacking IL-2 or IL-2R genes are deficient in CD4+CD25+ T-cells (111-113). Finally, adoptive transfer of CD4+ CD25+ T-cells in to IL-2 or IL-2R/-/- mice prevented the development of autoimmunity and inflammation. These studies suggest that IL-2 is critical factor for Treg development and function.

In humans, CD25 deficiency causes severe autoimmunity and allergy which is indistinguishable from IPEX (114). Mice lacking IL-2 succumb to a series of ailments, known as IL-2 syndrome, which is characterized by reduced numbers of Foxp3+ T-regs and dysfunction of Foxp3+ Tregs (115). Moreover, it has been demonstrated that neonatal mice administered with high doses of neutralizing antibody to IL-2 show significantly lower numbers of Foxp3+ Tregs and develop autoimmune conditions similar to the mice that lack Foxp3+ T-regs (116).

IL-2 has been shown to be important for sustained expression of Foxp3 and CD25 in natural Tregs and to increase their suppressive function at least in vitro (117, 118). Although other cytokines such as IL-7 and IL-17 have been shown to be important for Treg
development, their deficiency did not alter the Treg numbers. In contrast IL-2 and IL-15 double deficiency caused significant reduction in numbers of Foxp3+ T-regs (119). Thus IL-2 is essential for the development, survival and function of Tregs.

IL-2 has multiple targets including CD4+ and CD8+ T-cells, B cells and natural killer cells. It facilitates differentiation of CD4+ T-cells to Th1 and Th2 T-cells and expands CD8+ memory T-cells and natural killer cells. IL-2 also maintains Foxp3+ natural T-regs, expands them at high doses and promotes naive T-cells to become Foxp3+ Tregs (120). On the other hand, it induces apoptosis in antigen activated T-cells. The source of IL-2 is activated T-cells, and thus there is a negative feed-back control of immune responses via IL-2 VIZ IL-2 secreted by activated T-cells/non-regulatory T cells contributes to the maintenance, activation and expansion of natural Tregs, which in turn limits the expansion of non-regulatory T cells (88). Disruption of this loop in normal conditions promotes the development of autoimmunity/inflammatory disease. Furthermore, manipulation of this feed-back loop is critical in tuning the intensity of Treg mediated suppression, hence strength of immune responses.

Although IL-2 has been convincingly shown to be important for Treg homeostasis, involvement of other molecules or knowledge about other factors that are absolutely required for Treg expansion and homeostasis is very limited. For instance, the application of Tregs in clinical settings has been limited by the inability to continuously expand Tregs in vivo. Tregs from both humans and rodents do not proliferate robustly through prototypical activation (121-124). Nevertheless, several strategies have been developed for Treg expansion including using agonistic anti-CD3 and anti-CD28 Abs (125), (126), ligands targeting the 4-1BB receptor along with anti-CD3 and IL-2 (127, 128). These and other activation dependent methods require very pure Tregs because contaminating Teff can proliferate more robustly upon activation and distort the final composition of the culture (129). A combination of
surface markers like CD25 high and CD127 low are used in FACS based sorting to generate >95% pure Foxp3+ Tregs; with IL-2 supplementation (130) (129). Thus, identification of additional factors for selective expansion of Treg from naive T-cell whole populations would be extremely beneficial and also would help provide much better understanding of T-reg biology.

**D. Modulation of Dendritic cells for tolerance induction**

Dendritic cells are professional antigen presenting cells that can induce central and peripheral tolerance by mechanisms such as deletion, anergy and induction of regulatory lymphocytes (131). Due to the exceptional ability of these APCs to regulate immune response, much attention has been focused on them to stimulate T and B-cell responses. T-cell activation and clonal expansion by DCs involves a very complex signaling and is mediated by TCR ligation together with delivery of co-stimulation signals and cytokine production. Therefore, molecules that modulate DC function might be effective in modulating autoimmune responses. Much evidence has emerged recently concerning the inherent tolerogenic capacity of the DCs. Mechanisms by which DCs can promote peripheral tolerance are under intensive investigation. These studies have extensive applications for the therapy of autoimmune diseases and allograft rejection. DCs are equipped with properties that can activate both innate and adaptive immune response. Following antigen uptake and activation, DCs migrate from primary to secondary lymphoid organs, redistribute MHC-Ag complexes to the cell surface and up-regulate surface expression of co-stimulatory molecules, such as CD80, CD86, and a process known as "maturation". This process of maturation also involves production of high amounts of pro-inflammatory cytokines such as IL-12 and TNF-\(\alpha\) converting DC into very powerful T-cell priming APC.
In addition to the immunostimulatory capacity of mature DC, a vast majority of work describe the immunoregulatory capacity of immature and semi-mature DCs (11). Immature DCs induce T-cell anergy and are characterized by low surface expression of MHC class II, co-stimulatory molecules and pro-inflammatory cytokines IL-12 and TNF-\(\alpha\). Immature DCs has also been used for tolerance induction. Jonuleit et al have shown that *in vitro* stimulation of allogenic T-cells with immature DCs can lead to generation of IL-10 producing T-reg cells (132). In accordance with these findings it has been shown by another group (133) that subcutaneous injection of immature monocyte derived DCs pulsed with influenza matrix peptide into human volunteers, can lead to peptide specific IL-10 producing T-reg and Ag-specific inhibition of CD8+ T-cell killing activity.

In contrast, semi-mature DCs are characterized by high expression of co-stimulatory molecules, but low/negative production of pro-inflammatory cytokines. We and others have previously shown that semi-mature DCs have immunoregulatory functions and induce Tregs *in vitro* and *in vivo* in autoimmune disease models (14, 16, 134). For instance, Menges at al demonstrated that repeated injections of DCs matured with TNF-\(\alpha\) were maintained in semi-mature state (MHC class- II-high, co-stimulatory-high, pro-inflammatory cytokine-low), which in turn induced IL-10 producing peptide specific T-cells *in vivo* and antigen specific protection from EAE (135). A similar approach was used by Sato et al, who expanded DCs in the presence of IL-10 and TGF-\(\beta\) and matured them with LPS or TNF-\(\alpha\). These DCs were MHC class-II-high, co-stimulatory-low and low producers of pro-inflammatory cytokines (136). In a murine model of Graft Versus host disease (GVHD), it was demonstrated that host-matched semi mature DCs protected the mice from GVHD lethality and induced expansion of IL-10 producing CD4+ CD25+ T-reg (137).

We have shown that modulation of DCs by Granulocyte-macrophage colony stimulatory factor (GM-CSF) can lead to the suppression of experimental autoimmune
diseases including EAT, myasthenia gravis and IDDM (17, 18). Moreover, extensive in vitro and in vivo studies from our laboratory have shown that semi-mature CD8α-DCs, from GM-CSF treated but not untreated mice, when adoptively transferred into CBA/J mice were able to facilitate the induction of CD4+CD25+Foxp3+ Tregs and suppress EAT in the recipient mice. We have also shown that CD4+CD25+ Tregs suppressed the disease through the enhanced production of IL-10 (14, 16, 20). These observations have been substantiated by studies from Dr. Kong’s group which have shown that CD4+CD25+ T cells from Tg-tolerized mice can suppress mouse Tg-specific responses in vitro (138). Similarly others have shown that GM-CSF cultured autologous myeloid DCs decreased the incidence of Type-1 diabetes (139). Although the protective action of GM-CSF had been demonstrated in various autoimmune models in our lab, the specific factors/molecules on DCs contributing to increase in Tregs had remained elusive. Therefore, the current study was aimed at understanding the mechanism by which GM-CSF imparts this tolerogenic phenotype upon DCs and to identify specific molecules that are involved in the expansion/induction of Tregs with the hope that the study will provide us good insights in to Treg homeostasis.

In a separate study it has been shown that GM-CSF cultured immature myeloid donor DCs prolonged heart allograft survival in a mouse model (140). In separate instances, it has been shown that NF-κB decoy oligodeoxy ribonucleotides treated myeloid DCs, Fas ligand transfected myeloid DCs, Rapamaycin exposed alloantigen pulsed DCs from donor mice prolonged heart allograft survival in the recipient mice (141-143). Furthermore, it has been shown that treatment of NOD mice with adenovirus IL-4 transduced DCs and ex vivo IFN-γ stimulated autologous splenic DCs reduced the incidence of type-1 diabetes (144). Thus DC modulation has been extensively employed to demonstrate reduced incidence of autoimmune diseases and allograft survival.
**Exploiting co-stimulation for tolerance induction:**

The most popular co-stimulatory pathway on DCs that has been shown to profoundly affect cytokine balance and thus T-cell differentiation is B7-1/B7-2.CD28/CTLA4 pathway. *In vivo* blockade by CTLA4-Ig that blocks the interactions of B7-1 and B7-2 prevented the development of EAE (145). Mice treated with anti B7-1 antibodies at the time of induction of EAE were protected from developing the disease due to immune deviation as shown by the cytokine profile and qualitative nature of the immune response in EAE (146). A similar treatment of NOD mice with anti-B7-2 antibody was effective in ameliorating the development of diabetes, where as anti-B7-1 accelerated disease onset (147). Blockade of CD40-CD154 signaling pathway using anti-CD154 monoclonal antibodies has been shown to potentiate the capacity of DCs to induce long-term cardiac allograft survival (148).

Recently it has been shown that signalling via programmed death1 ligand (PD-L1) but not PD-L2 was required for conversion of Foxp3+ T-regs. Furthermore, *ex vivo* PD-L1/-/- DCs failed to support Foxp3 induction in the presence of TGF-β (36, 149). While the roles of some co-stimulatory molecules have been more clearly defined, roles of some of the other molecules such as OX40L remain un-resolved. Both OX40 and OX40L are members of the tumor necrosis factor super family with co-stimulatory function (150). Signaling through OX40 can promote survival and expansion of autoreactive effector T-cells (Teff), and blocking OX40L-OX40 pathway can diminish experimental autoimmune encephalitis (EAE) (151), inflammatory bowel disease (IBD) (152) and type-1 diabetes(T1D) (153). In contrast, others have suggested that OX40L-OX40 interaction contribute to both Treg expansion and function (154, 155).

One of the emerging co-stimulatory pathways gaining importance recently is Notch family ligands. The role of Notch signaling has been implicated in the development and differentiation of DCs and T-cells (49-52) and is required for the development and
maintenance of hematopoietic. In particular, Notch receptor and ligands are expressed in lymphoid progenitors throughout lymphoid development. Upon ligand binding to the Notch receptor, its intracellular portion is cleaved by a presinilin associated gamma secretase. The cleaved intracellular part translocates to the nucleus where it forms a heterodimeric complex with various co-activator molecules and acts as a transcriptional activator (156). Expression of specific ligands on DCs activates a specific T-cell response (157). For instance, it has been shown that APCs over-expressing Jagged-1 and hematopoietic progenitors expressing Jagged-2 can activate Tregs (56, 57). Conversely, it has been shown that ligation of Jagged-1 expressed on Tregs to Notch-1 receptors expressed on DCs can trigger the production of TGF-β, which in turn can render DCs to become tolerogenic leading to further expansion of Tregs (158). Furthermore, Notch signaling has been recently shown to drive the differentiation of peripheral CD4+ T-cells into T-regs (159). These and other studies show the reciprocal regulation of DC and T cell function via Notch signaling. Thus modulation of co-stimulatory molecules by various agents on DCs that can promote Treg expansion/function should be explored to treat autoimmune diseases.
III. MATERIAL AND METHODS

A. Animals

Six to eight week old CBA/J mice were purchased from the Jackson laboratory. Wild type (WT) C57/B6 and MHCII<sup>−/−</sup> mice were purchased from Taconic Farms. CD80<sup>−/−</sup>, CD86<sup>−/−</sup>, CD80<sup>−/−</sup> CD86<sup>−/−</sup> mice were kindly provided by Dr. Chenthamarakshan Vasu (Department of Surgery, University of Illinois (Chicago, IL). Mice were housed in the Biological Resources laboratory facility at the University of Illinois (Chicago, IL) and provided food and water ad libitum. All animal experiments were approved by the University of Illinois at Chicago animal care and use committee.

B. GM-CSF, antibodies and thyroglobulin

Recombinant GM-CSF, Carboxy fluorescein succinimidyl ester (CFSE) and neutralizing antibodies to TGF-β were purchased from Invitrogen (Carlsbad, USA). Pacific blue conjugated anti-CD4, mouse IgG1 isotype control was purchased from Caltag Laboratories (Invitrogen). PE conjugated anti-H-2K<sup>d</sup> (MHC class II), anti-CD25, anti-CD80, anti-CD86, anti-CTLA4, anti-IL-10, streptavidin; PE conjugated anti I-A<sup>B</sup> (MHC Class-II), , PE-Cy7 conjugated anti-GITR, FITC conjugated anti-CD8α and isotype control mAbs were purchased from BD Pharmingen (San Diego, CA, USA). APC conjugated anti-CD11c, anti-CD11b, anti-Foxp3, anti-IL17; Biotin conjugated anti-PDL1, anti-PDL2; anti-CD3 PE conjugated anti-OX40L, anti-Jagged1, anti-IL-4, anti- IFN-γ, anti-IL-12 and OX40 agonist (OX86), were purchased from eBioscience (San Diego, California). Anti-Notch3, Notch1 PE were obtained from Santa cruz Biotechnology (California). Mouse thyroids were obtained from Pel-Freeze (Rogers, AR) and thyroglobulin was prepared as described earlier (160). Recombinant TGF-β and neutralizing antibodies to OX40L, Jagged1, Notch3 and IL-2 were
purchased from R&D systems (Minneapolis, MN). Gamma secretase inhibitor (GSI) was obtained from Sigma-Aldrich (S-2188, St. Louis, MO).

C. Spleen, bone marrow (BM) derived DCs and T-cell subpopulation isolation

Bone marrow cells were cultured in complete RPMI containing 10% heat-inactivated FBS in the presence of 20ng/ml GM-CSF for 3 days. On days 4 and 6, fresh medium containing 20ng/ml GM-CSF was added. Non-adherent CD11c⁺ DCs were sorted using anti-CD11c coated magnetic beads according to the manufacturer’s directions (Miltenyi Biotec) on day 8. For some co-culture experiments sorting for specific sub population of BMDCs (>95% purity) was performed by Moflo cell sorter (Beckman Coulter Cyan ADP). Splenic CD11c⁺ DCs isolated using the same protocol were kept in culture in the presence (G-spDC) or absence (C-spDC) of GM-CSF for 2 days. CD4⁺, CD4⁺25⁻, CD4⁺25⁺ T-cell subpopulations were isolated using appropriate kits and Auto Macs according to the manufacturer’s directions (Miltenyi Biotec).

D. Priming mice with mTg and OVA:

Groups of CBA/J mice were immunized (3 mice per group for each experiment) subcutaneously with OVA (100μg/mouse) or mTg (100μg/mouse) emulsified in CFA on day 1 and day 10.

E. Treatment of mice with GM-CSF:

2 groups of 3 mice each were treated with PBS (control) or 2 μg of GM-CSF/mouse/day, for 5 consecutive days from days 1-5 and 12-16 and then euthanized and spleen isolated.
**F. In-vitro co-cultures of DCs and T cells**

Each *in vitro* experiment was conducted in triplicates with T-cells, spDCs and BMDCs pooled from 3 mice. BMDCs \((5 \times 10^4)\) and splenic CD11c\(^+\) DCs were cultured with CD4\(^+\), CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^+\) T-cells at the ratio of 1:2 for 5 days. For cultures involving activation of CD4\(^+\) T-cells, anti CD3 (2\(\mu\)g/ml) was added for 48 hrs or mTg (100 \(\mu\)g/ml) was added for 5 days. For proliferation assays T-cell subpopulations were labelled with CFSE at 10 \(\mu\)M according to manufacturer’s instruction (Invitrogen, Carlsbad, CA) before co-culturing them with DCs. Some cultures were supplemented with TGF-\(\beta\) (3 ng/ml), 1-methyl tryptophan (1-MT) (200 \(\mu\)M), anti-TGF-b (30 \(\mu\)g/ml), anti-PDL2 (5 \(\mu\)g/ml), anti-CD80 (5 \(\mu\)g/ml), anti-OX40L (up to 10 \(\mu\)g/ml) and OX40 agonist (OX-86, 5-10 \(\mu\)g/ml), GSI (5-10\(\mu\)M) anti-Jagged1 (10-20\(\mu\)g/ml). For blocking experiments, BMDCs were pre-treated with the antibodies alone for 30min at 37\(^0\)c before keeping them in co-culture with naive CD4\(^+\) T-cells.

**G. Transwell experiments**

In some experiments physical separation between DCs and T-cells was required. We used a transwell of 0.6\(\mu\)m pore size and plated either DCs / sorted populations of DCs and T-cells at the ration of 1:2. After the end of 5 day co-culture, cells were stained separately and analysed by FACS.

**H. BMDC transfection with SiRNA**

A 21bp siRNA sequence specific to Jagged1 \((5'-CTCGTAATCCTTAAATGTT-3')\) was synthesised and annealed by the manufacturer (Dharmacon) and was used as previously described (161). BMDC transfection was also performed as described in this study. Briefly, 3 ml of 20 \(\mu\)M annealed siRNA was incubated with 3 \(\mu\)l of GenePorter (Gene Therapy Systems)
in a volume of 94µl of serum-free RPMI 1640 at room temperature for 30 min. This was then added to each well containing BMDCs and incubated for 4 h at 37°C. Three µl of GenePorter alone were used as mock controls. After incubation, 500µl/well RPMI 1640 supplemented with 20% FCS was added to the cells. Twenty-four hours later, transfected or treated BMDCs were washed and used in subsequent experiments.

**I. Isolation of in vitro generated nTregs:**

Total CD4+ T-cells were co-cultured with BMDCs at the ratio of 1:2. After 5 days in co-culture, CD4+ T-cells were stained with Pacific blue (PB) Anti CD-25 and PE Anti-CD25. Cells were incubated on ice for 30 minutes and washed three times with FACS buffer. Cells were then sorted using Moflo cell sorter. The sorted population were collected in 10% FBS complete RPMI medium.

**J. Suppression assay**

CD4^+CD25^- effector T-cells were isolated from spleen of OVA/mTg treated mice, stained with CFSE and plated in to a 96well flat bottom wells at 0.5×10^6 cells/well in the presence of OVA/mTg (100 µg/ml) and splenic APCsCD4^-CD25^- effector T-cells were isolated from spleen of OVA treated mice, stained with CFSE and plated in to a 96well flat bottom wells at 0.5×10^6 cells/well in the presence of OVA (100 µg/ml) and splenic APCs. Isolated CD4^-CD25^- nTregs were then kept in co-culture with different ratios of CD4^-CD25^- T-cells isolated from OVA primed mice.

**K. Tetramethylrhodamine Methyl Ester (TMRM) and Propidium iodide (PI) Staining:**

Cells from the BMDC-CD4^+ T cell cultures were stained with tetramethylrhodamine methyl ester (100nM) for 15 min at 37°C. Total Cells were stained with fluorochrome labelled
anti-CD4 for the analysis of T-cells before incubating them with TMRM and PI. For TMRM staining, cells were then washed with ice-cold PBS and subjected to FACS analysis. Loss of Tetramethylrhodamine methyl ester staining was used as a marker of apoptosis as it determines mitochondrial depolarization. In our assay percentage of CD4+ T-cells from the co-culture retaining the TMRM stain were determined from FACS analysis as a measure of live cells. We also performed Propidium iodide staining of cells for the assessment of number of non-vital cells.

L. FACS

Freshly isolated and ex vivo cultured cells were washed with PBS-BSA-EDTA. For surface staining cells were labelled with appropriate FITC, PE, APC and PE-Cy7 conjugated Abs for 30 min. For cell proliferation assay, the cells were similarly stained with CFSE. Stained cells were washed three times and analysed by Cyan flow cytometer. For intracellular staining, surface stained cells fixed and permeabilized according to the manufacturer’s instructions (eBioscience) and incubated with appropriate Abs. Briefly, For experiments where intracellular staining was required, samples were washed with FACS buffer twice. Following washing, T-cells were stained with Anti-PB CD4 for 30 minutes on ice. Stained cells were washed three times and incubated in the fixative (1:3 ratio of the permeabilization concentrate and the diluent) for at least 45 minutes. Samples were washed 2 times with 1X permeabilization buffer following fixation. Cells were then stained for additional analysis of intracellular molecules for 30 min on ice. Samples were then washed two times in 1X buffer and analyzed by FACS.

M. REVERSE TRANSCRIPTASE-PCR

Total RNA was extracted using Trizol reagent (Invitrogen). First strand cDNA was synthesized with Superscript 2 (Invitrogen). Gene specific primers were used for semi
quantitative PCR amplification (0.5 min at 94°C, 0.5 min at 55°C, and 0.5 min at 72°C for 33 cycles) to detect relative amount of transcripts. The following primer sets were used to amplify the indicated products:

HPRT-F, GTTGGATACAGGCCAGACTTTGGT
HPRT-R, TACTAGGCAGATGGCCAGGACTA
IL-1β-F, AAGAGCTTCAGGCAGGCACTATCAA
IL1β-R, TAATGGGAACGTACACACCAGCA
IL-6-F, AACCGCTATGAAGTTCCTCTCTGC
IL-6-R, TAAGCCTCCGACTTGTGAAGTG
IL-10-F, TGCACCTCACCACAAGGCAG
IL-10-R, TGGCCTTGTAGACACCTTGT
IL-12-F, ACCTGCTGAAGACCACAGATGAC
IL-12-R, TAGCCAGGCAAACCTCGTTG
IFN-α-F, TGAAGGACAGGCAGGACTTTGGAT
IFN-α-R, TGGCAGCAAGTTGAACGGGAAGA
IFN-β-F, TCCAGCTCAAGGAAGGCAGGCAACA
IFN-β-R, AGAAACACTGTCTGGTG
IFN-γ-F, CTGCATCTTGGCTTTGCAGCTCTT
IFN-γ-R, TTCGCCCTTGCTTTGCTGAAGAAG
IDO-F, TGGGCCCATGACATACGAGAACAT
IDO-R, TGGCAAGGTGGAACCTTTCTCACAGA
TNF-α-F, TTCCGAATTCACTGGAGCCTCGAA
TNF-α-R, TGCACTCAGGGAAGAGATCTGGAA
TGF-β-F, TGATACGCTGAGTGGCTGTCTTT
TGF-β-R, TGTACTGTGTGGCCAGGCTCCAAA
OX40L-F, ATGTCTGCCTGCAAATCTCTCCT
OX40L-R, CTTTGAAAGCCAAAGAGGCGCCACCA
PDL1-F, ACTTGTACGTGGTGAGTATGGCA
PDL1-R, TGGCTGGATCCAGAAATTCTCTCT
Notch-1-F, TGTTAATGAGTGCACTCTCCAA
Notch-1-R, CATTCTGAGCCATCAATCTTGTC
Notch-2-F, TGGAGGTAAATGAATGCCAGAGC
Notch-2-R, TGTAGCGATTGATCGGC
Notch-3-F, ACACTGGGAGTTCTCT
Notch-3-R, GTCTGCTGGCCATGGGATA
Notch-4-F, CACCTCCTGCCATAACACCTTG

Notch-4-R, ACACAGTCATCTGGGTTCATCTCAC

N. Adoptive transfer of BMDCs

Three groups of 3 mice each were given 2 treatments 10 days apart with mTg (100µg/ml) emulsified in CFA. 10 days after the last treatment these mice received via i.v. injection either i.p) PBS, ii) 2 x 10^6 purified CD11c^+ DCs from untreated CBA/J mice or iii) 2 x 10^6 CD11c BMDCs purified and sorted populations from in vitro BM cultures. Two identical adoptive transfers were done for each group at 5 day intervals. Five-days after the last transfer, mice were sacrificed and spleens and lymph nodes were collected for various analyses.

O. Histological examination of thyroids:

Thyroids were collected from sacrificed mice were fixed in formalin, embedded in paraffin, sectioned across both lobes. Tissue sections were stained with hematoxylin and eosin, and subjected to microscopic examination. Thyroid pathology was evaluated and the extent of thyroid lymphocytic infiltration, as a marker of disease severity, was scored using a scale of 1+ to 5+. An infiltrate of at least 125 cells in one or several foci was scored 1+. Ten to twenty foci of cellular infiltration involving up to 25% of the gland was scored 2+. An infiltration involving up to 25–50% of the gland was scored 3+. Destruction of >50% of the gland was scored 4+, and near-complete destruction of the gland with very few or no remaining follicles was scored 5+. 
**P. Statistical analysis**

Mean, standard deviation, and statistical significance were calculated using the MS-Excel application software. Statistical significance was determined using the one tailed Students t-test. A $P$ value of $\leq 0.05$ was considered significant.
IV. RESULTS

A. GM-CSF derived bone marrow dendritic cells expand T-regulatory cells in vitro in an OX40L dependent but TCR independent manner

1. DCs derived from GM-CSF treated bone marrow precursors (BMDCs) can increase Foxp3\(^+\) Tregs in T-cell co-cultures

From the onset, we defined tolerogenic DC as a type of DC that would be able to expand or induce Foxp3\(^+\) Tregs. To test whether GM-CSF could differentially modulate differentiated spleen-derived CD11c\(^+\)CD8\(\alpha\)\(^-\) (spDCs) and confer a tolerogenic phenotype, DCs from 6-8 week old naïve CBA/J mice were cultured in the presence (G-spDCs) or absence (C-spDCs) of GM-CSF for 48 hours. CD4\(^+\) T cells from either naïve or mTg immunized mice were co-cultured with either G-spDCs, C-spDCs or BMDCs derived by culturing BM cells in the presence of GM-CSF for 7 days and analyzed for Foxp3\(^+\) Tregs (Fig 1A). When co-cultured for 5 days with CD4\(^+\) cells from mTg immunized mice in the presence of mTg (100 µg/ml) (Figure 1, upper panel) the G-spDCs showed only a modest increase (6.63±1.03\%, \(p<0.02\)) in the percentage of Foxp3\(^+\) cells compared to C-spDCs (3.97±0.94\%) (Figure 1, upper panel). However, the BMDC-CD4\(^+\) T cell co-culture showed a highly significant increase (15.40±0.48\%) in the percentage of Foxp3\(^+\) T cells (\(~4-5\) fold, \(P<0.001\)). The same response was observed even when the CD4\(^+\) T cells derived from naïve mice were co-cultured without the addition of any exogenous antigen (C-spDCs=3.52±0.88\%; G-spDCs=6.71±1.5\%, \(p<0.2\); BMDCs=15.66±0.96\%, \(p<0.001\)) (Figure 1, lower panel). These results indicated that GM-CSF imparts its tolerogenic effect primarily through differentiation of BM precursors into tolerogenic BMDCs. The effect of GM-CSF on mature lymphoid organ resident (splenic) DCs was minor in this context. Therefore, further studies were focused on understanding the mechanism of action of BMDCs in expanding/inducing Foxp3\(^+\) Tregs.
Since Foxp3+ Tregs share a common differentiation pathway with IL-17 producing TH17 type T-cells, we wondered if the above phenomenon was merely due to increased T-cell differentiation by BMDCs. To rule out any non-specific effect like increased naïve T-cell differentiation by BMDCs, we set up co-cultures of naïve T cells with C-spDCs, G-spDCs and BMDCs without exogenous antigen and analyzed for IL-17 secreting TH17 cells. After 5 days of co-culture we did not see any difference in the percentage of IL-17+ T cells between any of these groups (Figure 2a). In typical experiments, the percentage of Foxp3+ Tregs was reduced in the splenic DC co-cultures over the course of 5 days from its initial level, while it increased in BMDC co-cultures (Figure 2a). In contrast the percentage of IL-17+ T-cells remained similar for all the groups.

We also stained the BMDC/T-cell co-cultures for IFN-γ and IL-4 to determine skewing, if any, towards a Th1 or Th2 phenotype. We found that the percentage of IFN-γ or IL-4 producing T-cells was similar between the groups (Figure 2b). We measured the percentage of necrotic and apoptotic cells by staining with propidium Iodide and TMRM respectively and found that the Treg increase in co-cultures could not be attributed to non-Treg cell-death (Figure 2b). Based on these findings, we concluded that there was a specific increase in the numbers of Foxp3 Tregs in BMDC co-cultures not attributable to any non-specific phenomenon.
Figure 1: GM-CSF derived BMDCs can increase the percentage of Foxp3+ Tregs in cocultures: Splenic dendritic cells (spDCs) were isolated and cultured with or without GM-CSF for 48 hours. Bone marrow derived DCs (BMDCs) were generated in vitro with GM-CSF. Control and GM-CSF treated spDCs and BMDCs were co-cultured with CD4+ T-cells either from mTg primed mice in the presence of mTg (upper panel) or naïve mice without antigen (lower panel) and stained with FITC-labelled anti-CD4 and APC labelled anti-Foxp3 for FACS analysis. Each scatter plot is representative of five independent experiments.
Figure 2: CD4⁺ T-cells exhibit similar viability and Th1/Th2 cytokine profile in culture with splenic DCs and BMDCs: (a) Control and GM-CSF treated spDCs and BMDCs were co-cultured with CD4⁺ T-cells from naïve mice without antigen for 5 days and stained for the expression of CD4, Foxp3 and IL-17 by FACS. (b) Analysis of Th1 (IFNγ), Th2 (IL-4) cytokine expression in CD4⁺ T-cells (upper two panels) after co-culture with spDCs (cultured in the presence/absence of GM-CSF) and BMDCs. Analysis of CD4⁺ T-cell viability in the co-culture with PI and TMRM (lower two panels). Percentage of dead cells positive for PI staining is indicated. For TMRM staining total viable cells are indicated by the percentage of cells retaining the stain. Each scatter plot in all the experiments represents three separate analyses.
**2. GM-CSF does not act directly on Foxp3+ T-cells:**

In a recent study, it had been proposed that Tregs express a functional GM-CSF receptor alpha-chain CD116 and proliferate in response to GM-CSF (162). To see if the tolerogenic effect of GM-CSF was a consequence of its direct action on T cells, we set up naïve CD4+ T-cell cultures stained with CFSE with or without antigen presenting cells (splenocytes depleted of CD4+ T-cells served as APCs) and treated them with varying concentrations of GM-CSF ([Figure 3a](#)). In the absence of TCR stimulation and APCs, no increase in the CD4+ T-cell proliferation was noted ([Figure 3a](#)) after 5 days of culture. Although there was proliferation of both Foxp3+ and Foxp3- T cells in presence of anti-CD3 and splenic antigen presenting cells (APCs), ([Figure 3a, lower panel](#)), there was no significant difference in the percentages of Foxp3+ cells between GM-CSF treated and untreated cultures. When we co-cultured T cells with APCs in the absence of antigenic stimulus, or in the presence of anti-CD3 alone or anti-CD3 along with anti-CD28 ([Figure 3b](#)), we found no correlation between GM-CSF concentration and increase of Tregs *in-vitro*. These data supported the notion that GM-CSF does not directly cause selective expansion of Foxp3+ Tregs either in the presence or absence of APCs and/or TCR activation.
Figure 3: GM-CSF has no direct on Foxp3+ T-cells: CFSE labelled CD4+ T-cells were cultured in different concentrations of GM-CSF ranging from 0-2500 ng/ml in the presence and absence of (a) anti-CD3/APCs (b) anti-CD3/anti-CD28/APCs for 4 days and analyzed for Foxp3 expression by FACS. The numbers indicate the percentage of double positive (CD4+Foxp3+) T-cells. The experiment was repeated three times with similar results.
3. The capacity of CD11c⁺ BMDCs to increase Foxp3⁺ Tregs in T cell co-cultures is primarily contact dependent:

We wanted to test if the “tolerogenic phenotype” was a characteristic of all BM derived cells or that of GM-CSF-induced CD11c⁺ BMDCs alone. First, we followed the differentiation of BMDCs by scoring for the percentage of CD11c⁺ cells in BM cultures at days 2 (~5%), 4 (~12%), 7 (~51%) and 11 (~95%) (Figure 4a, upper panel). The CD11c⁺ BMDC population developed almost exclusively from the CD11b⁺ precursors and was found to be CD8α⁻ (Figure 4a, 4b-upper panel). Using BMDCs from each of these time points, we set up T cell co-cultures for 5 days without TCR stimulation and found a direct correlation between the percentages of Tregs and CD11c⁺ cells in BM cultures (Figure 4a, lower panel). Further, we separated CD11c⁺ cells from CD11c⁻ cells (Figure 4b, lower panel) and co-cultured them with naïve CD4⁺ T cells for 5 days without antigen. We found that co-cultures with GM-CSF derived CD11c⁺ BMDCs (G-BMDCs) resulted in an increase of Foxp3⁺ cells which was considerably more (17.6±1.0%) than that noted in the presence of GM-CSF derived CD11c⁻ cells (non-DCs, 10.7±0.6%, p=0.004) (Figure 4b, lower panel). These data suggested that although other bone marrow derived cells may have the capacity to expand/induce Tregs in co-cultures, CD11c⁺ BMDCs have a more potent ability to induce Tregs.

The BMDC induced increase in the percentage of Tregs could be contact dependent and/or cytokine driven. To address this question we set up co-cultures of BMDCs with naïve T cells either in direct contact or in transwells, where there is fluid exchange but no cell-to-cell contact. We found that the capacity of BMDCs to cause increase in Tregs without antigenic stimulation was contact dependent (12.6±0.7% Tregs) as BMDCs failed to exhibit this property when cultured in transwells (0.74±0.1% Tregs) (Figure 5, upper panel). However, when the T cells were stimulated with anti-CD3 in the presence of BMDCs in
transwell it was able to cause increase in Treg percentages (6.3±0.05% Tregs, p=0.002 vs. C-spDCs) (Figure 5, lower panel) although not to the same extent as BMDCs (10.10% Tregs) when in contact with T cells. This also suggested that cytokines secreted by these BMDCs could facilitate induction of Tregs upon TCR stimulation.
Figure 4: CD11c+ BMDCs increase the percentage Foxp3+ Tregs in co-cultures: (a) BM cells were analyzed for the expression of CD11b and CD11c on days 2, day 4, day 7 and day 11 (upper panel). BM cells obtained from the respective days were co-cultured with CD4+ T-cells and after five days the cells were analyzed for Foxp3 expression by FACS (lower panel). (b) CD11c and CD8α expression on cells from GM-CSF derived BMDCs (upper panel). CD11c+ and CD11c− cells from GM-CSF cultures were co-cultured with CD4+ cells from naïve mice and percentage of CD4+Foxp3+ T cells from the co-culture analyzed (lower panel). Experiments a and b were repeated at least three times with similar results.
Figure 5: CD11c⁺ BMDCs increase the percentage Foxp3⁺ Tregs in co-cultures primarily through a contact-dependent mechanism: Co-cultures of BMDCs with CD4⁺ T-cells either together or separated by transwell (TW) were analyzed for Foxp3 expression without anti-CD3 (upper panel) or with anti-CD3 (lower panel). In transwell co-cultures, CD4⁺ T cells were cultured in the bottom wells while the BMDCs were cultured in the top wells. Numbers indicate the percentage of double positive CD4⁺Foxp3⁺ T-cells. Experiment was repeated at least three times with similar results.
4. GM-CSF derived BMDCs can selectively expand Foxp3⁺ Tregs

To understand whether the increase in the percentage of Tregs in co-cultures of T cells with BMDC was due to an expansion of the pre-existing Foxp3⁺ Tregs or an adaptive conversion of Foxp3⁻ T cells to Foxp3⁺ cells, we stained total CD4⁺ T cells with CFSE and set up co cultures with BMDCs in the absence of antigenic stimulation. We found that in the presence of C-spDCs, neither the Foxp3⁺ nor the Foxp3⁻ population showed appreciable proliferation (less than 1%, Figure 6a). In contrast, when cultured with BMDCs, only the CD4⁻Foxp3⁺ population underwent robust expansion (11.4±0.7%, p=0.001). At least 7 divisions were observed during a 5 day culture (Figure 6a, lower panels). It is known that Tregs express the IL2-Rα (CD25) which is also a marker for T cell activation. Therefore, we asked if this selective expansion was dependent upon the state of activation of the T-cells. We found that a majority of the Foxp3⁺ Tregs after expansion was also CD25⁺ while only a small percentage of Foxp3⁻ cells were CD25⁺ (Figure 6b). We found that the Foxp3⁻ T-cells failed to proliferate irrespective of whether they were CD25⁺ or CD25⁻ (Figure 6b, upper panel). In contrast, both CD25⁻Foxp3⁺ and CD25⁻Foxp3⁺ cells proliferated (Fig 4B, middle and lower panels). However, we failed to observe any significant adaptive conversion of naïve or effector T cells to Tregs in BMDC co-cultures (Figure 7). From this we concluded that the ability of BMDCs to increase Tregs was primarily dependent on their ability to selectively expand pre-existing Foxp3⁺ T cells (natural Tregs) in a contact dependent manner.
Figure 6: BMDCs can directly and selectively expand nTregs in T-cell co-cultures: (a) spDCs and BMDCs were co-cultured with CFSE labelled CD4⁺ T-cells from naïve mice and analyzed for proliferation and Foxp3 expression. Small panels on the right show extent of CFSE dilution of Foxp3⁺ and Foxp3⁻ cells in the original histograms. (b) Extent of CFSE dilutions in different T cell sub-populations are measured by gating on Foxp3⁺ or CD25⁺ T-cells. Upper panel shows the position of the gate, middle panel shows the gated population and lower panel shows the extent of CFSE dilution of the gated population. Each scatter plot is representative of five separate experiments.
Figure 7: BMDCs do not cause induction of Foxp3+ Tregs in the absence of any TCR stimulation: CFSE labelled CD4^+CD25^- T-cells were co-cultured with control (spDCs) with or without BM culture supernatant (BM sup), BM cells (BM day 0) or BMDCs and analyzed for Foxp3 expression and CFSE dilution. Each scatter plot is representative of five separate experiments.
5. Expression of co-stimulatory molecules and cytokines by BMDCs and spDCs:

We characterized the BMDCs for their expression of co-stimulatory molecules and compared them with G-spDCs and C-spDCs to gain some insight into the basis for their increased tolerogenic phenotype (Figure 8). The percentage of cells amongst C-spDCs and G-spDCs expressing CD80, CD86, MHCII and PDL1 were comparable while G-spDCs showed a higher percentage of PDL2 expression relative to the C-spDCs. The BMDCs on the contrary had higher percentages of CD80, CD86 and lower percentage of PDL1 and PDL2 relative to G-spDCs. These data suggested that the differences in the expression of CD80/86 or PDL1/2 between G-spDCs and BMDCs could be contributing to the increased tolerogenic effect of BMDCs. Also we found reduced transcript levels of all tested pro-inflammatory cytokines (IL-1β, IL-12, TNF-α, IFN-γ and IL-6) in BMDCs as compared to C-spDCs or G-spDCs. Interestingly, the level of transcript for IDO, which has been implicated in Treg generation (163), was increased in G-spDCs, while it was lower in BMDCs. In contrast, we observed elevated levels of TGF-β transcripts in BMDCs.
Figure 8: Phenotypic and cytokine profile of BMDCs: (a) BMDCs and spleen derived DCs cultured in the presence or absence of GM-CSF were analyzed for the expression of CD80, CD86, MHC class-II, PDL1 and PDL2. Each scatter plot represents three independent experiments. (b) Reverse transcriptase PCR analysis of OX40L and PDL1 from BMDC and splenic DC cultures. The two bands in each category indicate the transcript levels at 31 and 33 PCR cycles. HPRT is shown as control.
6. Under antigenic stimulation supernatants from BMDC cultures can adaptively convert Foxp3⁺ T cells to Foxp3⁻ Tregs in a TGF-β dependent manner: To understand how BMDC-secreted cytokines could facilitate increase in Tregs upon anti-CD3 stimulation, we analyzed the transcript levels of various cytokines in day 7 BMDCs by semi-quantitative RT-PCR and compared them with C-spDCs and G-spDCs (Figure 9a). We found reduced transcript levels of all tested pro-inflammatory cytokines (IL-1β, IL-12, TNF-α, IFN-γ and IL-6) in BMDCs as compared to C-spDCs or G-spDCs. Interestingly, the level of transcript for IDO, which has been implicated in Treg generation (163), was increased in G-spDCs, while it was lower in BMDCs. In contrast, we observed elevated levels of TGF-β transcripts in BMDCs.

Since TGF-β has been shown to induce iTregs (34, 35), we wondered whether the TGF-β present in the BMDC-sup could be responsible for Treg differentiation and/or adaptive Treg generation in vitro. Therefore we labelled CD4⁺CD25⁻ cells from naive mice with CFSE and cultured them in the presence of BMDC-sup or TGF-β with and without TCR stimulation. In the absence of TCR stimulation (Figure 9b, top panel) neither BMDC-sup nor TGF-β caused any appreciable conversion of Foxp3⁻ T cells to Foxp3⁺ Tregs. However, upon anti-CD3 stimulation, there was significant adaptive conversion of Foxp3⁻ T cells to Foxp3⁺ Tregs in the presence of BMDC-sup (p<0.01) as well as TGF-β (p<0.01) (Figure 9b, bottom panel). In the presence of different concentrations of BMDC-sup and soluble anti-CD3 we found a dose dependent conversion of Foxp3⁻ cells to Foxp3⁺ Tregs (Fig 3C, upper panel). Further, in the presence of anti-CD3 and 4X BM sup, a TGF-β neutralizing antibody (anti-TGF-β) abrogated the adaptive conversion of Tregs not only at the recommended dose of 50 µg/ml but also when used at a lower concentration of 12.5 µg/ml (p<0.01 for both doses) without affecting the proliferation of Foxp3⁺ cells (Fig 3C, lower panel). A mouse IgG1 isotype control antibody failed to demonstrate this inhibition at a concentration of 20 µg/ml.
These data indicated that BMDC-sup mediated adaptive conversion of Foxp3⁻ T cells to Foxp3⁺ Tregs was primarily through TGF-β.

We found that the BM supernatant could also be used to adaptively convert mTg specific Tregs. When we cultured CD4⁺CD25⁻ T-cells isolated from mTg immunized mice in the presence of splenic APCs and mTg supplemented with 4X concentration of BM supernatant, about 1.5% Foxp3 cells were detected after 72 hours. Although the percentage of Foxp3⁺ T cells was very low, likely due to very low frequency of mTg specific T cells, nevertheless, these data suggest that it might be possible to induce antigen specific Tregs.
Figure 9: Contact independent induction of adaptive Tregs in vitro by BM supernatant is TGF-β dependent. (a) CFSE labelled CD4⁺CD25⁺ T-cell cultures supplemented with TGF-β or BM culture supernatant (1X) were stained with APC labelled anti-Foxp3 in the absence (upper panel) and presence (lower panel) of anti-CD3 (b) CFSE labelled CD4⁺CD25⁺ T cells were cultured with different concentrations of BM supernatant and anti-CD3. The induction of Foxp3⁺ in T-cells in the presence of increasing concentrations of BM supernatant (upper panel) and its inhibition by different concentrations of anti-TGF-β (lower panel) is shown. Experiments a and b were repeated three times with similar results.
Figure 10: BM supernatant can induce Tregs in T-cell isolated from mTg immunized mice: Induction of iTregs only from the culture of CD4⁺CD25⁻ T-cells isolated from mTg immunized mice supplemented with BM sup (4X) in the presence splenic APCs. Induction of Tregs is shown by the increase in percentage of Foxp3⁺ T-cells. Each scatter plot represents three separate analyses.
7. The contact mediated expansion of Foxp3+ cells in-vitro is OX40L dependent

We used blocking antibodies and inhibitors to determine the relative importance of some of these molecules in Treg expansion (Figure 11a). Our earlier study had shown a preferential ligation of CD80 resulted in IL-10 dependent Treg induction (164). Therefore, we blocked CD80 using an appropriate antibody and this blockage appeared to show only a partial abrogation of Treg expansion (from 14.10% to 9.25%). The PDL1 expression was high in CspDCs (95.1%) relative to BMDCs (64.6%) and therefore, we assumed that this molecule is unlikely to play a role in Treg induction by BMDCs. Although the percentage of cells expressing PDL2 was comparable in C-spDCs (39.0%) and BMDCs (42.2%), an earlier study had implicated PDL2 as a negative regulator of T-cell activation (165). Hence we used a blocking antibody to PDL2 to further investigate its role in Treg expansion. Both blocking PDL2 or addition of 1-methyl-Tryptophan (1-MT), a negative regulator of indole deoxygenase and hence tryptophan catabolism, failed to inhibit Treg induction. Since BMDCs secrete high levels of TGF-β, a cytokine implicated in the differentiation of activated T-cells into Foxp3+ Tregs, we also used a TGF-b blocking antibody to rule out any cytokine dependent effect. As expected, it did not abrogate BMDCs mediated Treg expansion.

Since BMDCs selectively expanded Tregs in a contact dependent manner, we asked whether it depended on the interaction of a Treg specific molecule with the corresponding ligand on DCs. One such receptor ligand pair is OX40/OX40L. Tregs constitutively express OX40 on their surface (166, 167) while the OX40 ligand (OX40L) is not constitutively expressed on DCs but can be induced (168). Therefore, we analyzed the expression of OX40L transcripts in BMDCs and spDCs (Figure 11b). We found that BMDCs expressed OX40L transcripts while spDCs did not. Direct staining of OX40L on the surface of spDCs and BMDCs (Figure 11c) confirmed that only a small fraction of spDCs expressed OX40L (4.5±0.7%) while a significantly higher percentage of BMDCs expressed OX40L (29.7±2.8%,...
p<0.01). Interestingly, the expression of OX40L in CD11c^- cells in the BM cultures was also negligible (2.5±0.2%).

Based on the above findings, we used a blocking antibody against OX40L at three different concentrations (lo=2.5 µg/ml; mid=5µg/ml; hi=10µg/ml) to see if it could abrogate Treg expansion by BMDCs. While the BMDC positive control predictably drove the expansion of nTregs in co-cultures (10.1±0.5% dividing cells as measured by CFSE dilution), when supplemented by the OX40L blocking antibody, the expansion was inhibited (lo=3.4±0.4%, med= 2.3±0.2%; hi=1.1±0.1%) in a dose dependent manner (p<0.01 in all cases) (Figure 12a) while leaving the percentage of non-dividing nTregs unaffected (4.3 to 5.9%). Further, when we added back an OX40 agonist at two different concentrations (lo=5 µg/ml; hi=10µg/ml) in combination with the OX40L blocking antibody, we observed significant reversal of the inhibition of Treg expansion (Figure 12b). While the anti-OX40L reduced the proliferation of Tregs from 11.0±0.5% in control to 2.1±0.1%, increasing concentrations of the OX40 agonist (OX86) revived the proliferation to 3.4±0.3% (lo, p<0.02 vs. anti-OX40L) to 8.2±0.4% (hi, p<0.001 vs. anti-OX40L). These data strongly indicated that OX40-OX40L signalling is required for the expansion of nTregs by BMDCs.
Figure 11: BMDC dependent Treg expansion is not mediated by PDL-2 and CD80:
(a) Co-culture of BMDCs and CD4⁺ T-cells in the presence of various blocking and neutralizing antibodies to anti-inflammatory cytokines or cell surface molecules were stained and analyzed for Foxp3 expression. (b) Reverse transcriptase PCR analysis of OX40L and PDL1 from BMDC and splenic DC cultures. The two bands in each category indicate the transcript levels at 31 and 33 PCR cycles. HPRT is shown as control. (c) Analysis of surface expression of OX40L in spDCs, CD11c⁺ and CD11c⁻ BMDCs. Each scatter plot represents five separate experiments.
Figure 12: The selective expansion of Tregs in BMDC co-cultures is OX40L dependent:
(a) Co-culture of BMDCs and CFSE labelled CD4+ T-cells in the presence of increasing concentrations of a neutralizing antibody to OX40 ligand were analyzed for CFSE dilution and Foxp3 expression in T-cells. (b) Co-cultures of BMDCs and CD4+ T-cells in the presence of either anti-OX40L antibody alone or in combination with OX40 agonist at two different concentrations (lo=5 μg/ml and hi=10 μg/ml). Each scatter plot represents five separate experiments.
8. Expansion of nTregs by BMDCs requires IL-2

Although we observed antigen independent but OX40-OX40L interaction dependent Treg expansion in BMDC co-cultures, we did not know if this interaction required TCR activation. Additionally, since Foxp3+ Tregs are known to be dependent on IL-2 for survival in vitro, we wanted to determine the source of IL-2. First, we found that the BMDC mediated Treg expansion in vitro could be abrogated by an anti-IL-2 antibody (Figure 13a) which indicated that IL-2 was required (p<0.01). We then proceeded to sort for CD4+CD25+ T-cells from naïve mice (which constituted the bulk of the natural Tregs) and set up co-cultures with BMDCs with and without IL-2 (Figure 13b). While the BMDCs predictably expanded Tregs in vitro from CD4+ cell co-cultures (8.9±0.8% divided cells vs. 6.3±1.1% undivided cells) as measured by CFSE dilution, they could not efficiently expand the sorted CD4+CD25+ subset (8.2±0.5% divided cells vs. 63.8±4.5% undivided cells). However, when IL-2 was added to the co-cultures, it not only increased proliferation of Tregs in CD4+ T-cell co-cultures (18.6±1.7% divided cells vs. 5.0±0.7% undivided cells; p<0.01 vs. splenic APCs with IL-2) but also restored efficient expansion of sorted CD4+CD25+ subsets (73.2±1.2% divided cells vs. 22.7±1.7% undivided cells; p<0.01 vs. splenic APCS with IL2). We concluded that the CD4+CD25+ T-cells were incapable of making IL-2 which is essential for their efficient expansion. The BMDCs were themselves not able to provide this required IL-2 as the CD4+CD25+ subset did not expand efficiently unless exogenous IL-2 was provided. However, in total CD4+ T-cell co-cultures, they were able to proliferate as the IL-2 was most likely produced by the CD4+CD25- T-cells. Interestingly, addition of exogenous IL-2 caused minor Treg expansion in splenic APC co-cultures with both CD4+ (2.4±0.1% divided cells vs. 9.0±0.8% undivided cells) and CD4-CD25+ T-cells (9.0±0.8% divided cells vs. 67.3±2.8% undivided cells). We observed some loss of Foxp3 expression in the CD4-CD25+ T-cell co-
cultures that were not supplemented with IL-2. These results showed that IL-2 was necessary for the maintenance of Foxp3^+ status and expansion of Treg cells \textit{in-vitro}.
Figure 13. BMDC mediated Tregs expansion is dependent on IL-2 but does not require TCR interaction: (a) Abrogation of Tregs proliferation by anti-IL-2: BMDCs were co-cultured with CD4+ cells without or with anti-IL-2. (b) Treg expansion is IL-2 dependent: BMDCs and control splenic APCs were co-cultured with total CD4+ (upper panel) and sorted CD4+CD25+ (lower panel) T-cells in the presence or absence of IL-2. On day 4 all the cultures were analyzed for the CFSE dilution of Foxp3 expressing T-cells by FACS. Experiments were repeated 3 times with similar results.
9. Expansion of nTreg by BMDCs is TCR independent, but requires OX40L stimulation

We wanted to investigate the role of TCR in this BMDC mediated Treg expansion. Since TCR interactions of CD4+ T-cells require presentation of antigen in the context of MHC class-II molecules, we decided to use BMDCs from MHC class-II deficient mice. GM-CSF cultured BMDCs from these mice were similar to BMDCs from CBA/J mice with respect to the expression of most surface molecules we tested (Figure 14a) except for MHC class-II which was not detected. Since the MHC class-II deficient mice were on C57/B6 background, at first, we set up co-cultures of wild type C57/B6 BMDCs with native CD4+ Tregs and found that they selectively expanded Tregs in vitro just as we saw in case of BMDCs from CBA/J mice (Figure 12b). We then proceeded to set up BMDC co-cultures either with total CD4+ T-cells or sorted CD4+CD25+ T-cells from naïve wild type C57/B6 mice. We found that MHC class-II-/- BMDCs failed to expand Tregs either in CD4+ T-cell (0.5±0.03% divided vs. 5.2±0.6% undivided) or CD4+CD25+ Treg co-cultures (6.5±0.3% divided cells vs. 62.4±1.7% undivided cells). However adding exogenous IL-2 restored Treg expansion in both cases (10.3±1.2% divided cells vs. 1.5±0.1% undivided cells in CD4+ co-cultures and 79.8±4.2% divided cells vs. 13.31.2% undivided cells in CD4+CD25+ T-cell co-cultures; p<0.01 vs. BMDC co-cultures without IL-2) (Figure 14b, upper panel). This indicated that the production of IL-2 by the CD4+CD25- cells required MHC class-II-TCR interaction, but the Treg expansion itself did not. Furthermore, addition of anti-OX40 ligand to the IL-2 supplemented cultures significantly reduced the proliferation of Tregs for both total CD4+ co-cultures (3.3±0.1% divided cells vs. 3.5±0.4% undivided cells; p<0.01 for dividing cells with respect to corresponding co-culture without antibody) and CD4+CD25+ T-cell co-cultures (32.3±1.6% divided cells vs. 52.2±2.1% undivided cells, p=0.001 for dividing cells w.r.t corresponding co-culture without antibody (Fig 14b, lower panel). In contrast, we found little
or no proliferation of Tregs when in culture with MHC class-II deficient splenic APCs. These data conclusively showed that the specific expansion of Tregs from a population of total CD4+$^*$ T-cells \textit{in vitro} by GM-CSF cultured BMDCs is independent of TCR/antigen presentation, but was dependent on the production of IL-2 by CD4$^+$CD25$^-$$^*$ T-cells present in the CD4 population which in turn required TCR/MHC class-II interaction.
Figure 14: OX40L dependent and TCR independent expansion of Tregs by BMDCs: 
(a) BMDCS from C57/B6 mice can also expand Tregs: Bone marrow derived DCs (BMDCs) from WT C57/B6 mice were used in co-cultures with CD4\(^+\) T-cells also derived from WT C57/B6 mice. Splenic APCs were used as negative control. (b) Treg expansion by BMDCs is TCR independent: BMDCs from MHC class-II\(^{-/}\) mice were generated in vitro with GM-CSF co-cultured with CFSE labelled CD4\(^+\) cells (upper panel) or CD4\(^+\)CD25\(^+\) cells (lower panel) in the presence or absence of IL-2. In some cultures, anti-OX40L antibody was added as indicated. Experiments were repeated 3 times with similar results.
10. The in vivo tolerogenic effect of GM-CSF is mediated by a special class of CD8α− DCs that differentiate from BM precursors

We have seen that BMDCs can expand Tregs in vitro. However we did not know if these tolerogenic DCs are mobilized in vivo upon GM-CSF treatment. Therefore, we treated mice with GM-CSF to see if it led to an increase in CD11c+ CD11b^Hi cells in the spleen (Figure 15a). Indeed, the percentage of CD11c+CD11b^Hi cells was much higher in GM-CSF treated mice (0.5±0.04%) than PBS treated controls (0.2±0.04%). When we gated on the double positive population we found that almost all of them (92-98%) were CD8α−. The percentage of Foxp3^+ T cells was also higher in GM-CSF treated mice (12.7±0.9%) than in PBS treated controls (8.9±0.8%, p< 0.01). These data suggested that GM-CSF most likely acted on BM precursors and mobilized the development of a class of DCs (CD11c^+CD11b^HiCD8α−), which then populated the lymphoid organs and might have contributed to the expansion of nTregs. Therefore, we wanted to see if the BMDCs could also expand Tregs in vivo.

We first immunized 3 groups of mice with mTg and confirmed mTg specific IgG response in the sera. 10 days after the last treatment these mice were adoptively transferred i.v. with either i.p.) PBS (buffer), ii) 1 x 10^6 purified CD11c^+ DCs from untreated mice (spDC) or iii) 1 x 10^6 purified CD11c^+ BMDCs. A total of 3 identical adoptive transfers, 5 days apart, were done for each group. Five days after the last transfer, mice were sacrificed and spleens were analyzed for T-reg percentages. Relative to buffer (10.4±0.1% Tregs) the spDC treatment did not lead to an increased percentage of Tregs (10.1±0.5% Tregs), while the BMDCs did (14.4±0.3%, p<0.01 against both groups) (Figure 15b).
Figure 15: GM-CSF treatment leads to the development of CD11b\(^+\)CD11c\(^+\) tolerogenic DCs \textit{in vivo}: (a) Mice were treated with GM-CSF for five consecutive days for two weeks and spleen cells were stained with FITC labelled anti CD11c, APC labelled anti CD11b, FITC labelled anti CD4, APC labelled anti Foxp3 and analyzed by FACS. The second panel indicates the percentage of double positive CD11b\(^+\)CD11c\(^+\) cells that are CD8\(\alpha\)-. The third panel indicates the percentage of CD4\(^+\)Foxp3\(^+\) T-cells from the control mice and GM-CSF treated mice. Each scatter plot represents five different experiments. (b) Bar graph indicates the percentage of Foxp3\(^+\) Tregs in mice immunized with antigen (mTg+CFA) followed by adoptive transfer of buffer, splenic DCs or BMDCs. Each column represents the mean ± SD of an experiment conducted with 3 animals in each group. *, Statistically significant value.
**B. OX40L and Jagged-1 expression on BMDCs is required for the expansion of Tregs and suppression of Experimental autoimmune thyroiditis.**

1. **OX40L alone is not sufficient for the in vitro expansion of Tregs by BMDCs**

In the first part of the study, we have demonstrated that *in vitro* expansion of Foxp3+ Tregs is predominantly mediated by OX40L+ DCs (Figure 12). In order to further confirm if OX40L is sufficient for the expansion of Tregs *in vitro*, we went a step ahead and set up co-cultures with sorted populations of OX40L+ and OX40L- BMDCs with naive CD4+ T-cells. As expected, only OX40L+ BMDCs drove the proliferation of Foxp3+ Tregs (10.1±0.6%) efficiently than OX40L- BMDCs (0.5±1%, p=0.002) (Figure 16). Also, supplementation of co-cultures of OX40L- BMDCs/splenic DCs and CD4+ T-cells with a functional OX40 agonist did not completely revive the proliferation of Foxp3+ Tregs (2±1%) proliferating Tregs) as compared to OX40L+ BMDCs (16±0.7%, p<0.001) (Figure 17). These results suggested to us that other factors in addition to OX40L expression on BMDCs may be involved in the *in vitro* expansion of nTregs by BMDCs. Thus, a second part of the study was initiated to study the role of other molecules that are involved in the proliferation of T-regs by BMDCs. Therefore, observations from our initial study suggested that although OX40L is required for the *in vitro* proliferation of nTregs by BMDCs, it was not sufficient.
Figure 16: BMDC ex-vivo expansion of nTregs is dependent on OX40L: Splenic dendritic cells (spDCs) and total or sorted OX40L+ and OX40L- Bone marrow derived DCs (BMDCs) were cocultured with naïve CFSE labelled CD4+ T-cells without exogenous antigen. After 5 days in coculture, T-cells were stained for FOXP3 and analyzed by FACS. Each scatter plot is representative of five independent experiments.
Figure 17: OX40L likely acts co-ordinately with a second membrane bound ligand on BMDCs: CFSE labelled CD4\(^+\) T-cells were cultured with control or sorted OX40L+ BMDCs (L+BMDCs), OX40L- BMDCS (L-BMDCs) treated BMDCs, supplemented in some cases with an OX40 agonist (+agonist low/high). Figure shows summary of cell proliferation data analyzed by FACS. The experiment was repeated three times with similar results. * statistically significant value.
Other BMDC specific molecule/s acting co-operatively with OX40L in Treg expansion is/are also membrane protein/s

The other molecule on BMDCs involved in the increased proliferation of Tregs could be cell surface molecule and/or a soluble mediator. To verify if BMDCs secreted a molecule that could co-operate with OX40L in Treg expansion we performed transwell experiments. We set up experiments, with splenic APCs and CD4+ T-cells along with OX40 agonist, with BMDCs in the other well. The transwell allowed free movement of soluble factors released by the BMDCs to reach the other chamber. There was no observed proliferation of Tregs (0.2±0.1%) compared to the positive control: co-culture of CD4+ T-cells and total BMDCs (10.3±0.7%) (Figure 18a). Also, it had been recently proposed by Ruby et al (45) that OX40 agonist can activate Foxp3+ T reg expansion only in the absence of certain cytokines such as IFN-γ and IL-4. In order to investigate if skewing of cytokines in the co-cultures caused any change in the proliferation of Tregs, we supplemented the BMDC, CD4+ T-cell co-culture with IFN-γ and IL-4 and saw that there was no negative impact on Treg proliferation (Figure 18b). Thus, our experiments suggested that BMDC specific soluble factors were not involved in the BMDC induced Treg expansion and that it may be contact dependent.

Our earlier study had shown a preferential ligation of CD80 resulted in IL-10 dependent Treg induction (164). In order to further delineate the role of CD80/86 co-stimulation, and as a further attempt to identify the other molecule/s involved in the proliferation of Foxp3+ Tregs by BMDCs, we used BMDCs from CD80 and CD86 knockout mice for co-culture with naive CD4+ T-cells. Upon co-culture, we observed that absence of either CD80 or CD86 on BMDCs did not affect the proliferation of Foxp3+ Tregs (7.3±1%) and was comparable to the frequency noted when wild type BMDCs were used (7.5%±0.6%) (Figure 19). These data strongly suggested that OX40L is necessary but may not be sufficient for the expansion of
nTregs by BMDCs and that other BMDC specific molecule/s co-operating with OX40L in Treg expansion is/are also surface bound.
Figure 18: OX40L likely acts co-ordinately with a second membrane bound ligand on BMDCs: (a) CD4+ T-cells cells from naïve mice were co-cultured with wild type BMDCs either together or in transwells, in some cases supplemented with splenic DCs, BM supernatant and an OX40 agonist. After 5 days in co-culture, T-cells were stained and analyzed by FACS. (b) Co-cultures of BMDCs with CFSE labelled CD4+ T-cells were supplemented with IL-4 or IFN-γ and analyzed by FACS. Experiment a and b were repeated at least three times with similar results.
Figure 19: *Ex vivo* Treg expansion by BMDCs is not mediated by CD80/86: BMDCs from CD80, CD86 and CD80/86 deficient mice were co-cultured with naïve CFSE labelled CD4$^+$ T-cells without exogenous antigen and analyzed by FACS (lower panel). Each scatter plot is representative of three experiments.
3. Role of Notch signalling in the in vitro proliferation of Foxp3+ Tregs by BMDCs

As per our results, the receptor for the ligand should be constitutively expressed on Tregs as BMDCs selectively expanded Tregs, and the ligand should be present on OX40L+ BMDCs since only OX40L+ BMDCs were able to expand Tregs. Since we had ruled out a role for some important co-signalling molecules such as CD80, CD86, PDL1, we decided to look for additional molecules that may be relevant to BMDC function. As discussed earlier, the role of Notch signalling in T cell regulation is becoming more apparent and it has been shown to activate a specific T-cell response based on the specific Notch ligand expressed on APCs. Notch activation is typically a twostep process: 1) upon ligand binding the intracellular portion of the Notch receptor is cleaved by a gamma secretase enzyme. 2) The intracellular portion of the Notch by itself acts as a transcription factor which then translocates to the nucleus and complexes with other transcription factors, which then initiate transcription. Jagged ligands have been shown to direct the naive T-cell response to a Th2 and Treg type and Delta like ligands towards a Th1 response (169). Further, it has been shown that APCs over-expressing Jagged1 and hematopoietic progenitors expressing Jagged-2 can activate Tregs (56, 57). Also, it has been shown by Elyaman et al that in an mouse model for EAE, administration of immunized mice with Jagged1Fc led to an increase in Ag specific IL-10 producing cells (170). Conversely, it has been shown that ligation of Jagged-1 expressed on Tregs to Notch1 receptors expressed on DCs can trigger the production of TGF-β, which in turn can render DCs to become tolerogenic leading to further expansion of Tregs (158). These and other studies showed the reciprocal regulation of DC and T cell function via Notch signalling. Owing to the involvement of Notch signalling in Treg activation and tolerance induction in autoimmune disease models, we wanted to investigate if Notch signalling had any role in the in vitro proliferation of nTregs by BMDCs. Another study that strongly
prompted us to consider the role of Notch signalling is by Anastasi et al, which showed that CD4+CD25+ T-regs constitutively expressed Notch3 receptor. Furthermore Bleck (48) et al recently demonstrated that up-regulation of OX40L and Jagged1 on human myeloid dendritic cells drive a Th2 type of response. This study also demonstrated that co-stimulation by two molecules is required for T-cell activation. Taking into consideration that Notch signalling can affect T-cell activation, Notch receptor is constitutively expressed on Tregs and a possible role of a Notch ligand Jagged1, with OX40L in T-cell activation, we decided to investigate the role of Notch signalling in the in vitro expansion of T-regs.

In order to verify if Notch signalling was involved in the in vitro expansion of Tregs by BMDCs, we used a gamma secretase inhibitor (GSI) in the BMDC/T-cell co-cultures. This inhibitor prevents the cleavage of all Notch receptors to their respective enzymatically active intracellular domains (NICD). Interestingly, blocking Notch signalling completely abrogated the proliferation of Tregs (1±0.1%) in the presence of BMDCs in a dose dependent manner compared to the positive control (8.1±0.2%, p<0.001) (Figure 20a). Furthermore, it had no effect on Tregs and did not alter cell survival, as assessed by PI staining of Tregs co-cultured with BMDCs (Figure 20b). These data suggested that Notch signalling played a part in BMDC mediated Treg expansion.
Figure 20: Notch signalling is critical in Treg expansion by BMDCs: (a). Co-cultures of BMDCs with CFSE labelled CD4+ T-cells were supplemented with Gamma-secretase-inhibitor (GSI), an inhibitor of Notch signalling, and analyzed by FACS. The data shows that proliferation of Tregs is inhibited by blocking Notch signalling. (b) Summary of FACS data from Propidium Iodide staining of co-cultures from GSI experiment showing cell necrosis across all co-cultures was at similar level.
4. Role of Notch ligand Jagged1 in the in vitro proliferation of Foxp3+ Tregs by BMDCs

We know that BMDCs can cause proliferation of Tregs, but spDCs supplemented with an OX40 agonist cannot. Thus, we speculated that BMDCs may express a critical Notch ligand not expressed by spDCs. Direct staining of Notch ligands revealed that only a smaller fraction of spDCs expressed the Notch ligand Jagged1 (2±0.5%), while a significantly higher percentage of BMDCs expressed it (20±2.8%, p<0.01) (Figure 21). In contrast, spDCs showed higher levels of expression of all other Notch ligands, except Jagged 1, than BMDCs. Thus we speculated that Jagged-1 may be the membrane bound factor co-operating with OX40L in causing Treg expansion.

Based on the differential expression of Notch ligand Jagged1 on BMDCs, we used a blocking antibody against Jagged1 (lo=10µg/ml; hi=20µg/ml) to see if it could abrogate the Treg expansion by BMDCs. Although the BMDC-positive control drove the expansion of nTregs (13±1%) in co-cultures, when Jagged1-blocking antibody was added, the expansion was inhibited (lo=9.9±0.5%, high=6.9±0.2%) in a dose dependent manner (p<0.01 in all cases), while leaving the percentage of non-dividing nTregs unaffected (Figure 22a). Blocking either OX40L (hi=3.7±0.2%) or Jagged1 (hi=5.3±0.3%) suppressed the proliferation of Tregs. Moreover, blocking both molecules simultaneously in the co-cultures significantly abrogated (=0.5±0.1%, p<0.001 Vs BMDC-T cell co-culture) the proliferation of Tregs by BMDCs (Figure 22b). These data indicated that Notch signalling induced by Jagged1, along with OX40L, may be required for the induction of proliferation of Tregs.
Figure 21: Notch ligand Jagged1 is expressed only on BMDCs: Phenotypic characterization of splenic DCs and BMDCs comparing the surface level expression of different Notch ligands using FACS Analysis. Numbers indicate the percentage of cells expressing the ligand. Experiment was repeated three times with similar results.
Figure 22: OX40L and Jagged-1 are required simultaneously for BMDC mediated Treg expansion: (a) Co-cultures of BMDCs with CFSE labelled CD4+ T-cells were supplemented with two concentrations of a Jagged-1 neutralizing antibody and analyzed by FACS. (b) Co-cultures of BMDCs with CFSE labelled CD4+ T-cells were supplemented with neutralizing antibodies to Jagged-1 and OX40L, either alone or in combination and analyzed by FACS. Experiments a and b were repeated three times with similar results.
5. OX40L and Jagged1 on BMDCs are required for the in vitro expansion of Tregs

Since we had noted that OX40L+ BMDCs had a superior ability to increase Tregs relative to OX40L- BMDCs (Figure 14), we wanted to investigate the relationship between surface expression of OX40L and Jagged1 on BMDCs. We analysed the surface expression of OX40L and Jagged1 by gating on positive and negative populations for each with respect to the other. Interestingly, BMDCs that were OX40L- were also Jagged1-. On the other hand, about half of OX40L+ BMDCs were Jagged1+ (53±0.5%, p<0.02) (Figure 23). In order to investigate if both ligands were required for the expansion of Tregs by BMDCs, we sorted BMDCs based on their OX40L and Jagged1 expression into two subpopulations: OX40L+Jagged1+ and OX40L+Jagged1- BMDCs and used in co-cultures with naive CD4+ T-cells. Interestingly, only OX40L+Jagged1+ BMDCs were able to efficiently expand Tregs (12.5±0.2%) compared to OX40L+Jagged1- BMDCs (1.4±0.1%, p<0.001) (Figure 24). Blocking of both ligands (Anti-OX40L=10g/ml, Anti-Jagged1=20µg/ml) on OX40L+Jagged1+ BMDCs abrogated the proliferation of Tregs (0.7±0.1%, p<0.01 vs. OX40L+Jagged1+ BMDCs).

To further substantiate the relative importance of these ligands, we performed SiRNA mediated knock down of Jagged1 (Figure 25a) on BMDCs and co-cultured with CD4+ T-cells. SiRNA mediated silencing of Jagged1 on BMDCs significantly reduced the expression of Jagged1, without altering the expression of OX40L (2.1±1%, p<0.01) (Figure 25a, lower panel). These DCs were used in co-culture with naive CFSE labelled CD4+ T-cells. After 5 days in co-culture, T-cells were stained and analyzed for proliferation of Foxp3+ Tregs (Figure 25b). The proliferation of Foxp3+ Tregs was significantly reduced when Jagged1 silenced BMDCs (1.47±0.5%) were used in the co-cultures compared to the normal
BMDCs (8.09±1%). Proliferation of Tregs was further reduced when OX40L blocking antibody (hi=10µg/ml) was used (0.2±0.1%) along with Jagged1 SiRNA treated BMDCs. Therefore, absence of either OX40L or Jagged1 significantly reduced the proliferation of Foxp3+ Tregs by BMDCs. These data suggested that signalling mediated by both OX40L and Jagged1 are required for the in vitro proliferation of Foxp3+Tregs by BMDCs.
Figure 23: Half of OX40L+ BMDCs are also Jagged1: BMDCs were analyzed for surface expression of OX40L and Jagged1. OX40L+ cells are gated and analyzed for Jagged-1 expression and vice versa using FACS analysis: Upper panel shows that about 50% of OX40L+ BMDCs (gated population) were Jagged1+, while OX40L- BMDCs were also Jagged1-. Lower panel shows that most of the Jagged1+ BMDCs (gated population) were also OX40L+ where as Jagged1- BMDCs were also OX40L-. 
Figure 24: OX40L and J-1 are required simultaneously for BMDC mediated Treg expansion: CFSE labelled CD4+ T-cells were co-cultured with either total or OX40L+Jagged-1+ or OX40L+Jagged-1- BMDCs. Some cultures were supplemented with anti-OX40L and anti-Jagged1 antibodies. After 5 days in co-culture, CD4+ T-cells were stained and analyzed for CFSE proliferation. Each scatter plot is representative of five separate experiments.
Figure 25: SiRNA mediated knock down of Jagged1 on BMDCs abrogates the proliferation of Tregs: (a) BMDCs treated with Jagged1 SiRNA were stained with Anti-Jagged1 and Anti-OX40L to confirm the knock down of Jagged1. Upper panel shows the expression of ligands on BMDCs not treated with the SiRNA. Lower panel shows the expression of ligands in SiRNA treated BMDCs. (b) Jagged1 SiRNA treated BMDCs were co-cultured with CFSE labelled CD4+ T-cells and analyzed by FACS at the end of five day culture. Numbers indicate the percentage of Foxp3+ T-cells.
6. BMDCs stimulate Treg expansion by activating Notch pathway

Notch signalling has been implicated in the differentiation of Naive CD4+ T-cell response to Th1, Th2 and Treg response. It had been previously shown that constitutive expression of activated form of Notch3 in mice had increased levels of CD4+CD25+ Tregs in the spleen and lymph nodes and are protected from the onset of Type 1 Diabetes in NOD mice (171). This study also showed that wild type naive CD4+CD25+ T-cells had enhanced expression of Notch3 compared to the CD4+CD25- T-cells. Similarly, Jagged-2 mediated activation of Notch3 by hematopoietic progenitors resulted in expansion of Tregs (56). Therefore, we speculated that Notch3 may be activated by Jagged1 signalling that in turn facilitated the expansion of Tregs by BMDCs. We analyzed the mRNA expression patterns of all four Notch receptors in Foxp3+ (Treg; GFP+) and Foxp3- (Teff; GFP-) cells from Foxp3-GFP mice. Our RT-PCR results showed that while Notch1 and Notch4 were similarly expressed in Teffs and Tregs, Notch2 was predominantly expressed in Teffs while Notch3 was predominantly expressed in Tregs (Figure 26). Thus, like OX40, we found Notch3 was preferentially expressed on Tregs making it likely that it was the critical Notch receptor involved in Treg expansion by BMDCs.
Figure 26: Notch3 mRNA transcripts are specifically expressed on Tregs: GFP+ and GFP- cells isolated from Foxp3-GFP mice were analyzed for expression of Notch receptors by RT-PCR. Each scatter plot represents three independent experiments.
7. BMDCs stimulate Treg expansion by activating Notch3 pathway

Since BMDC selectively expanded Tregs, it is possible that a Treg specific molecule such as Notch3 interacted with the corresponding Notch ligand Jagged1 on DCs. First, we wanted to investigate if there was activation of Notch receptors upon stimulation with BMDCs. Activation of Notch occurs with the gamma secretase specific cleavage of the Notch Intracellular Domain (NICD). FACS staining for different Notch receptor specific NICDs revealed that while Notch1 specific NICD was not detected (Figure 27a) Notch3 specific NICD was present in the Tregs only in BMDC-CD4+ T-cell co-culture but not in cultures where they were supplemented with Anti-Jagged1 blocking antibody (Figure 27b upper panel). We further gated on non-proliferating and proliferating Tregs in the co-cultures based on their CFSE dilution and found that the presence of Notch3 specific NICD occurred only in proliferating Tregs. This demonstrated that Notch3 activation correlated with Treg proliferation. To link this correlation with causation, we added blocking antibodies to Notch3 and Jagged1 to the BMDC-T cell co-culture in our subsequent experiments to examine if proliferation of Tregs is a direct consequence of Jagged-1-Notch3 interaction. Blocking Jagged1 had abrogated the proliferation of Tregs earlier (Figure 22). Blocking Notch 3 signaling also inhibited the proliferation of Tregs (hi=1.97±0.2%, lo=3.43±0.1%, p<0.02) by BMDCs in a dose dependent manner (Figure 28). These data suggested that Notch3, selectively expressed in Tregs, is activated by Jagged1 expressed by BMDCs and was required for the proliferation of Tregs. Collectively, our data suggested that OX40L and Jagged1 on BMDCs initiate simultaneous co-signalling through OX40 and Notch3 on Tregs leading to their proliferation.
Figure 27: BMDCs activate Notch3 in Tregs: Co-culture of spDCs and BMDCs with CD4⁺ T-cells analyzed for activation of Notch1 and Notch3. (a) Analysis of Notch1 specific NICD in Foxp3⁺ T-cells in BMDC/T-cell co-cultures analyzed by FACS. (b) Analysis of Notch 3 specific NICD in Foxp3⁺ T-cells (upper panel). CFSE dilution is used as measure of cell-proliferation. lower panel: Cells are gated on diluted CFSE or undiluted populations and analyzed for Notch3 specific NICD.
Figure 28: Blocking Notch3 signalling abrogates the proliferation of Foxp3+ Tregs: Co-culture of BMDCs and CD4+ T-cells in the presence of neutralizing antibodies to Notch3 analyzed for proliferation of T-cells at the end of 5 day co-culture using FACS analysis. Each scatter plot represents three separate experiments.
C. OX40L+Jagged1+ BMDCs can suppress EAT

1. Ex vivo expanded nTregs by OX40L+Jagged1+ BMDCs can suppress effector T-cell proliferation

**T-cell proliferation in vitro**

*Ex vivo* generated Tregs through BMDC mediated signalling are likely polyclonal with respect to TCR specificity. This brings to question their capacity to suppress antigen specific T-effector proliferation. If natural Tregs (nTregs) are self antigen specific, then these Tregs may suppress autoantigen specific proliferation of Teff while being ineffective against proliferation of T-eff specific to foreign antigens. Alternatively, they may broadly suppress Teff proliferation through bystander suppression mediated likely through cytokines. In order to investigate if the nTregs expanded *ex vivo* by OX40L+Jagged1+ BMDCs could suppress effector T-cell proliferation from both self and non-self antigens we immunized mice with mouse thyroglobulin (mTg), a self antigen, and chicken ovalbumin (OVA), a non-self antigen. Mice were immunized with 100 µg mTg/OVA to induce an antigen specific effector T cell response, which we monitored through the emergence of serum antibodies to mTg/OVA. Total T-cells from naïve mice were used to set up BMDC/T-cell co-cultures. In the absence of TCR stimulation, the expanded nTregs were a major fraction of the CD25+ T-cells and were therefore isolated on the basis of CD25 expression. We then isolated CD4+CD25- T cells from the above mentioned immunized animals, stained them with CFSE and set up co-cultures with splenic APCs in the presence of mTg/OVA with or without sorted nTregs (CD4+CD25+). CD25- cells from OVA treated mice and mTg treated mice proliferated only in the presence of OVA and mTg respectively. The OVA induced proliferation was much more robust as compared to mTg induced proliferation as expected. However, both mTg and OVA-induced proliferation was suppressed when CD25+ nTregs were added at either 1:1, 1:2, 1:4 ratio of Tregs:Teffs. **(Figure 29)**. Thus, the *ex vivo* expanded Tregs appear to be equally potent in suppressing self and non-self antigen specific proliferation.
Figure 29. In vitro generated Tregs suppress effector T-cell proliferation: CD4+CD25+ T-cells were sorted from the co-culture of OX40L+Jagged1+ BMDCs and T-cells. The sorted Tregs were set up in co-culture with CFSE labelled effector T-cells isolated from OVA and mTg immunized mice at different ratios. After 5 days in culture, CD4+T-cells were analyzed for CFSE proliferation using FACS.
2. Adoptive transfer of OX40L+BMDCs in to immunized mice can lead to increased Foxp3+Tregs:

We know that approximately 50% of the OX40L+ BMDCs are also Jagged1+. Our in vitro data also indicated that only OX40L+Jagged1+ BMDCs, and not OX40L+Jagged1- or OX40L-Jagged1- BMDCs, have the capacity to expand Tregs ex vivo (Figure 24). We wanted to investigate if this specific subset of DCs can also expand Tregs in vivo. Furthermore, we wanted to confirm if this capacity for expanding Tregs can be used therapeutically to suppress Experimental Autoimmune Thyroiditis (EAT). To verify this, we adoptively transferred OX40L+ Jagged1+ BMDCs (L+J1+BMDCs) and OX40L+Jagged1- (L+J1-BMDCs) into mice immunized with mTg to induce EAT (5 mice in each adoptive transfer group) on days 1 and 10. About 2 million OX40+Jagged1+ and OX40L+Jagged1- BMDCs were adoptively transferred to each mouse in the respective groups on days 17 and 22. Mice were sacrificed on day 35 and analysed for Foxp3+Tregs in the spleen and lymph nodes. For cytokine analyses, CD4+ T-cells isolated from different groups were restimulated with APCs in the presence of mTg for 3 days. After 3 days in culture T-cells were intra cellular stained for different cytokines and analysed by FACS. Immunized mice that received OX40L+ BMDCs had increased percentages of Foxp3+ Tregs (15±0.5%) compared to the immunized mice (9.2±1%) and mice that received OX40L+Jagged1- BMDCs (9±0.5%, p<0.01 Vs OX40L+ BMDCs in all cases) (Figure 30).
**Figure 30: Immunized mice treated with OX40L+ BMDCs show increased Tregs**:

Three groups of BMDCs were used viz. L+BMDCs (also Jagged1+), OX40L+ Jagged1- BMDCs for treating mTg immunized mice. Mice were sacrificed and isolated T-cells from spleen were analyzed for Foxp3+ Treg percentages by FACs. Results are representative of three separate experiments.
3. Adoptive transfer of OX40L+BMDCs into immunized mice leads to increased IL10+, IL-4+ CD4+ T-cells in spleen and lymph nodes:

The splenocytes and thyroid draining lymph nodes from various recipient groups of mice were also analysed for other tolerance markers like IL-10 secretion. Immunized mice that were treated with OX40L+Jagged1+ BMDCs showed decreases in the percentage of Th1 specific pro-inflammatory cytokine (IFNγ, IL-12) expressing T-cells and increase in IL-10 (0.8±0.1%) and IL-4 (1.8±0.2%) expressing CD4+ T-cells (Figure 31) in the spleen. The cytokine profile of T-cells in the lymph nodes following immunization and adoptive transfer showed that mice that received OX40L+Jagged1+ BMDCs showed higher percentage of IL-4 (1.9±0.3%) and IL-10+ CD4+T-cells (5.8±0.1%) (Figure 32). This is a very interesting observation as T-cells in co-culture with BMDCs in vitro do not show skewing towards Th2 type cytokine profile in the absence of antigenic stimulation (Figure 2). Moreover, in two separate studies it had been shown that Jagged1 can stimulate IL-4 and IL-10 production in T-cells (161, 170). Owing to the history of the role of OX40L in T-effector activation in disease conditions (45), and as only the immunized mice that received OX40L+ Jagged1+ BMDCs had shown increased IL-4+IL-10+ T-cells, it is possible that under antigenic stimulation, Jagged1 may be playing two important roles: driving proliferation of Tregs and activating IL-10 production in T-cells; thereby maintaining an anti-inflammatory environment enabling OX40L to expand Tregs and thereby conferring protection against EAT. However, the role of Jagged1 in the activation of IL-10+IL-4+ T-cells needs further investigation.
Figure 31: Immunized mice treated with OX40L+Jagged1+ BMDCs show increased expression of IL-4, IL-10+ T-cells in spleen: Bar graphs showing percentage of IL-12;IFNγ (upper panel), IL-4+;IL-10 producing T-cells (lower panel) in the spleen of differently treated mice analyzed by FACS. Bar graphs show percentage of IL-4 (left) and IL-10 (right) producing cells in the spleen of differently treated mice analyzed by FACS. (**P<0.04 versus immunized control mice).
Figure 32: Immunized mice treated with OX40L+ BMDCs show increased expression of IL-4, IL-10+ T-cells in spleen: Bar graphs showing percentage of IL-12;IFN-γ (upper panel), IL-4;IL-10 producing T-cells (lower panel) in the draining lymph nodes of differently treated mice analyzed by FACS. (***P<0.01).
4. mTg immunized mice treated with OX40L+ BMDCs show decreased lymphocytic infiltration in thyroid lymph nodes.

The pathology of EAT has been established before (16). H&E staining of thyroid sections routinely reveal extensive infiltration of follicles by lymphocytes. The degree of tissue infiltration by lymphocytes can be used to numerically score for disease severity. To analyze if adoptive transfer of OX40L+Jagged1+ BMDCs could prevent thyroid infiltration by T-effectors, immunized mice that were treated with OX40L+Jagged1+ BMDCs and OX40L+ Jagged1- BMDCs were sacrificed on day 35 to isolate thyroids. The cross sections of thyroids were stained with hematoxylin and eosin to evaluate the lymphocytic infiltration. Mice that received OX40L+Jagged1+ BMDCs had reduced infiltration of lymphocytes compared to the group of mice treated with OX40L+Jagged1- BMDCs or immunized control mice (p=0.02 in both cases, Figure 33). Our results showed that OX40L+Jagged1+ BMDCs can suppress EAT.
Figure 33: mTg immunized mice treated with OX40L+ BMDCs show reduced infiltration of lymphocytes: H&E stained sections of thyroid tissue from treated mice showing extent of tissue infiltration by lymphocytes. Results shown are representative of three independent experiments. Bottom bar graph panel shows the severity of the disease in different groups of mice.
V. GENERAL DISCUSSION

T-lymphocytes play a key role in the initiation and regulation of adaptive immune responses to native and foreign antigens. T-cell activation is achieved by a two signal mechanism: signal one comes from the interaction of MHC bound antigenic peptides on APCs with T cells bearing the cognate TCR, and signal 2 is mediated through the interaction of co-stimulatory ligands expressed on APCs with their cognate receptors on T-cells (e.g. CD80/86 expressed on APCs interact with CD28 expressed on T cells) (172). Activation leads to clonal expansion and proliferation of T-cells. Lack of co-stimulation either leads to T-cell anergy or suboptimal response. Interestingly the co-stimulation is not only required for activation of T cells but certain other co-stimulatory molecules are involved in down modulating T-cell responses (e.g. interaction of CD80/86 on APCs with CTLA4 expressed on T cells). Therefore, depending upon the nature of co-stimulation the T cell responses can be either up-regulated or down-regulated. The best characterized and perhaps the most critical co-stimulatory pathways are between CD28 and CD80/CD86 and between CD40 and CD154, members of the TNF:TNF receptor family. Autoimmune diseases are usually caused by many factors such as genetic susceptibility, environmental factors and deregulation of immune response. However, it is now well established that immune response to self-antigens does not occur or occurs weakly in the absence of appropriate co-stimulation. Therefore, significant efforts over the years have been targeted at manipulating co-stimulatory signalling to suppress or treat autoimmune diseases. While the potential involvement of new molecules in autoimmune diseases are being discovered, role of CD28, CTLA4, OX40 (CD134), program death-1(PD-1), inducible co-stimulator (ICOS), CD40L are fairly well understood. Therefore, these pathways are now being targeted to inhibit the progression of autoimmune conditions or to treat tumours by enhancing T-cell activation. Signalling mediated by CD28, ICOS, OX40 have been implicated in the activation of T-cell response. While ICOS has been
shown to be important for memory T-cell activation, CD28 is required for naive T-cell activation (173-175). The role of OX40 is still quite controversial; some of the earlier studies (176) demonstrated that OX40 is essential for naive T-cell activation. However, recent investigations revealed that it is required for Treg expansion and function in various autoimmune models (46). CTLA4 and PD-1 are considered inhibitory T cell co-stimulator molecules. Soluble CTLA-Ig has been commonly used to block /disrupt B7 pathway and promote B7/CTLA4 interaction. Therapy mediated by PD-1 has been achieved by its natural ligand PDL1 (145, 149). It is now believed that targeting co-stimulation for treating autoimmune conditions preserves the normal repertoire of T-cells, while suppressing autoreactive T-cell response.

Recently, the role of Notch ligands in Treg activation has been an emerging field of investigation. Notch signalling is evolutionarily well conserved and Notch signalling has been shown to be important in determining cell fate decisions (53). APCs over expressing Jagged1, can inhibit antigen specific T-cell immunity and induce mucosal tolerance. Similarly, over expression of Jagged1 in human B-cells induced Tregs as did constitutive expression of active Notch receptor Notch3 in transgenic mice (171). It has also been shown that Jagged1 and Delta1 differentially regulate T-cell activation in an animal model of EAE (170). Expression of a specific Notch ligand on APCs has been attributed to a specific T-cell response. Therefore, Notch signalling can be exploited to tweak the immune response in autoimmune models and activation of T-cell response in tumour models.

The above discussion clearly illustrates the complex signalling that takes place between APCs and T cells and how specific interactions can either lead to immune suppression or autoimmune pathology. Since it is hard to individually manipulate one signal at a time and obtain the desired effect, some investigators have used different growth factors to manipulate DCs. Although fully matured DCs can elicit a robust immune response
including pathogenic immune responses against self-antigens, antigen presentation by immature DCs can cause anergy. In contrast to these two types of DCs, the semi-mature DCs can induce tolerance. This approach to modulating immune response may be more effective because it affects the earliest steps of the immune response and therefore likely to impact upon all subsequent steps. Over the years agents that modify or alter the maturation process of DCs and thus the expression of co-stimulatory molecules on DCs have been widely used to suppress autoimmune conditions. For instance, recently it was demonstrated that G-CSF derived hematopoietic progenitors expressing Notch ligand Jagged-1 can prevent progression of spontaneous Diabetes (56). Also, FLT3L derived BMDCs could expand nTregs in a contact dependent mechanism and prevented death in mice by Graft versus host disease (GVHD) (177). Similarly, a recent study by Yangliang et al demonstrated the selective role of PDL2 on mesenteric lymph node DCs in oral tolerance (165).

We have successfully used GM-CSF for the treatment of a range of autoimmune conditions in mice including EAT (16), experimental autoimmune myasthenia gravis (EAMG) (17) and type-1 diabetes (18) involving CBA/J, C57/B6 and NOD strains of mice respectively. In all cases we have observed a significant increase in splenic Treg population. Results from adaptive transfer of DCs from, and naïve T cells from WT mice into, GM-CSF treated and untreated scid mice led us to conclude that the increase in Tregs was mediated through CD8α⁺ DCs from GM-CSF treated, and not untreated, mice (20). Furthermore, we found that GM-CSF derived BMDCs were efficient in expanding Tregs in vitro in the absence of any antigenic stimulation. Therefore, we hypothesized that GM-CSF has its effect primarily on BM cells and that GM-CSF derived BMDCs are more effective in increasing Tregs in a TCR independent manner.

In the first part of the study, we have shown that GM-CSF effect was primarily on BM cells which then migrated to the spleen. Moreover, GM-CSF derive BMDCs were
more effective in increasing the proportion of Tregs in DC/T-cell co-cultures in an OX40L dependent manner independent of any TCR stimulation. Results from our transwell experiments exposed two distinct mechanisms of action of BMDCs that cause Treg expansion (Figure 5). The supernatant from BM cultures alone could induce Tregs in a cytokine dependent manner, but only upon TCR stimulation (Figure 9). This effect was primarily mediated through the enhanced production of TGF-β by BMDCs relative to spDCs. It has been shown that Foxp3⁺ Tregs and IL-17⁺ TH17 type T-cells arise from reciprocal developmental pathways in both of which TGF-β plays a critical role (178). However, in the co-presence of IL-6 the activated T-cells differentiate into TH-17 cells. Our RT-PCR results clearly showed that BMDCs produced far less IL-6 than spDCs (Figure 8). Thus a combination of high TGF-β and lower IL-6 production by BMDCs likely favoured the differentiation of TCR stimulated CD4⁺ cells into Foxp3⁺ adaptive iTregs. The reason for the comparatively lower percentage of Foxp3⁺ Tregs in CD3 stimulated co-cultures than antigen-free cultures is difficult to speculate. It is however possible that upon strong TCR stimulation, T-effectors proliferated more robustly than Tregs, thereby contributing to lower Treg percentages.

We have ruled the involvement of CD80, PDL2 co-stimulatory molecules and any soluble mediators such as cytokines in the in the expansion of Tregs by BMDCs (Figure 9). BMDCs caused a significant increase in Foxp3⁺ nTregs in co-cultures, primarily through proliferation, by a contact dependent mechanism that did not require TCR stimulation. Based on these findings, we speculated that a ligand expressed on the BMDCs is binding to its cognate receptor on Tregs and causing T cell signalling. Therefore, we investigated the role of OX40 which is constitutively expressed on Tregs. Both OX40 and OX40L are members of the tumor necrosis factor superfamily with co-stimulatory function (150, 179). Signalling through OX40 can promote survival and expansion of autoreactive effector T-cells, and blocking
OX40-OX40L pathway has been shown to diminish experimental autoimmune diseases including EAE (151), inflammatory bowel disease (152) and type-1 diabetes (153). In contrast, in our current study, addition of increasing concentrations of neutralizing antibody to OX40L in BMDC/T-cell co-cultures correlated with decreased proliferation of Tregs. The apparent contradiction between the earlier studies and our current findings is most likely due to experimental context. For example, in an interesting study Ruby et al (45) suggested that signalling through OX40 can result in divergent outcomes depending upon the overall signalling context including the cytokine milieu. Our observation is consistent with this notion because OX40 stimulation, in the absence of cytokines like IL-6 and IFN-γ likely resulted in the expansion of Tregs. However, we believe that the role of some of these cytokines is secondary to the contact dependent mechanism as BMDCs were capable of Treg expansion even in the presence of blocking antibodies to TGF-β and IL-6 (Figure 11). Interestingly, the expression of OX40 ligand transcript only in BMDCs, and not in C-spDCs, as indicated by RT-PCR (Figure 11), may partly explain why the nTreg expansion did not occur with C-spDCs.

We found that BMDCs could not expand sorted CD4+CD25+ Tregs as efficiently as they could in total CD4+ co-cultures, but could do so upon IL-2 addition (Figure 13). This indicated that the required IL-2 was being produced by the CD4+CD25- T-cells in the total CD4+ co-cultures. Although IL-2 was necessary for Treg expansion, it was not sufficient as spleen derived APCs failed to efficiently expand Tregs either from total CD4+ T-cell co-cultures or isolated CD4+CD25+ T-cell co-cultures even in the presence of IL-2 (Figure 13). Using BMDCs from MHC class-II−/− mice in similar co-cultures (Figure 14), we found evidence for TCR requirement as the Treg specific expansion failed to occur even in total CD4+ co-cultures. However, addition of exogenous IL-2 restored the expansion to WT levels showing that the involvement of TCR was most likely required only for the production of IL-
2. Additionally, this expansion in the presence of IL-2 was dramatically reduced by the anti-OX40L antibody, showing that IL-2 was required for the maintenance of the Tregs while the expansion was likely a TCR independent phenomenon that required OX40-OX40L interaction. Taken together, these data suggested that the specific expansion of Tregs in CD4^{+} co-cultures involves two separate mechanisms; the MHC class-II-TCR interaction (between BMDCs and CD4^{+}CD25^{-} cells) that results in IL-2 production, and expansion of the CD4^{+}CD25^{+} Tregs that occurs through a TCR independent interaction which, among other things, involves OX40-OX40L ligation. Our observation is very similar to a recent study which showed that BMDCs developed in vitro with Flt3L can expand Tregs in vitro and in vivo in an IL-2 dependent but TCR independent manner (177).

Although OX40L appears to be essential for the BMDC mediated Treg expansion in vitro, we do not think it is sufficient, as addition of a functional OX40 agonist to co-cultures of spleen derived APCs and T-cells did not lead to Treg expansion. It is possible that in addition to the OX40-OX40L interaction, the Treg expansion may require other interactions as part of a more complex immunological synapse. It is of particular interest because the study by Ruby et al demonstrates that under antigenic stimulation, OX40 agonist exacerbates the disease. Therefore we wanted to explore the involvement of other molecule/s involved in the expansion of nTregs by BMDCs as it may provide us a better understanding of the nTreg homeostasis and the therapeutic efficacy of BMDCs in suppressing autoimmune thyroiditis. Therefore, apart from OX40, we investigated the role of Notch3 shown to be expressed by Tregs alone (171).

Role of Notch signalling has been shown to be important for haematopoiesis and cell fate determinations. As antigen presenting cells (APCs) over-expressing Jagged-1 and hematopoietic progenitors expressing Jagged-2 can activate Tregs (56, 57), a role for Notch signaling in BMDC mediated Treg expansion became apparent. Notch receptors, encoded by
genes Notch 1-4 and five surface bound ligands encoded by Jagged1 and Jagged2, and Delta-like1, 3 and 4 (Dll1, Dll2 and Dll3) have been described (49-52). Ligand binding to Notch results in the activation of presenilin associated gamma secretase, which cleaves Notch and releases its intracellular domain (NICD). The NICD then translocates to the nucleus and forms a heterodimeric complex with various co-activator molecules and acts as a transcriptional activator (156). The type of cell and the specific ligand/receptor mediated signals regulate the final outcome (53, 180). Although Jagged family Notch ligands have been shown to enhance Tregs (56, 57), specific role for a particular Notch mediated signaling in Treg expansion, in the absence of canonical TCR mediated signaling, has not yet been established. Although much is known about TCR mediated T cell activation and proliferation (181), signaling required for Treg proliferation in the absence of TCR stimulation remains unknown. Using BMDCs from MHC class-II deficient mice we have shown that OX40L mediated ex vivo expansion of Tregs did not require TCR stimulation per se although it was critically depended on exogenous IL-2. Thus, the mechanism of BMDC induced expansion of Tregs is unique and needs to be elucidated.

We hypothesize that the OX40L/OX40 interaction is required for Treg expansion and constitutes only one of the signals and the other molecule involved is a Notch ligand. To verify if Notch signaling is indeed involved in Treg proliferation, we blocked Notch signaling using a gamma secretase inhibitor (GSI) and found that in the presence of GSI, the expansion of CD4+Foxp3+ T-cells was blocked (Figure 20). This suggested a role for Notch signaling in BMDC mediated Treg expansion. More specifically we found that BMDCs had higher levels of expression of Notch ligand Jagged1 and blocking the ligand in co-cultures abrogated the proliferation of Tregs by BMDCs (Figures 21 and 22). Since we had noted that OX40L+ BMDCs had a superior ability to increase Tregs relative to OX40L- BMDCs (Figure 16), we wanted to investigate the relationship between OX40L and Jag-1. Interestingly, the BMDCs
that were OX40L- were also Jagged-1-. On the other hand, about half of OX40L+ BMDCs were Jag-1+ (Figure 21). Additionally, we found that only the OX40L+Jagged-1+, and not the OX40L+Jagged1-, BMDCs were capable of inducing significant Treg expansion (Figure 24). Collectively, our studies suggested that OX40L and Jagged-1 are essential for BMDC mediated Treg expansion.

Upon ligand binding, Notch receptors undergo two proteolytic cleavages. The first cleavage catalysed by ADAM-family metalloproteases is followed by the gamma secretase mediated release of Notch intracellular domain (NICD) which then translocates to the nucleus to promote transcription. Using FACS staining we detected Notch3 specific NICD in the proliferating Tregs, while Notch1 specific NICDs were not detected (Figure 24). Notch3 mediated signalling has been reported to sustain regulatory phenotype on Tregs (182). Constitutive activation of Notch3 signalling in the thymocytes and T cells from N3-tg (transgenic mice expressing Notch3 intracellular domain) mice are associated with expansion of peripheral CD4+CD25+ cells (183). Similarly, Jagged-2 mediated activation of Notch3 by hematopoietic progenitors resulted in expansion of Tregs (56). Therefore, we speculated that Jagged-1 expressed on BMDCs likely activates Notch3 expressed on Tregs and contribute to Treg expansion. Our subsequent experiments have shown that blocking/knock down of Jagged-1 on BMDCs specifically inhibits the activation of Notch3 on T-cells (Figure 27).

Our in vitro data indicate that only OX40L+Jag-1+ BMDCs, and not OX40L+Jag-1- or OX40L-Jag-1- BMDCs, have the capacity to expand Tregs. In order to test the therapeutic efficacy of a specific subset of BMDCs, we adoptively transferred the OX40L+ BMDCs and OX40L+Jagged-1+ BMDCs into mTg immunized mice. Mice that received OX40L+ BMDCs had increased percentage of FOXP3+ T-regs compared to mice treated with OX40L-BMDCs and OX40L+Jagged-1- BMDCs (Figure 30). Also, mice that received OX40L+BMDCs showed increased levels of IL-10+CD4+ T-cells and decreased percentage of IFN-γ+CD4+ T-
cells in their lymph nodes. The OX40L+BMDCs also suppressed EAT as indicated by little or no thyroid infiltration of lymphocytes compared to mice treated with OX40L-BMDCs and OX40L+Jagged-1- BMDCs. Therefore, treatment of mice with OX40L+ BMDCs led to suppression of ongoing EAT likely through the expansion of Tregs and increase in percentage of IL-10+ CD4+ T-cells. This observation is in accordance with our initial finding that the protective effect of GM-CSF may be mediated by skewing the immune response towards a Th2 type of T-cell response and by activating IL-10+CD4+Foxp3+T-regs. Therefore, since GM-CSF primary acts on BM precursors it is likely that GM-CSF mobilized OX40L+Jagged-1+ BMDCs. These DCs caused expansion of Tregs and activation of IL-10 production in T-cells, thereby suppressing the autoimmune thyroiditis.

Although GM-CSF has been identified as a DC growth factor in vitro and DCs have been routinely enriched from mouse bone marrow cultures using GM-CSF (184, 185). GM-CSF null and GM-CSF-receptor null mice have normal levels of DCs indicating that GM-CSF may not be essential for DC development per se (186, 187). In contrast, mice lacking Fms-like tyrosine kinase 3 ligand (Flt3L) have impaired DC development (188). Therefore, it has been proposed that while Flt3L is physiologically essential for the steady state DC development from precursors, the GM-CSF may control the development of monocyte-derived ‘inflammatory’ DCs characterized by an intermediate level of expression of CD11c, high expression of CD11b, surface MAC3 expression (a glycoprotein also found on activated macrophages) and the absence of CD4 or CD8 (189, 190). Consistent with this concept we observed an increase in the CD11b+CD11c+CD8α+ DCs in the spleen of GM-CSF treated mice, which also correlated with a higher percentage of Foxp3+ Tregs (Figure 15). Additionally, adoptive transfer of ex vivo differentiated BMDCs into recipient mice treated with mTg lead to an increase in Treg percentages. This increase could be the result of a combination of TCR independent nTreg expansion and TCR dependent iTreg induction.
through mTg presentation. Therefore, in our animal models of GM-CSF mediated suppression of autoimmunity, it is likely that GM-CSF treatment caused a faster turnover of myeloid differentiation in the BM that led to an accumulation semi-mature CD11b⁺CD11c⁺ DCs in the spleen that then caused Treg expansion and differentiation. We do not yet know if this phenomenon is simply the result of increased GM-CSF mediated differentiation in the bone marrow and therefore an aberration of “physiological DC homeostasis” or there really exists a GM-CSF directed (and Flt3L independent) DC development pathway leading to increased tolerance to self antigens.

Our observations have strong implications for the treatment of various autoimmune diseases. Deficiency of naturally occurring Tregs has been shown to contribute to a variety of autoimmune conditions (191, 192) and adoptive transfer of polyclonal or antigen selected nTregs can be sufficient to overcome autoimmune and allergic conditions (25, 193, 194). Our data also shed some light on the molecules that may be important for Treg homeostasis and has to be determined in the future studies if the ligands in combination can be directly used for treating autoimmune condition. BMDCs more specifically OX40L+Jagged-1+ BMDCs provide a simple and efficient method of selectively expanding polyclonal Tregs *ex vivo* which can then be adoptively transferred as a potential therapy for human autoimmune conditions.
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