THE ROLE OF B CELL SUBSETS IN PERIPHERAL T CELL TOLERANCE INDUCTION

By

Erin Martin

A THESIS/DISSERTATION

Presented to the Department of Microbiology and Molecular Immunology
and the Oregon Health & Science University
School of Medicine
in partial fulfillment of
the requirements for the degree of
Master of Science

December 2010

School of Medicine

Oregon Health & Science University

Certificate of Approval

This is to certify that the Masters Dissertation of

Erin A. Martin

"THE ROLE OF B CELL SUBSETS IN PERIPHERAL T CELL TOLERANCE INDUCTION"

Has been approved

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Dissertation Advisor – David Parker

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Committee Member – Jagdeep Obhrai

_____

Committee Member – Keith Bahjat

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ACKNOWLEDGEMENTS

First and foremost, I'd like to thank my mentor, David Parker. Thank you to the present and past members of the Parker Lab for all of your support: Susan Murray, Yoshinobu Koguchi, Fanny Polesso, Jennifer Gardell, Timothy Thauland, Alex Rowe, and Katelynne Gardner-Toren. Thank you to Mandy Boyd, Dorian LaTocha, and Pamela Canaday for cell sorts. Thank you to the members of my committee, Ann Hill, Jagdeeb Obhrai, Keith Bahjat and David Parker. A special thanks to Katelynne Gardner-Toren, who helped perform the work in Chapter 2, experiment 1 and 2, and Susan Murray who performed unpublished experiments mentioned in Chapter 3.

ABSTRACT

The process of central tolerance eliminates self-reactive B and T lymphocytes as they develop in the bone marrow and thymus. Although stringent, this process is not complete because some auto-antigens that are not expressed during central tolerance are present in the periphery. Therefore, peripheral tolerance mechanisms are in place to silence any self-reactive B and T cells that escape central tolerance. New bone marrow B cell emigrants, termed transitional B cells, undergo anergy and eventual apoptosis upon encounter with self-antigen. Mature T cells may undergo anergy, apoptosis, or develop into regulatory T cells that suppress immune responses. In this study, we examine the role of transitional and mature B cells in T cell tolerance induction. More specifically, we use an *in vivo* model to provide evidence that transitional and mature follicular B cells are capable of inducing antigen-specific T cell anergy. Further, we examine the capacity of three B cell subsets in converting naïve T cells to regulatory T cells *in vitro.* All together, these data implicate B cell subsets in peripheral T cell tolerance induction.

CHAPTER 1. INTRODUCTION

B-2 B cell development in the bone marrow

Mature B cells fall into three distinct subsets. Follicular (FO) and Marginal Zone (MZ) B cells are primarily located in the spleen and lymph nodes, while B-1 B cells are found in both the spleen and peritoneal cavity (PC). The subsets can be further distinguished according to developmental timing; B-1 B cells have fetal origins and are largely self-renewing, while FO and MZ B cells belong to the B-2 subset that is continuously renewed throughout life.

B-2 cells originate from hematopoetic stem cells in the bone marrow (BM), defined by expression of stem cell factor receptor, CD117 (c-kit). As the cells that are destined to become B cells develop through the early lymphoid progenitor and common lymphoid progenitor stages, they gradually lose surface CD117 and gain terminal deoxynucleotidyl transferase (TdT), Rag1 and Rag2 expression. Next, heavy chain rearrangement occurs, and this defines the pro-B stage. When they reach the pro-B cell stage, cells have begun to express low levels of B220 as well as the fate-determining transcription factor Pax5. Notably, Pax5 drives CD19 expression, a commonly used indicator of B lymphocyte lineage (Hardy, 2007).

Next, the cell enters the early and late pre-B cell stages, during which light chain rearrangement leads to the production a functional B cell receptor (BCR) composed of a paired light and heavy chain. Engagement of the BCR on the late pre-B cell induces re-expression of the Rag1/2 genes and additional light chain rearrangements, with the goal of changing the specificity away from self-reactivity.

This process is called receptor editing (Ravetch, 2000). Pre-B cells that fail to produce a non-autoreactive BCR fail to develop and die before they can migrate to the periphery (Thien, 2004). In fact, auto-reactivity among BCRs initially expressed by B cells is very common. To determine the prevalence of self-reactive B cells in the bone marrow, Wardemann et al. cloned antibodies from single BM-derived B cells of three healthy human donors and tested them for reactivity against a panel of defined antigens (Wardemann, 2003). Indeed, 75.9% of antibodies cloned from pre-B cells and 40.7% from Tr B cells in the blood were reactive against a HEp-2 extract of 248 nuclear and cytoplasmic antigens. Due to the high likelihood of self-reactivity leading to BCR ligation and apoptosis, it is estimated that no more than 5% of newly formed B cells will enter the mature B cell compartment (Allman, 2004).

Transitional B cells

The small proportion of cells that acquire a functional, non-autoreactive BCR enters the "transitional" (Tr) B cell developmental stage. At this point, the B cells leave the BM, enter the blood stream and migrate to the spleen. Historically, Tr cells have been identified as $CD19^{+}$ B cells that express high levels of CD93 and HSA (heat shock antigen) (Allman, 1993). At any given time, Tr B cells make up 5-10% of the spleen and may become functionally mature, $CD93^{lo}HSA^{lo}$ B cells within 4 days. Tr B cells have three stages of development that can be identified by the expression levels of B220, IgM, CD93, and CD23 (Allman, 2001). Transitional 1 (T1) is the first developmental stage, defined by cell surface staining that is $CD93^{hi}$ IgM^{hi} $CD23^{lo}$. All T1 cells will develop into transitional 2 (T2) cells as they increase surface CD23

expression. From the T2 stage, cells lower their surface expression of IgM and may develop into mature $CD23^{+}CD21^{lo}$ FO or $CD23^{lo}CD21^{+}$ MZ B cells. However, in the context of BCR ligation and insufficient survival signals, Tr cells will develop into functionally anergic transitional 3 (T3) cells (Merrell, 2006).

Early studies indicated a clear difference in response to BCR stimulation between immature, Tr, and mature B cells. Whereas immature B cells undergo receptor editing and mature B cells enter the cell cycle, Tr B cells become functionally silent and eventually undergo apoptosis in response to BCR ligation (Allman, 1992). It has been established that B cells located in the periphery decrease surface BCR expression and partially uncouple downstream signaling pathways in response to chronic BCR ligation (Goodnow 2001). However, Gross et al. found that Tr B cells are significantly more sensitive to BCR stimulation than FO B cells, a phenomenon associated with the Lyn-CD22-SHP-1 signaling pathway (Gross, 2009). Lyn is a Src family kinase that phosphorylates ITAMs (immunoreceptor tyrosine-based activation motifs) located on the BCR Igα/Igβ chains following antigen binding as well as ITIMs (immunoreceptor tyrosine-based inhibition motifs) associated with CD22. Because Tr B cells have lower levels of Lyn, they cannot efficiently phosphorylate ITIMs on CD22, resulting in an inability to inhibit BCR signaling (Gross, 2009). Thus, chronic ligation of Tr BCRs leads to cell desensitization, in which the NFκB and JNK signaling pathways are uncoupled from the BCR (Hodgkin, 1995). As a result, the BCR loses signaling capacity as measured by intracellular Ca^{2+} flux and tyrosine phosphorylation (Eris, 2004).

Several mouse models have been utilized to study the induction of anergy in autoreactive Tr B cells. In the widely utilized hen egg lysozyme (HEL) transgenic mouse model, the MD4 genotype encodes a BCR with high affinity for HEL (Goodnow, 1988). MD4 B cells that develop in mice that express membrane-bound HEL (mHEL) encounter HEL in the BM, cannot undergo productive receptor editing, and experience a developmental block at the pre-B cell stage (Mason, 1992). However, when MD4 mice are crossed with mice that express soluble HEL (sHEL) (ML5), B cells first encounter their cognate antigen in the periphery and experience a developmental block at the Tr B cell stage (Goodnow, 1988). In addition to BCR desensitization, Tr B cell anergy includes poor collaboration with T cells.

Research utilizing the HEL transgenic model reveals that Tr B cell anergy is not due to poor antigen uptake or presentation (Lund, 2010; Eris, 1994). One study utilized HEL peptide, which loads directly on to MHC class II on the B cell surface and bypasses the necessity of antigen receptor engagement. By comparing non-tolerant MD4 cells loaded with HEL protein versus tolerant MD4xML5 cells, one can compare the efficiency of antigen presentation to T cells. In both cases, the antigen-specific T cells enlarge but fail to produce cytokines, proliferate, or promote B cell blasts (Ho, 1994). However, in both anergic and non-anergic B cell cultures, the addition of stimulatory anti-CD28 overcomes poor T cell activation. Indeed, one of the early responses to acute BCR signaling is surface display of the CD28 ligand, B7.2. Anergic B cells do not maintain increased B7.2 on the cell surface and therefore fail to ligate CD28 on T cells. Although reconstituting B7.2 in anergic B cells does not amend the BCR signaling defect, it does enable clonal expansion and antibody production in

response to T cell help (Rathmell, 1998). Using the HEL transgenic system, Cook et al showed that tolerant B cells can be induced to produce antibodies in response to membrane preparations from activated T cells (Cooke, 1994). The preparations contain CD40L, which acts together with IL-4 and IL-5 to overcome Tr B cell anergy and deliver the activating signals required for productive T-B collaboration. Taken together, these data indicate that poor T-B collaboration experienced by anergic Tr B cells is due, at least in part, to their decreased B7.2 expression and not to an inability to produce antibodies following CD40 ligation.

Without B cell survival signals delivered by T cells in response to co-stimulation, anergic B cells are vulnerable to Fas ligand (FasL) induced cell death. When transferred to a new host with T cells that recognize HEL, anergic B cells from MD4xML5 mice die while non-tolerant B cells from wild type mice produce antibodies (Rathmell, 1995). However, when the MD4xML5 B cells are deficient in CD95 (Fas), they are able to proliferate and produce antibodies upon interaction with antigen-specific T cells. CD95 ligation is not required for autoreactive B cell elimination in the BM, suggesting that anergic Tr B cells are uniquely vulnerable to FasL-mediated apoptosis (Rathmell, 1995). This characteristic has been attributed to desensitized BCR signaling, which may render anergic Tr cells unable to transmit signals necessary for the suppression of Fas-mediated cell death. Additionally, B7.2 expression on Tr B cells may be insufficient to elicit necessary survival signals from T cells (Rathmell, 1995). Indeed, Rathmell et al. found that enhanced expression of B7.2 can rescue autoreactive B cells from Fas-L induced cell death (Rathmell, 1998).

Another important factor in the developmental "fork in the road" that determines Tr B cell fate is availability of BAFF (B cell activating factor, also known as Blys). The first indication that a survival factor may play a role in Tr B cell survival came from B cell competition studies. In mice that are transgenic for only heavy chain of HEL-specific BCR, random rearrangement of the light chain produces a BCR that can recognize HEL on about 1% of B cells (Lesley, 2004). When crossed to mice that express soluble HEL (ML5), the 1% HEL-specific B cells disappear in the periphery (Cyster, 1994). However, in MD4 x ML5 mice, all B cells express HEL-specific BCRs and are present in the periphery in numbers within 2-3 fold of wild type (Manson, 1992) These studies indicate that tolerant Tr B cells experience poor survival when they are among a polyclonal B cell population, and improved survival among B cells with a set BCR repertoire. Indeed, Cyster et al. reported that B cells undergo competition based on the level of BCR self-reactivity, leading to exclusion of the more autoreactive cells to the outer PALS (periarterial lymphatic sheath) region of the spleen (Cyster, 1994). Because follicular exclusion is associated with a short half-life, occurs in mice deficient in follicular homing molecule CCR7, and is independent of CD95 ligation, limited availability of an unknown trophic factor was implicated (Ekland, 2004; Cyster, 1995; Phan 2003).

Nearly ten years later, a report by Thien et al. identified BAFF as the trophic factor responsible for rescuing autoreactive B cells from anergy and facilitating their migration into the B cell follicle (Thein 2004). Furthermore, chronic BCR ligation and subsequent pro-apoptotic signaling experienced by anergic Tr B cells can be overcome by sufficient levels of BAFF (Lesley, 2004). Tolerant B cells that receive

BAFF have elevated levels of the pro-survival kinase Pim2 (Thien, 2004). In the context of the previously described HEL transgenic model with a set heavy chain, the 99% of cells that have rearranged their light chains in such a way that does not recognize BAFF will experience less BCR signaling than the 1% of B cells with a HEL-specific BCR, and therefore require less BAFF to develop into mature B cells. Therefore, the length of time to death is related to the strength of the BCR signal and the time required for acquisition of the BAFF signal to save the cell. It follows that the half-life of tolerant HEL-specific B cells is 3-4 days, and the half-life of non-tolerant B cells is 4-5 weeks (Hodgkin, 1995).

Mice that are transgenic for excess BAFF expression exhibit systemic autoimmunity accompanied by elevated levels of circulating autoantibodies, increased peripheral B cell numbers, and immune complex-mediated glumerulonephritis (Mackay 1999). The B cells responsible for this phenotype have escaped central tolerance in the BM, as BAFF responsiveness is acquired during the Tr developmental stage (Hsu, 2002). However, once B cells enter the T3 developmental stage, they have hit what is considered a developmental "dead end" and cannot be rescued by excess BAFF (Teague, 2007). T3 B cells do not undergo apoptosis immediately, but remain at the junction of T cell and B cell areas of the outer PALS region of the spleen, possibly to induce T cell tolerance to the auto-antigens that rendered the anergic.

Mature B cell subsets

Of the $\sim 2 \times 10^7$ surface IgM+ cells produced in the murine BM each day, it is estimated that only 10% enter the spleen and 2-5% reach maturity (Teague, 2007).

Most of the cells that reach maturity will enter the major splenic B cell population, the FO subset. This subset is named for the follicular regions of the lymph nodes and spleen, but is not static and migrates throughout the blood and lymphatic system (Rolink, 2004). FO B cells have a diverse BCR repertoire and participate in T-dependent antibody responses that yield IgM and IgG antibodies in early extra-follicular responses. FO B cells are the major B cell subset participating in the germinal center reaction, where they interact with T_{FH} cells, which induce somatic hypermutation and affinity maturation. These processes allow FO B cells to acquire BCR of high affinity for specific antigen. Some FO B cells are selected to become long-lived plasma cells and high affinity memory cells with isotype-switched BCRs (Jacob, 1993; McHeyzer-Williams, 1993).

In addition to the FO B cell subset, Tr B cells may join the mature MZ B subset. These cells reside in the marginal zone, an area of the spleen located at the periphery of the PALS and follicular area where arterial blood empties into open sinuses. The MZ is an ideal environment for MZ B cells to collaborate with resident macrophages to filter the blood (Kraal, 2002). MZ B cells preferentially express a BCR repertoire that is biased toward bacterial cell-wall constituents and senescent self-components such as oxidized low-density lipoproteins (Lopes-Carvalho, 1994). These components may be taken up by scavenger receptors on MZ-resident endothelial cells and macrophages. Due to increased surface expression of the integrin LFA-1 (leukocyte function-associated antigen-1), MZ B cells are held in the marginal zone of the spleen by interactions with endothelial cells expressing LFA-1 ligands ICAM-1 and VCAM-1 (intercellular adhesion molecule-1 and vascular cellular adhesion

molecule-1, respectively) (Lu, 2002). In support of this theory, MZ B cells disappear in mice after treatment with anti-integrin antibodies (Lu, 2002). It is believed that upon LPS or BCR stimulation, MZ B cells can down-regulate LFA-1 surface expression and migrate to the T-B border in order to participate in T-dependent antibody responses (Lo, 2003). Although MZ B cells can form T-dependent germinal centers and produce high affinity antibodies through somatic hypermutation, they are perhaps best known for their ability to respond to TLR ligands and thymus-independent ligands (Song, 2003).

The third commonly studied subset of B cells is termed B-1. All B-1 B cells are derived from $Lin^{-}CD45R^{lo}CD19^{+}$ progenitors that are found in the neonate liver or adult BM, while B-2 progenitors are Lin^{-} $CD45R^{+}CD19^{-}$ and exclusively located in the adult BM (Montecino-Rodriguez, 2006). Just as B-2 B cells can be subdivided into FO and MZ subsets, two subsets of B-1 B cells have been identified, based on surface expression of CD5. The first B cells to develop during fetal hematopoesis are $CD11b^{+}IgM^{hi}CD5^{hi}$ B-1a B cells, which are self-replenishing population following birth (Herzenbert, 2006). The most distinctive feature of $CD5^{+}$ B-1a B cells is production of auto-antibodies that include specificities for branched carbohydrates, glycolipids, and glycoproteins, including phosphorycholine, phospatidycholine, the Th-1 glycoprotein, viral coat proteins, and bacterial cell-wall constituents (Baumgarth, 2005). These self-reactive antibodies are not pathogenic, but rather termed “natural auto-antibodies”. $CD11b^{+}IgM^{hi}CD5^{lo}$ B-1b cells develop from B-1 progenitors from the BM throughout life, and are required for protection against

certain parasites and bacteria (Haas, 2005). Collectively, B-1a and B-1b subsets account for about 5% of B cells in the mouse.

B cell—T cell interactions in the periphery

As antigen presenting cells (APCs), B cells play an important role in peripheral T cell homeostasis as well as the initiation of adaptive immune responses. One facet of adaptive immunity is the B cell antibody response, which generally follows a four-step model (Eris, 1994). Initially, antigen that has been captured by the B cell BCR is internalized, processed it into peptide fragments, associated with MHCII, and presented on the cell surface (Lanzavecchia, 1985). B cells are particularly good APCs under conditions of limiting availability, owing to their antigen-specific BCRs that can take up and present antigen in a highly efficient manner. Utilizing the F(ab')2 fragment of normal rat Ig and T cells specific for the fragment, Tony et al. showed a 10,000-fold shift in the dose response curve when B cells internalize and present antigen (Tony, 1985). Once the peptide is presented on the B cell surface, the second step in T-B collaboration can occur as antigen-specific TCR on the T cell interacts with the peptide-MHCII (pMHC) complex. Following this initial stimulation of the naïve T cell, secondary stimulation, or co-stimulation, must be delivered from the B cell. This typically occurs in the form of B7.1 and B7.2 presented by the B cell, which ligate CD28 on the T cell (Hathcock, 1993). Next, CD40L is expressed on the T cell surface. In the final step, the B cell undergoes proliferation and antibody secretion in response to ligation of CD40 by CD40L and cytokines released by the T cell (Lane, 1992).

T cell activation by antigen presenting B cells requires co-stimulation. If the T cell encounters pMHC in the absence of co-stimulation, functional anergy may result. T cell anergy is a passive form of tolerance, featuring failure to differentiate to effector function or survive as memory cells (Rocha, 1995). Early studies utilizing *in vitro* stimulation of T cell clones in the absence of co-stimulation led investigators to conclude that APCs expressing low levels of B7, such as small resting B cells, are capable of inducing T cell tolerance *in vivo* (Jenkins, 1990). More recent studies indicate that APCs, such as local dendritic cells *in vivo* may provide other co-stimulatory signals necessary to avoid T cell tolerance (Lohr, 2005). Therefore, T cell fate *in vivo* appears to be dependent upon availability of a third stimulatory signal in the form of OX40L, IL-12, or other cytokines (Huddleston, 2006). Without the third signal, T cells may be rendered functionally silent or tolerant.

FoxP3+ Regulatory T cells

When signal three comes in the form of IL-2 and TGFβ, the $CD4^+$ T cell will acquire a regulatory phenotype. As opposed to passive tolerance in the form of anergy, regulatory T cells (Tregs) inhibit inappropriate immune responses in a dominant manner. Conventional Tregs express the transcription factor FoxP3, which conveys the regulatory phenotype. Deficiency in functional FoxP3 leads to hyperactivation of $CD4^+$ T cells, overproduction of pro-inflammatory cytokines, and spontaneous autoimmunity (Ochs, 2005). This phenotype is best illustrated in humans with the genetic disease IPEX (immune dysregulation, polyendicrinopathy, enteropathy, X-linked syndrome), owing to mutations in the FOXP3 gene. Prior to the identification

of FoxP3, Sakaguchi et al. identified CD25 (IL-2Rα) as a marker for T cells with regulatory potential (Sakaguchi, 1995). CD25 was later found to be crucial to the development of Tregs, making it a useful marker that is often used in place of or addition to FoxP3. Although the focus of the current study is $FoxP3^+$ Tregs, recent studies have also identified $FoxP3^-$ T cell populations with a regulatory phenotype (Fujio, 2010).

Naturally occurring Tregs (nTreg) originate in the thymus, while induced Tregs (iTregs) are derived from naïve $CD4^+$ T cells in the periphery. In both cases, T cells that express an autoreactive T cell receptor (TCR) are preferentially selected into the Treg compartment (Josefowicz, 2009). The effect of antigen dose on nTreg development is illustrated in TCR transgenic mice that also express antigen in thymic stromal cells at differing levels. When antigen is expressed at high levels, the majority of thymocytes expressing antigen-specific αβ TCR that survive negative selection become Tregs (Jordan, 2001). Despite a bias toward self-reactivity, sequence analysis of TCRs expressed by $FoxP3^+$ and $FoxP3^-$ T cells reveals overlap in antigen specificity (Hsieh, 2006). These results indicate that the healthy T cell repertoire is made up of potentially pathogenic T cells as well as suppressive Tregs that recognize the same self-antigens.

The dependence upon CD25 (IL-2Rα) for Treg development and survival indicates a critical role for IL-2 in FoxP3 induction. Indeed, mice lacking IL-2 or CD25 exhibit approximately 50% decrease in $FoxP3^+$ thymocytes, whereas mice lacking the common gamma chain (γc) shared between IL-2, IL-7, and IL-15 are completely

devoid of FoxP3$^+$ cells (Fontenot, 2005). Recent studies indicate that conditional thymocyte deletion of STAT5, which is downstream of γc receptors and binds the FoxP3 gene, results in drastically reduced numbers of Tregs (Burchill, 2007). In addition to the FoxP3 promoter, STAT5 interacts with the FoxP3-CNS2 (conserved non-coding sequence 2). Methylation states of the FoxP3-CNS2 as well as the FoxP3 promoter has been highly studied of late, and has been shown to effect Treg stability (Josefowicz, 2009b). FoxP3-CNS2 function is dependent upon demethylation of the CpG island located at this region, which is typically unmethlyated in nTregs but remains highly methylated in iTregs, possibly contributing to decreased stability in iTregs. In fact, ablation of the Dnmt1 (DNA methyltransferase 1) gene, which is responsible for CpG motif demethylation, has been shown to increase the induction and stability of FoxP3 expression.

In addition to decreased stability, iTregs differ from nTregs in their induction requirements. For example, CD28 cross-linking inhibits peripheral Treg induction, indicating suboptimal co-stimulation is ideal (Kim, 2006). Additionally, peripheral Treg induction requires CTLA-4 and TGFβ signaling, both of which are not necessary for FoxP3 expression in thymocytes (Zheng, 2006). Recent studies have shown that TGFβ is required for the binding of NFAT (Nuclear factor of activated T cells) to the FoxP3-CNS1 (conserved non-coding sequence 1) and may help recruit Dnmt1 to the FoxP3 locus (Josefowicz 2009a; Josefowicz, 2009b). In the presence of TGFβ and IL-6, activated CD4$^+$ T cells differentiate into Th17 cells, but the addition of IL-2 inhibits Th17 differentiation and promotes Treg induction (Sakaguchi, 2008). In addition to IL-2 and TGFβ, retinoic acid (RA) facilitates FoxP3$^+$ iTreg differentiation

(Benson, 2007). The requirement for a specific mileau of cytokines limits Treg induction *in vivo* to particular environments, such as gut-associated lymphoid tissue (GALT). Previous studies indicate that $CD103^+$ DCs in the GALT and gut-draining mesenteric lymph nodes take up and present gut-associated antigens derived from food and commensal microbiota (Josefowicz, 2009). In addition to presenting antigens that could be potentially allogenic, $CD103^+$ DCs produce TGFβ and RA, allowing for the induction of $FoxP3^+$ iTregs.

In addition to the GALT, peripheral draining lymph nodes are potentially a site of Treg induction and/or maintenance. For example, APCs in draining lymph nodes may interact with $CD4^+$ T cells expressing TCRs that recognize tissue-specific antigens (Sakaguchi, 2008). Indeed, $FoxP3^+$ Tregs specific for tissue antigens are enriched in regional lymph nodes, and can be categorized into subsets based on expression of homing receptors, adhesion molecules, and chemokine receptors. For example, $CCR7^+CD62L^{high}$ Tregs are capable of migrating to secondary lymphoid tissues and preventing the development of autoimmune diabetes in mice (Szanya, 2002). Additionally, $CD103^+$ Tregs have been correlated with a memory-like phenotype, are more suppressive than $CD103^-$ Tregs, and can control colitis (Uhling, 2006).

In this study, we analyze the effects of different B cell subsets in peripheral T cell tolerance. Both B and T cells undergo tolerance mechanisms at various times throughout development. Auto-reactive T cells that escape central tolerance in the thymus must be silenced, either by functional anergy or development of a regulatory

phenotype, in order to avoid potentially pathogenic effects. Herein we examine the roles of mature FO, MZ, and B-1 B cells in Treg induction, as well as the role of Tr B cells in peripheral T cell tolerance induction.

CHAPTER 2. REGULATORY T CELL INDUCTION BY MATURE B CELL SUBSETS

Introduction

The role of co-stimulatory molecules presented by APCs in the conversion of naïve $CD4^+$ T cells to Tregs has been widely studied. While it remains unclear whether co-stimulation from B7.1 and B7.2 is a requirement for iTreg development, it is apparent that the expression level of these molecules on the APC has some impact on cell fate. For example, in conditions favoring Treg induction *in vitro*, B cells are superior to dendritic cells (DCs) at stimulating Treg conversion despite their weaker APC potential (Chen 2007). The impact of co-stimulation is further evidenced by activated B cells, which express higher levels of B7 than resting B cells, but are less efficient at inducing $FoxP3^+$ Tregs *in vitro* (Benson, 2007). These findings have been extended to *in vivo* systems. Jia-Bin Sun et al. utilized cholera toxin B conjugated to ovalbumin peptide (OVA/CTB) to "educate" B cells (Sun, 2008). In this model, CTB mediates uptake by all B cells regardless of BCR specificity, which then present the antigen to OVA-specific T cells. When B cells are exposed to OVA/CTB, they are considered educated and promote expansion of $FoxP3^+$ Tregs. Interestingly, educated B cells have decreased levels of surface B7 and express higher levels of IL-10 mRNA.

Confounding our understanding of how B7.1 and B7.2 participate in Treg conversion is the disparate stimulatory role of their shared ligands, CTLA-4 and CD28. While CD28 ligation results in T cell proliferation and IL-2 secretion, CTLA-4 inhibits IL-2 production and cell cycle progression (Fife 2008). Previous studies

have shown that blocking CD28 ligation enhances iTreg function, while blocking CTLA-4 suppresses iTreg development (Zheng, 2004). However, the necessity of CTLA-4 for FoxP3 induction has been called into question by CTLA-4 deficient mice, which develop functional Tregs (Fife 2008). Nonetheless, CTLA-4 does appear to play a role in autoimmune homeostasis, as mice deficient in the molecule develop multi-organ disease and die within three weeks. Additionally, some studies imply that at least minimal CD28 signaling is necessary for FoxP3 induction by naïve $CD4^+$ T cells in the periphery and may be required for optimal Treg proliferation (Josefowicz, 2009; Tang, 2008; Tang, 2003).

Programmed death ligand 1 (PD-L1) is another B7 family member and negative signaling molecule implicated in Treg development. Expressed on leukocytes, non-hematopoetic cells, and non-lymphoid tissues, PD-L1 binds to Programmed death 1 receptor (PD-1) on activated T and B cells (Fife, 2008). It is believed that PD-1 destabilizes the synapse by competing for signaling components, therefore TCR engagement and signaling is inhibited in cis by PD-1. The impact of PD-1 expression and signaling by Tregs has been illuminated by mice deficient in the molecule, which develop late-onset, progressive arthritis and lupus-like glumerulonephritis (Greenwald, 2005). Due to the delayed onset of autoimmune phenotype in PD-1 deficient mice as well as the more broad expression of PD-L1, one theory holds that CTLA-4 signaling is responsible for Treg induction in the lymphoid tissue, while PD-1 expression in peripheral tissue maintains Treg homeostasis (Fife, 2008). In support of this theory, Fife et al. found that PD-1/PD-L1 interactions but not CTLA-4 interactions are necessary for long-term tolerance in a diabetic mouse model (Fife,

2006). Later, the same group showed that PD-1/PD-L1 interactions encourage T cell motility and reduce T cell-DC interactions that lead to loss of T cell tolerance, but CTLA-4 has no effect on T cell motility (Fife, 2009). These and other reports show that PD-L1 expression by APCs plays a role in both iTreg induction and maintenance (Francisco, 2009).

B cells play a pivotal role in Treg homeostasis (Chen, 2007; Benson, 2007). This is evidenced by B-cell deficient uMT mice, which have reduced numbers of splenic FoxP3$^+$ Tregs compared to wild type mice (Sun, 2008). B cells have been utilized as APCs in several studies investigating the role of co-stimulation in iTreg induction. B cells fall into subsets based on differences in cell surface markers, homing, BCR specificity, and lifespan. For example, B-1 B cell expresses more surface B7.2 than the other subsets (Tumang, 2004). The relevance of co-stimulatory molecule expression has been reported by Li Wang et al, which found that increased PD-L1 expression by CD8α^+ DCs is responsible the subset's superior Treg induction capabilities *in vitro* (Wang, 2008). This finding led us to investigate the expression of co-stimulatory molecules MZ, FO and B-1 B cell subsets and their relative capacities to induce FoxP3+ Tregs *in vitro*.

Similar to Wang et al. who studied DC subsets, we found differences among the Treg inducing capabilities of the B cell subsets that correlate with their expression of B7 and PD-L1. Herein, we provide evidence for superior Treg induction by the FO B cell subset, which has the lowest expression levels of B7.1 and PD-L1. In contrast, the B-1 B cell subset expresses increased levels of co-stimulatory molecules and is the

least efficient Treg inducer. However, B-1 B cells were more efficient at Treg induction as their concentration was lowered. While both subsets had decreased efficiency of Treg induction following PD-L1 blockade, B7 blockade hampered Treg conversion by FO B cells but increased the B-1 subset's efficiency at higher B-1 cell concentrations. From these results, we conclude that the levels of B7 and PD-L1 expressed on B cell subsets effect their capability to induce $CD4^+$ $FoxP3^+$ Tregs *in vitro*.

Materials and Methods

Mice. All mice were housed under specific pathogen free-conditions. These studies were approved by the Institutional Animal Care and Use Committee at the Oregon Health & Science University. BALB/c, C57BL/6 and CD45.1 congenic mice were obtained from Jackson laboratories (Bar Harbor, ME). D011.10 $Rag2^{-/-}$ mice were obtained from Taconic Farms (Germantown, NY).

Antibodies and Reagents. Easy Sep mouse CD4+ enrichment and B cell enrichment kits were from Stemcell Technologies (Vancouver, Canada). PE/Cy7-anti-CD19, APC-anti-Strepavidin, APC-anti-CD93 (C1fqRp), FITC-anti-CD23 (FceRII), PE-anti-CD21/CD35, biotin-anti-CD25 (IL2Rα, p55), PE/Cy7-anti-CD4 (L3T4), AF647-anti-FoxP3, PE-anti-CD44 (Pgp-1, Ly24), PerCP/Cy5.5-anti-CD45.2, biotin-anti-PDL1 (B7-H1, CD274), mouse anti-IL6 and IL-10 ELISA kits were purchased from eBioscience (San Diego, CA). Fitc-anti-B220 (CD45R), biotin-anti-CD11b, PE-anti-CD11c, PB-anti-CD25 (PC61) were purchased from BD Biosciences (San Jose, CA). PE/Cy7-anti-PD1, PE-anti-CD152 (CTLA-4), LEAF purified anti-CD80 (clone 16-10A1), anti-CD86 (clone GL-1) and anti-PD-L1 (B7-H1; clone 10F, 9G2), and FoxP3 fix/perm buffer set were purchased from Biolegend (San Diego, CA). Live/Dead fixable aqua dead cell staining kit and Cell Trace CFSE kits were purchased from Invitrogen (Carlsbad, CA). Ovalbumin (OVA) peptide (323-339) was from AnaSpec, Inc. (Fremont, CA).

In-vitro Treg conversion. BALB/c B cells were enriched by magnetic negative selection from peritoneal cavity (PC) lavage or pooled spleen and lymph node samples. PC samples were sorted (BD Influx or Vantage cell sorters, BD Biosciences,

San Jose, CA) for $CD11c^{-}CD11b^{+}B220^{lo}CD19^{+}$ B-1 B cells; spleen/LN samples were sorted for $CD19^{+}CD93^{-}CD21^{+}CD23^{lo}$ MZ and $CD19^{+}CD93^{-}CD21^{lo}CD23^{+}$ FO cells. DO11.10 $Rag^{-/-}$ spleens were enriched for CD4+ T cells by magnetic negative selection and CFSE labeled. 100 $x10^{3}$ CD4+ T cells were cultured with 100$x10^{3}$, 50 $x10^{3}$, 25 $x10^{3}$ or 12.5 $x10^{3}$ sorted B cells at 37°C in the presence of 1μM antigenic peptide (OVA 323-339), 100U/ml IL-2, 20ng/mL TGF-β1 and, when specified, 10μg/mL anti-CD80, anti-CD86 or anti PD-L1. Cells were incubated in RPMI (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 10% tumor cocktail (dextrose, essential and non-essential amino acids, sodium pyruvate, sodium bicarbonate, gentamycin), 1% glutamine/penicillin/streptomycin and 1% 2-mercaptoethanol. Cells were stained in PBS with 2% fetal bovine serum and 0.1% sodium azide, analyzed with an LSR II Flow Cytometer (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star Inc, Ashland, OR).

Results

B cell subsets differ in expression levels of co-stimulatory molecules. To investigate their antigen presentation capacities, B cell subsets in steady state were analyzed by flow cytometry for surface expression of B7.1, B7.2, I-A^b, ICAM-1 (Inter-cellular adhesion molecule 1), and PD-L1. As shown in Fig.1, we found clear differences in expression levels among the subsets, with a trend toward the highest co-stimulatory molecule expression on B-1, intermediate levels on MZ, and lowest on FO B cells. The differences in expression levels among the subsets were especially apparent with B7.1, ICAM-1, and PD-L1. The median fluorescence intensity (MFI) of B7.1 was highest on B-1 B cells at 1,582.3 ±1, intermediate on MZ cells at 71.4±3, and lowest on splenic FO cells at 21.3±5 (Fig 1A). B-1 and MZ subsets had similar expression of PD-L1, levels more than three times higher than the FO subset. Although B-1 B cells expressed the highest levels of B7.1, ICAM-1, and PD-L1 among the subsets, MZ cells were greatest for B7.2 and I-A^b expression. The FO subset had the lowest expression of all co-stimulatory molecules. Overall, the widest range of co-stimulatory expression among the subsets in steady state is between B-1 and FO subsets, with MZ B cells displaying an intermediate phenotype.

B cell subsets differ in their ability to induce CD4+FoxP3+ Tregs. We next examined the relative abilities of the B cell subsets to induce Tregs *in vitro*. Sorted FO, MZ, and B-1 B cells were cultured with CFSE-labeled naïve TCR transgenic CD4+ T cells in the presence of antigenic OVA peptide, TGFβ, and IL-2. 100, 50, 25 or 12.5 thousand B cells of each subset was cultured with 100 ,000 T cells (1:1, 1:2, 1:4 and 1:8

dilutions). After three or five days, the T cells were analyzed for FoxP3 expression and CFSE dilution by flow cytometry.

The FO subset, which expressed the lowest levels of B7.1, ICAM-1, and PD-L1, were superior Treg inducers at day 3 (Fig. 2B). At least 40% of $CD4^+$ T cells cultured with FO B cells expressed FoxP3, regardless of dilution. The MZ subset, which had intermediate co-stimulatory molecule expression, also had intermediate Treg inducing capability. This trend was also apparent with total cell numbers (Fig. 2C). At 1:1 dilution, the FO subset induced 38,000±8 $CD4^+$ $FoxP3^+$ T cells, MZ induced 13,000±8, and B1 induced 13,000± 9. The elevated numbers of Tregs induced by FO B cells correlates with more overall $CD4^+$ T cell division, especially apparent at 1:1 dilution (Fig. 2A). CFSE division profiles indicate similar division among all subsets at day three (Fig. 2A).

In contrast to FO and MZ subsets, less than 10% of $CD4^+$ T cells cultured with B-1 B cells at 1:1 and 1:2 dilutions expressed FoxP3 (Fig. 2B). Interestingly, as the concentration of B-1 B cells decreased, both number and percentage of Tregs increased. At 1:1, 7.58 percent of $CD4^+$ T cells cultured with B1 B expressed FoxP3, compared to 30.3 percent at 1:8 dilution. This was not due to more division at lower dilutions, in fact the percent of $CD4^+$ T cells that remained undivided increased from 23.2 percent at 1:1 to 41.6 percent at 1:8 (Fig. 2A). Of note, there were more $CD4^+FoxP3^+$ T cells in culture with B-1 B cells with OVA than no peptide controls, indicating that 1:1 and 1:2 B-1 dilutions are not at background Treg induction levels.

At day five of *in vitro* culture, the FO B cell subset remained highest in percentage of FoxP3 expression, but the MZ and B-1 subsets caught up or overtook in total number of Tregs (Fig. 2D,E). At 1:1 dilution, there remained a clear distinction between the subsets in percentage of $FoxP3^+$ T cells, but at 1:8 dilution all subsets had induced a similar percentage of Tregs. The B-1 trend to induce more Tregs at lower concentrations was more pronounced at day five. In fact, the B-1 cells at 1:8 had at least two times more total $CD4^+$ T cells than FO and MZ subsets, regardless of dilution (Fig. 2E). The B-1 subset experienced a distinct reduction in both overall $CD4^+$ T cell proliferation as well as FoxP3 induction at 1:1 dilution, as compared to B-1 at 1:8 and the other subsets at all dilutions. There were no clear differences in IL-10 or IL-6 in the supernatants of all cultures, regardless of B cell subset or dilution (Suppl. Fig. 1). In summary, Treg induction by the subsets is inversely proportional to their levels of expression of co-stimulatory molecules, in that FO B cell subset is superior to MZ and B-1 in both cell number and FoxP3 expression at day 3 and has the lowest levels of co-stimulatory molecules (Fig. 1). Interestingly, the B-1 B cell subset, which expresses the highest levels of B7.1 and PD-L1, shows increased capacity to induce Tregs at higher concentrations, but is less efficient than the other subsets at all concentrations.

PD-L1 expression differs among B cell subsets and plays a role in FoxP3+ induction. We next wanted to determine if the variations in co-stimulatory molecule expression among the subsets is responsible for their differences in Treg induction. First, we analyzed expression of B7.1 and PD-L1 on the subsets following *in vitro* culture. After three days in culture with $CD4^+$ T cells, B-1 B cell expression of B7.1 and PD-L1

remained consistently higher than the FO subset at all dilutions (Fig. 3A, 4A). The surface expression of PD-1 and CTLA-4 by $CD4^+$ T cells was also consistent regardless of culture condition or subset (Suppl. Fig. 2).

To assess the contribution of PD-L1 signaling by B-1 and FO B cells in our system, the subsets were cultured with naïve $CD4^+$ T cells in the presence of PD-L1 blocking antibody for three days. Among three independent experiments, we saw a consistent reduction in the percent of $CD4^+$ T cells induced to express FoxP3 by FO B cells with the addition of PD-L1 block (Fig. 3B). This reduction is especially apparent in the total number of $FoxP3^+$ T cells in FO cultures, due in part to the reduction in total number of $CD4^+$ T cells, indicating a role of PD-L1 presented by FO B cells in T cell survival (Fig. 3C,D).

In contrast to the FO subset, the low efficiency of Treg induction by B-1 B cells at 1:1 and 1:2 dilutions was unaffected by PD-L1 blockade (Fig. 3B). However, the 1:4 and 1:8 dilutions, which result in increased FoxP3 induction without blockade, experienced a reduction in both percentage and total numbers of Tregs (Fig. 3B,C). Unlike the FO subset, the effect of PD-L1 block did not appear to effect overall $CD4^+$ T cell survival in cultures with B-1 B cells. There was, however, a reduction in overall $CD4^+$ T cell division in B-1 B cell cultures with PD-L1 block at 1:8 dilution. Of note, the percentage of $FoxP3^+$ and $FoxP3^-$ dead cells in all B-1 and FO cultures did not change (data not shown). Together, these data indicate that PD-L1 expressed on the FO and B-1 subset plays a role in $FoxP3^+$ induction, and on the FO subset in $CD4^+$ T cell survival.

The role of B7 molecules in Treg induction by FO and B-1 B cells. At steady state, B-1 B cells express more B7.1 than FO and MZ B cell subsets, and this is also true following three days of culture in our *in vitro* system (Fig. 4A). Thus, we next investigated the role of co-stimulation by B7 expressed on the subsets. To this end, B1 or FO B cells were cultured for three days with naïve $CD4^+$ T cells in the presence of blocking antibodies against B7.1 and B7.2.

To confirm the efficacy the B7 blocking antibodies, purified naïve $CD4^+$ T cells were co-cultured with purified whole splenic B cells in the presence or absence of OVA and the B7 blocking antibodies (Suppl. Fig. 3). In the presence of antigenic peptide, CFSE labeled $CD4^+$ T cells divided, but this effect was blunted by B7 blocking antibodies and in cultures without peptide.

Surprisingly, B7 blockade did not have a consistent effect on Treg induction by the FO B cell subset (Fig. 4B). Although the experiment in Figure 4B shows a reduction in percent FoxP3 at 1:1 dilution of FO B cells, two other experiments had inconsistent results in regards to the FO subset. However, the B-1 B cell results shown in Figure 4B are representative of three experiments. That is, B7 blockade consistently led to an increase in FoxP3 induction by B-1 B cells at all concentrations. The effect of B7 block was seen both in the percentage of total $CD4^+$ T cells that were $FoxP3^+$ and in total Treg cell numbers (Fig. 4C,E). From these data we conclude that B7 blockade of B-1 B cells improved their efficiency of Treg conversion, but had no consistent effect on FO B cell capability of FoxP3 induction.

Having seen such drastic differences in Treg induction by FO and B-1 subsets, we examined their ability to convert $CD4^+$ T cells to Tregs when cultured together in order to determine if the FO subset could compensate for the poor Treg induction by B-1 B cells. To this end, we cultured equal numbers of B-1, FO, and $CD4^+$ T cells together with OVA peptide for three days. When cultured with both subsets, 7.3±3 percent of $CD4^+$ T cells expressed FoxP3, compared to 26.8±2 for the FO subset alone and 4.4±1 for the B-1 subset alone (Fig. 4D). The addition of B7 blockade increased the percentage of Tregs induced in the presence of both subsets, similar to the slight increase that occurs in B-1-only conditions (Fig. 4D). From these data, it is clear that B-1 B cells are dominant in the culture and interfere with Treg induction at higher concentrations.

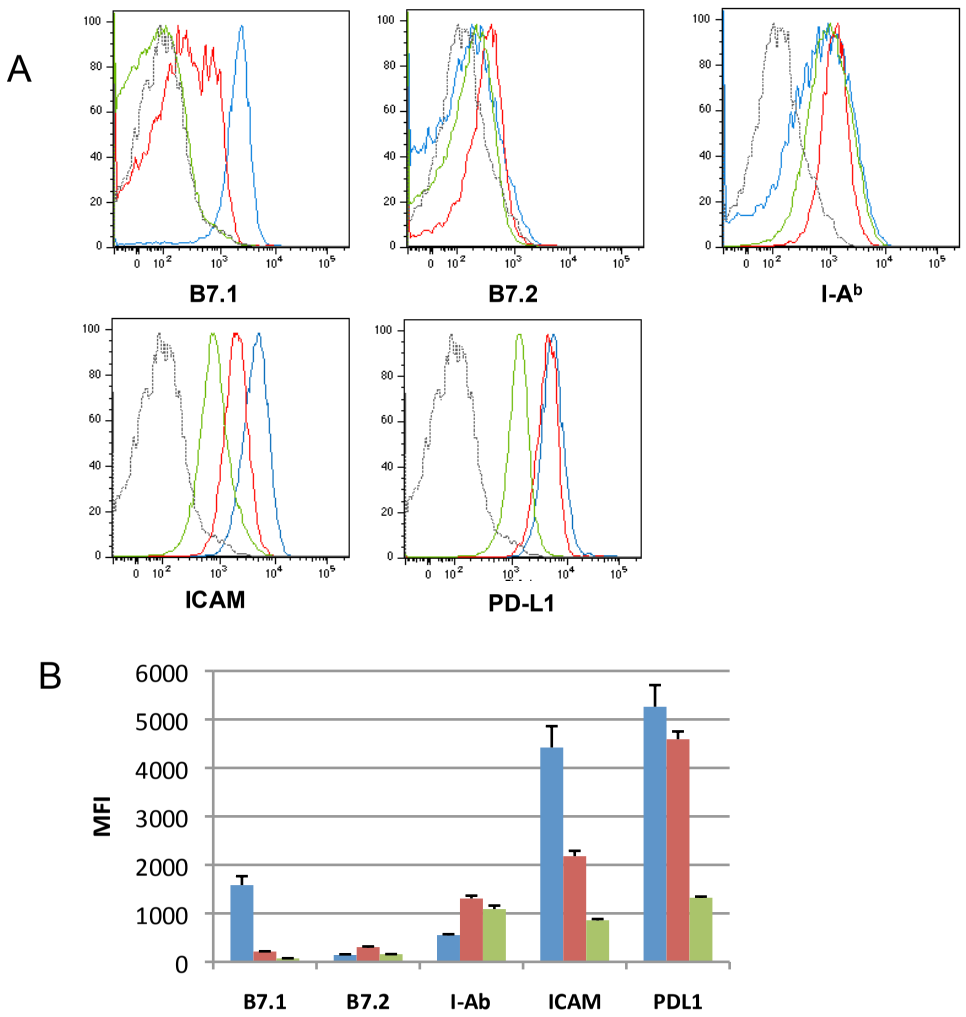


Figure 1-1. B cell subsets differ in expression levels of co-stimulatory molecules. B cells were isolated from the PC (B-1) or spleen (FO, MZ) and analyzed by flow cytometry (A) Graphs for B7.1, B7.2, I-A^b, ICAM-1, and PD-L1 on B-1 (blue, $CD11c^-$ $CD11b^+B220^{lo}CD19^+$), MZ (red, $CD19^+CD93^-$ $CD21^+CD23^{lo}$) and FO (green, $CD19^+CD93^-CD23^+CD21^{lo}$) B cell subsets. Black shows staining on $CD19^-$ lymphocytes (B) Median Flourescence Intensity of co-stimulatory molecules for B-1 (blue), MZ (red) and FO (green) subsets. Data representative of two independent experiments, with three animals per experiment. Data in B are the mean ±SEM. One experiment performed by Katelynne Gardener-Toren.

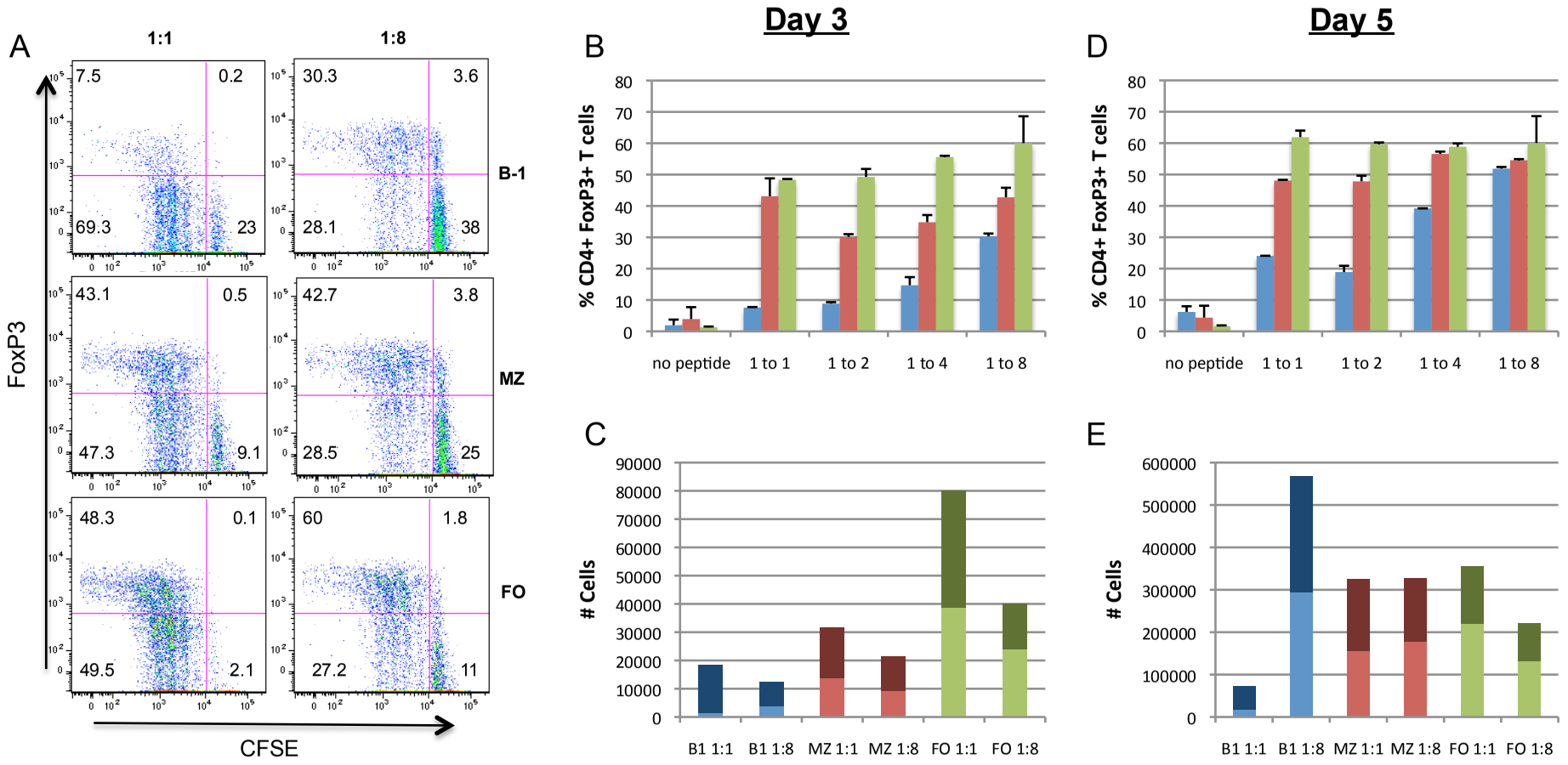


Figure 1-2. B cell subsets differ in ability to induce CD4+FoxP3+ T cells. 100,000 naïve CFSE-labeled $CD4^+$ T cells were co-cultured with B-1 (blue), MZ (red), or FO (green) B cells at varying dilutions in the presence of OVA, TGFβ and IL-2. Cells were analyzed by flow cytometry on day three (A-C) and day five (D-E). (A) Representative plots for 1:1 (left) and 1:8 (right) dilutions of B-1 (top), MZ (center) and FO (bottom) indicating FoxP3 expression of CFSE-labeled $CD4^+$ T cells on day 3. Numbers indicate mean values for one experiment. (B,D) Percent of total $CD4^+$ T cells expressing FoxP3. (C, E) Total numbers of $CD4^+$ T cells for each subset as indicated, $FoxP3^+$ (bottom/light color) and $FoxP3^-$ (top/dark color). Data in B-E represents triplicate wells for each condition. B, D data are the mean ±SEM. Data corresponds to one experiment which is representative of at least two independent experiments. One experiment performed by Katelynne Gardener-Toren.

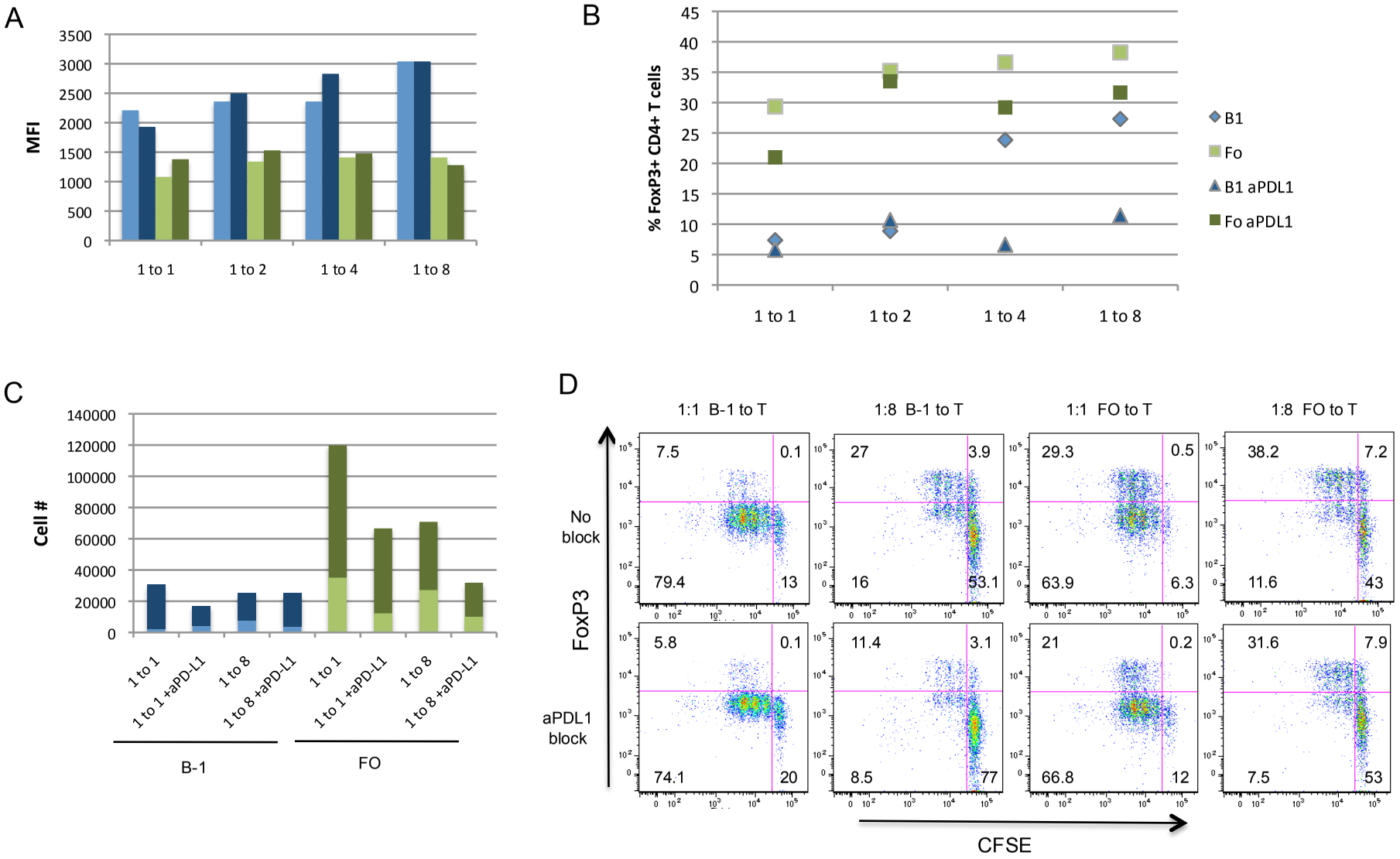


Figure 1-3. PD-L1 expression in FoxP3+ Treg induction. 100,000 naïve CFSE-labeled CD4+ T cells were co-cultured with B-1 or FO B cells in the presence of anti-PD-L1. Cells were analyzed by flow cytometry on day three for (A) PD-L1 expression by B-1 (blue) and FO (green) with block (dark) or without (light); (B) Percent of total $CD4^+$ T cells expressing FoxP3; (C) Total numbers of $CD4^+$ T cells for each subset as indicated, $FoxP3^+$ (bottom/light color) and $FoxP3^-$ (top/dark color). (D) representative FACS plot indicating CFSE division for $FoxP3^+$ or $FoxP3^-$ cells without (top) or with (bottom) PD-L1 block for the subset and dilution indicated. Numbers indicate mean values for one experiment. Data in B-C represents triplicate wells for each condition. Data corresponds to one experiment which is representative of at least two independent experiments

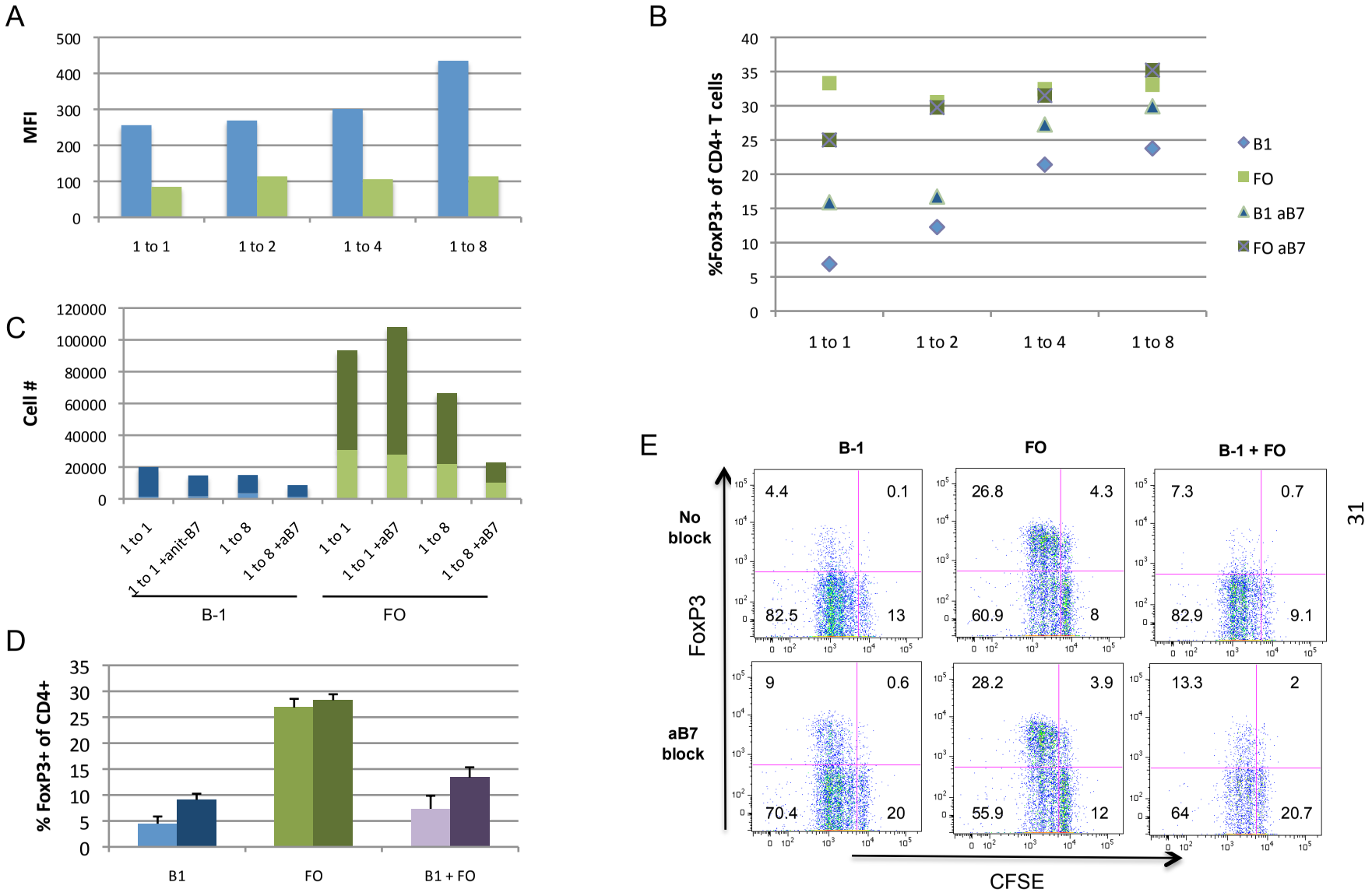
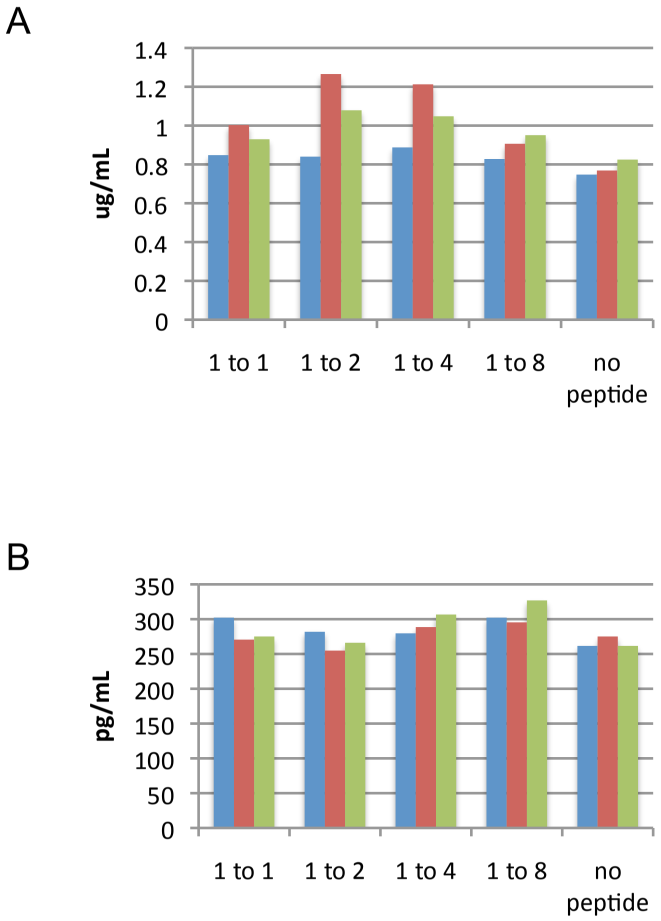
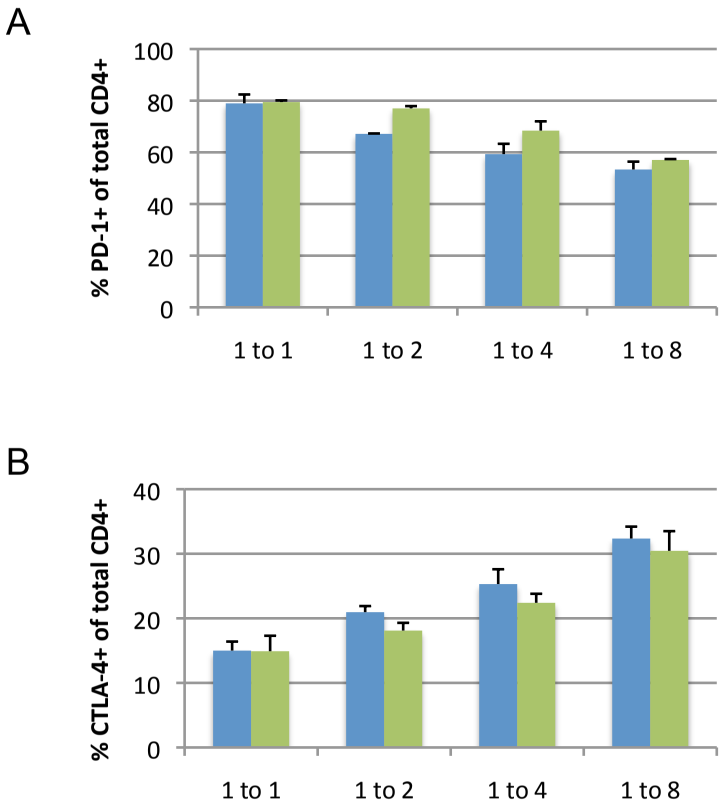


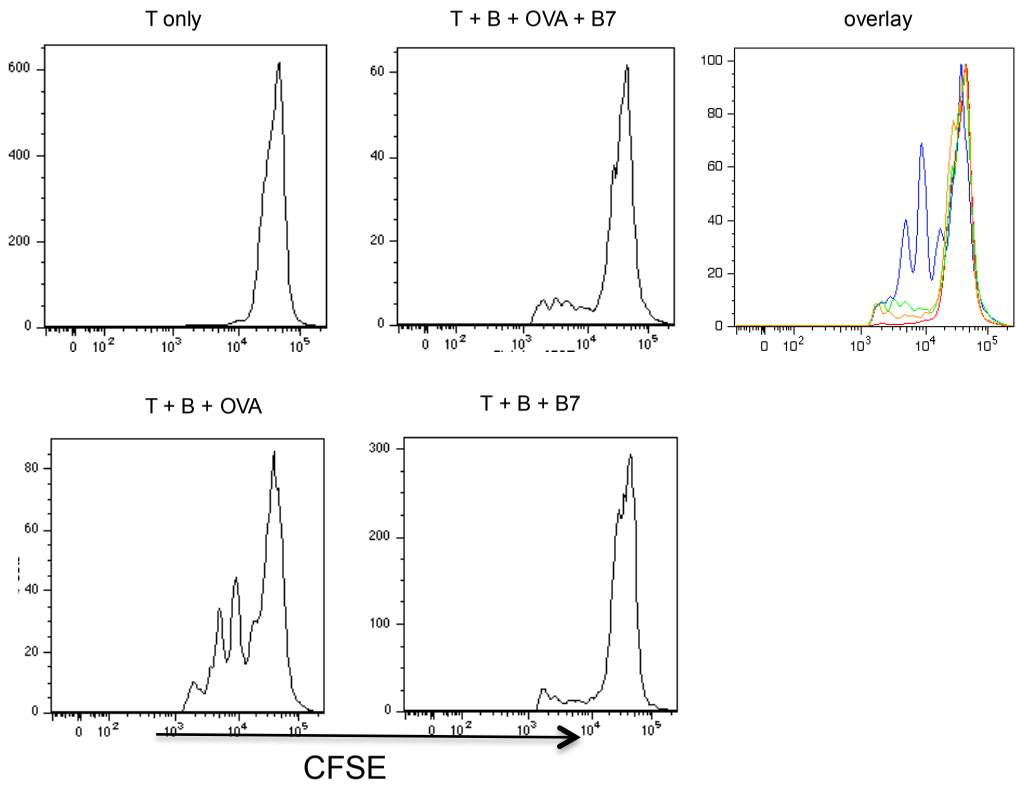
Figure 1-4. The role of B7 molecules in Treg induction by Fo and B-1 B cells. (A-C) 100,000 naïve CFSE-labeled T cells were co-cultured with B-1 or FO B cells in the presence of B7.1 and B7.2 blocking antibodies. Cells were analyzed by flow cytometry on day three for (A) B7.1 expression among B-1 (blue) and FO (green) cells; (B) percent of total CD4$^+$ T cells expressing FoxP3; (C) percent of CD4$^+$T cells that remained undivided for B-1 (blue) and FO (green), without block (light color) and with B7 block (dark color). (D) CFSE-labeled T cells were co-cultured at equal concentrations with B1 (green), FO (blue), or both (purple), without (light) or with (dark) B7 block. T cells were analyzed on day 3 for FoxP3 expression. (E) Representative plots for T cells from B, D. Numbers indicate mean values for one experiment. Data in B-D represents duplicate wells. Data in D are the mean ±SEM. Data corresponds to one experiment which is representative of at least two independent experiments.



Supplemental Figure 1-1. IL-6 and IL-10 ELISA data indicates no differences between B cell subsets. 100,000 naïve CFSE-labeled T cells were co-cultured with B-1 (blue), MZ (red), or FO (green) B cells at varying dilutions in the presence of OVA, TGFβ and IL-2. Supernatants were analyzed for IL-6 (A) and IL-10 (B) via ELISA. IL-6 Elisa level of detection is 0.0625ug/mL. Data representative of two independent experiments



Supplemental Figure 1-2. PD-1 and CTLA-4 expression by CD4+ T cells is not different among subsets. $CD4^+$ T cells were analyzed by flow cytometry 3 days after *in vitro* culture with B-1 (blue) or FO (green) B cells plus IL-10, TGFβ, and OVA peptide for surface expression of PD-1 (A) and CTLA-4 (B). Data representative of at least two independent experiments. All data are the mean ±SEM



Supplemental Figure 1-3. B7 blockade of CD4+ T cell stimulation. CFSE labeled T cells were cultured for three days with whole splenic B cells with or without OVA peptide and/or B7.1/B7.2 blockade. Cells were analyzed for CFSE dilution. Data are representative of one experiment.

Discussion

To our knowledge, the relative efficiency of B cell subsets in Treg induction was unknown prior to this study. Herein we reveal differences between MZ, FO, and B-1 B cells in their ability to induce Tregs *in vitro*. Similar to previous reports, our results indicate a clear correlation between expression of co-stimulatory molecules on APC and efficiency of FoxP3 induction in naïve $CD4^+$ T cells (Wang, 2008; Sun, 2008). The FO B cell subset expresses significantly lower levels of B7.1 and PD-L1, yet is consistently superior at converting naïve $CD4^+$ T cells to Tregs in our system. In contrast to FO B cells, B-1 B cells are much less efficient at Treg induction and have significantly higher expression of the co-stimulatory molecules. The MZ subset is intermediate in both co-stimulatory molecule expression and FoxP3 induction.

That MZ B cells have an intermediate phenotype between FO and B-1 B cells is interesting, in that MZ B cells share features of both subsets in regards to BCR specificity, location, and lifecycle. The B-1 subset is self-renewing in the PC and has a somewhat restricted V-gene repertoire enriched for specificities for T-independent (TI) antigens (Allman, 2004). In direct contrast, FO B cells are derived from hematopoetic precursors throughout life and enriched with specificities for T-dependent antigens. While MZ B cells have a similar developmental origin to that of FO B cells, they share many functional properties with B-1 B cells, including TI antigen specificity and self-renewal once they are recruited to the MZ phenotype (Allman, 2004; Hao, 2001).

In our study, FO B cells had the highest conversion rates when cultured at 1:1 dilution with naïve T cells, while B-1 B cells became more efficient as their concentration was lowered (Fig. 2). Initially, it was surprising that FO and B-1 subsets have divergent dose-response curves for Treg induction. While one might expect decreased efficiency as the APC is diluted, our B-1 subset data resembles results published by Chen et al. (Chen 2007). In that study, total splenic $CD4^+$ T cells were cultured at different ratios with allogenic B cells. When the ratio of B:T cells was highest, there was increased overall T cell proliferation but impaired Treg expansion. Indeed, in our system, T cells cultured with B-1 B cells at 1:1 underwent more total T cell proliferation than at the 1:8 dilution, despite having at least 50 percent fewer $FoxP3^+$ cells at day 3 (Fig. 2B,C).

Chen et al. found that the increased efficiency of Treg induction in cultures with a lower ratio of B:T cells was reduced by stimulatory anti-CD28 (Chen 2007). In our studies, B7 blockade lead to an increase in Tregs induced by B-1 B cells at all concentrations (Fig. 4). Despite no significant difference in B7.2 expression among the subsets, B-1 B cells have much higher B7.1 MFI than FO B cells (Fig. 1). It follows that limiting the signaling through CD28 by blocking B7 should and did enhance Treg induction by the B-1 subset. Interestingly, co-culturing both B-1 and FO subsets with $CD4^+$ T cells resulted in greatly reduced Treg induction compared to FO subset alone. Treg induction was further increased by B7 blocking antibodies (Fig. 4D). Together, these results indicate that excess B7 in the culture inhibits Treg induction, likely due to CD28 stimulation. This analysis corresponds well to the CTLA-4 ligation competition model proposed by Fife et al. (Fife 2008). In this model, CTLA-4

ligation prevails over CD28 in conditions with limiting B7. In our system, limiting B7 occurred in cultures with FO B cells, low concentrations of B-1 B cells, or B-1 B cells in the presence of B7 blockade. The out-competition for B7 binding by CTLA-4 is due to its higher binding affinity. It follows that when there is excess B7, CD28 signaling would prevail over CTLA-4, leading to increased $CD4^+$ $FoxP3^-$ T cell division and decreased FoxP3 induction.

That said, previous studies indicate that signaling through CD28 is necessary for FoxP3 induction in naïve $CD4^+$ T cells (Golovina, 2008). Additionally, Tregs that express TCRs that react with self-antigen have been shown to respond in a co-stimulation dependent manner to maintain homeostasis (Tang 2003). In contrast to our findings with the B-1 subset, we found that B7 blockade had no consistent effect on number or percentage of Tregs induced by FO B cells. It is unclear whether the small amount of B7.1 on FO B cells is signaling through CD28, CTLA-4, both or neither. However, our results indicate that B7 expression by FO B cells is minimal enough to limit CD28-driven $CD4^+FoxP3^-$ T cell proliferation. Future experiments will be necessary to determine the role of B7 in the mixed B cell experiments. For example, utilizing B7 knock-out mice, it would be interesting to co-culture B7 deficient B-1 B cells with wild type FO, and vice versa, to determine the impact of B7 expressed by each subset in Treg induction.

In addition to B7.1, B-1 B cells express the highest levels of PD-L1 among the subsets. Blockade of PD-L1 in B-1 cultures caused a drastic decrease in number and percentage of FoxP3+ Tregs, an effect that only occurred at 1:4 and 1:8 dilutions

(Fig. 3B). The FO subset, which expresses ten times less PD-L1 at steady state, experienced a reduction in Treg conversion at all dilutions with PD-L1 block. Utilizing PD-L1 coated beads and CD4+ T cells in an *in vitro* culture system, Francisco et al. found that PD-L1 regulates not only FoxP3 expression, but also maintenance and function of Tregs (Francisco 2009). These results call into question the role of PD-L1 in our system. For example, it remains unclear whether PD-L1 on each B cell subset is responsible for Treg induction, survival, or both.

I proposed above that increased expression of B7 on B-1 B cells is responsible for poor Treg conversion by the subset due excess CD28 signaling. Once the B7 concentration in the culture is lowered either by altering the dilution of B-1 B cells or utilizing B7 block, PD-L1 signaling may come into play with regards to induction and/or maintenance of Tregs. Further studies will be required to confirm this theory. To this end, PD-L1 knock out mice might be utilized for further *in vitro* experiments. For example, one might co-culture purified iTregs with PD-L1 knockout or wild-type B cells of each B cell subset and look for survival and maintenance of FoxP3 expression.

One main source of iTregs *in vivo* is the gut-associated lymphoid tissue (GALT) as a consequence of oral tolerance mechanisms. Indeed, the role FoxP3+ Tregs in ameliorating immune-mediated disorders of the intestine including Crohn's disease and ulcerative colitis has been an area of intense study. These disorders are associated with inflammatory bowel disease (IBD), and share a disordered $CD4^+$ T cell response to commensal enteric bacteria (Strober, 2002). Therefore, it is an

interesting possibility that Tregs engaged in suppression of IBD may be induced or maintained by the B cells that are located in the GALT (Brandtzaeg 2005, Fagarasan 2003). Although our study utilized B cells from the spleen and PC, the subsets analyzed can also be found in the GALT. For example, the small intestine represents a major source of IgA^+ plasma cells, approximately half of which originate from PC B-1 B cells (Shimomura 2008a). In addition to plasma cells, IgM^+ B cells exist within the normal intestine and $CD19^+$ $CD23^+$ FO-like B cells are located in isolated lymphoid tissues (Shimomura 2008a, Velazquez 2005).

Several studies have implicated B cells in Treg induction in the GALT. Utilizing B cell deficient uMT mice, Gonnella et al. found that both CD86 and B cells are important for the production of TGFβ that is required for oral tolerance in the GALT (Gonnella, 2006). Indeed, distinct regions of the GALT up-regulate TGFβ, IL-4, and IL-10 in response to orally administered OVA. Interestingly, Shah et al. found that while RNA for TGF-β1 is increased upon B cell activation, resting B cells express the less-studied TGF-β3 (Shah, 2008). Similar to previous studies, Shah et al. found that resting B cells are superior at expanding Tregs *in vitro,* which was overcome by addition of exogenous TGF-β3 to stimulated B cell cultures. This is in contrast to DCs, which have been shown to provide TGF-β1 to naïve $CD4^+$ T cells during FoxP3 induction (Luo, 2007). From these data, it is apparent that there may be several mechanisms of immune regulation occurring in the GALT. Supporting a role for B cells in GALT regulation, Wei et al. protected mice from $G\alpha i2^{-/-}$ $CD4^+$ T cell-induced colitis by transferring mesenteric lymph node B cells (Wei, 2005). In this model,

protection required the transfer of B cells in conjunction with regulatory FoxP3^{-} CD8α^{+} T cells.

The role of B cell subsets in Treg induction and maintenance is defined by not only antigen presenting potential but also migratory patterns. Previous reports have indicated that expanding human Treg cells with allogenic APCs can yield a population that is enriched for alloantigen specificity (Cohen, 2002). Upon transfer, these APC-expanded Tregs have more therapeutic activity and decreased potential to induce nonspecific immunosuppression in animal models (Trenado, 2003). This, paired with previous studies identifying Treg subsets based on homing receptors, leads one to the possibility of using B cell subsets to induce Tregs with homing properties (Szanya, 2002; Uhling, 2006). To this end, future studies might investigate the efficiency of expanding Tregs *in vitro* using the B-1 B cell subset for the purpose of inducing a PC-homing suppressive population. In support of this, a recent report by Chen et al. shows that human FoxP3^{+} Tregs can be efficiently expanded by allogenic B cells and are capable of inhibiting pathogenic T cells at very low Treg to T effector cell ratio (Chen, 2009).

The increased autoreactive BCR repertoire in the B-1 B cell compartment may potentially impact Treg induction and homeostasis. Previous studies have clearly demonstrated that antigen-specific Tregs are significantly more efficient than polyclonal Treg cells in suppressing autoimmunity (Masteller, 2006). Similarly, mouse alloantigen-specific Treg cells are proven to be more effective in preventing GVHD than are polyclonal Treg cells (Cohen, 2002; Trenado, 2003). Therefore, naïve

T cells specific for glycoproteins and peptides associated with branched carbohydrates may be converted to FoxP3$^+$ Tregs by B-1 B cells specific for these antigens and, in conjunction with natural antibodies produced by the B-1 B cells themselves, play a role in immune homeostasis. This theory is supported by a recent study from Shimomura et al., in which the hygiene hypothesis is tested by housing colitis-prone TCRα knockout mice in either a specific pathogen-free (SPF) or conventional facility (CV) (Shimomura 2008b). Interestingly, the study showed decreased incidence of colitis in the CV facility. Colitis in the SPF housed mice could be ameliorated by adoptive transfer of B-1 B cells (Wei, 2005). Although Shimomura et al. implicated natural autoantibodies as the cause of decreased colitis in the CV facility mice, it is possible that in wild type mice, B-1 B cells are responsible for the induction or maintenance of Tregs that suppress pathogenic T cells involved in colitis. Although our study shows decreased Treg induction by B-1 B cells as a whole subset, the proficiency of Treg conversion increased as the concentration lowered. Physiologically, the lower dilutions may be more relevant. Further studies that investigate the B-1 B cell subsets are necessary. The possible role of CD5$^+$ B-1a B cells in Treg induction is particularly interesting, as this B-1 subset has been implicated in autoimmune disorders and is the primary source of natural antibodies.

In conclusion, our study elaborates on previous reports that implicate B cells in Treg homeostasis. We find that, in addition to expression of B7 and PD-L1, the concentration of each subset effects the efficiency of Treg induction.

CHAPTER 3. THE ROLE OF TRANSITIONAL B CELLS IN T CELL TOLERANCE

Introduction

T cell tolerance occurs throughout the lifespan of the lymphocyte and involves different outcomes. Autoreactive thymocytes undergo clonal deletion, and in the periphery the T cell may undergo induction of a dominant Treg phenotype, apoptosis or functional anergy. The latter form of tolerance results in a silenced, or inactivated T cell that might otherwise partake in pathogenic immune responses. Studies indicate that T cell anergy occurs as a result of TCR stimulation in the absence of co-stimulation induced by infection or tissue damage (Jenkins, 1990). It follows that healthy tissue is tolerogenic rather than immunogenic. APCs activated by infection and/or tissue damage provide co-stimulation required for T cell activation (Yuschenkoff, 1996). Co-stimulation from DCs, macrophages, and activated B cells induces clonal expansion, differentiation, and survival of T cells. In contrast, low expression of co-stimulatory molecules on resting B cells engenders potent tolerizing capability (Eynon, 1992; Lin, 1991). Yuschekoff et al. studied the role of reduced co-stimulation on resting B cells by utilizing mice transgenic for membrane-bound human μ-chain (Yuschenkoff, 1996). The transfer of μ-chain expressing B cells to wild type mice led to T cell tolerance to further challenges with human μ-chain plus adjuvant. When B cells that expressed both μ-chain and B7.1 were transferred, the T cells were not tolerant, and induced an antibody response following challenge.

Further studies have shown that CD40 and other TNF receptor (TNFR) family molecules impacts T cell tolerance (Buhlmann, 1995; Lopes-Carvalho, 2005). Lathrop et al. utilized 102S transgenic mice that express antigenic cytocrome c (CytC) on MHCII. Upon transfer to 102S transgenic mice, antigen-specific AND TCR transgenic T cells proliferate and accumulate in the spleen as anergic T cells (Lathrop, 2004). However, the administration of an agonistic antibody against TNFR-family member OX40 causes the T cells to differentiate into pathogenic effector cells. This and other studies indicate that co-stimulation through the B7-CD28 axis is insufficient for an effective T cell response. T cell stimulation through TNFRs, including OX40, 4-1BB, CD27, CD30, and HVEM can contribute to T cell activation, proliferation, and survival (Croft, 2009). Although the ligands for most of these molecules are not constitutively expressed by resting or immature APCs, they are induced following activation and are potentially available to responding T cells within several days of antigen encounter (Croft, 2003). Additionally, soluble cytokines including IL-12, IL-6, IL-1, and others support T cell activation. All together, these studies indicate that availability of B7 co-stimulation, membrane-bound cytokines of the TNF family, and soluble cytokines determine T cell fate.

In addition to small resting B cells, tolerant Tr B cells are the perfect candidate for T cell tolerance induction. For example, Tr B cells that have become anergic are more likely to have an autoreactive BCR, and have encountered antigen that can be taken up and presented to T cells. In fact, maintenance of B cell anergy requires constant antigen receptor occupancy and signaling, indicating that anergic Tr B cells have continuous access to antigen (Gauld, 2005). Further, Cyster et al. found that anergic

Tr B cells are excluded from splenic follicles, to the outer PALS region, where they may have increased opportunity to interact with T cells along the T-B boundary (Cyster, 1994). Finally, although Tr B cells can effectively take up antigen by their BCRs and present it on MHCII to T cells, they are incapable of productive T-B collaboration due to defective BCR signaling and insufficient B7.2 expression (Rathmell, 1998). Taken together, these features of Tr B cells may allow them to induce T cell tolerance in a healthy individual.

Several models have emerged to study Tr B cells. Along with the previously mentioned MD4/ML5 HEL transgenic model, transgenic B cells specific for azophenyl-arsonate (Ars) were utilized in the present study. In the Ars transgenic model, B cells have moderate affinity for the Ars hapten, but the BCR cross-reacts with 100 times lower affinity to single stranded DNA (Benschop, 2001) Because DNA is readily available in the periphery due to cell death, Ars B cells encounter self-antigen during their Tr B cell stage. For this reason, they progress through T1 and T2 stages, and hit a developmental block at T3 (Merrell, 2006). Therefore, the peripheral B cell population in Ars mice is greatly enriched in T3 cells.

To study the role of Tr B cells in T cell tolerance, we utilized the 102S transgenic model in which antigenic peptide CytC is covalently linked to MHCII (IE^k) and expressed at low levels on all MHCII positive cells (Lathrop, 2004). The transfer of 102S B cells into an IA^B host ensures that AND T cells specific for CytC on IE^k do not recognize antigen taken up by endogenous APCs. Seven days following adoptive transfer of small amounts of pre/pro B cells expressing CytC-IE^k and purified naïve

antigen-specific AND TCR transgenic T cells, we harvested the T cells for analysis. By day seven, pre/pro B cells had developed through T1, T2, and T3 or FO stages. T cells from animals with CytC-IE^k B cells underwent division and were tolerant following secondary stimulation *in vitro.* To confirm the tolerance was induced by Tr rather than FO B cells, we utilized Ars transgenic B cells expressing CytC-IE^k, which produce few FO B cells (Merrell, 2006). B cells derived from Ars mice induced anergy in antigen-specific T cells, indicating that Tr B cells are indeed capable of interacting with T cells and inducing tolerance.

Methods

Mice. All mice were housed under specific pathogen free-conditions. These studies were approved by the Institutional Animal Care and Use Committee at the Oregon Health & Science University. Thy1.1, ML5, C57B6 and CD45.1 congenic mice were obtained from the Jackson laboratories (Bar Harbor, ME). CytC-IE^{k} (102S) transgenic mice were generated and bred to C57BL/6 in-house (Lathrop, 2004). AND TCR transgenic mice (Kaye, 1992) specific for CytC-IE^{k} were obtained from Steve Hendrick via P. Marrack and bred in house to C57BL/6 Rag-1$^{-/-}$ mice from the Jackson laboratories (Bar Harbor, ME). ArsA1 (ALK) transgenic mice were generously provided by Lawrence Wysocki. MD4 transgenic mice were generously provided by Jason Cyster.

Antibodies and Reagents. Easy Sep mouse CD4+ T cell and B cell enrichment kits a re from Stemcell Technologies (Vancouver, Canada). Biotin-anti-E4 (ArsA1 BCR) was a generous gift from L. Wysocki. PE/Cy7-anti-CD19, APC-anti-SA, APC/AF750-anti-strepavidin, APC-anti-CD93 (C1fqRp), FITC-anti-CD23 (FceRII), PE-anti-CD21/CD35, PE/Cy7-anti-CD4 (L3T4), PerCP/Cy5.5-anti-CD45.2, PerCP/e710-anti-CD4 (L3T4), APC-anti-Thy.12, PE/Cy7-anti-Thy1.1, PerCP/Cy5.5-anti-CD19, PB-anti-CD21 were purchased from eBioscience (San Diego, CA). FITC-anti-B220 (CD45R), biotin-anti-CD11b, PE-anti-CD11c, PE-anti-Vβ3, biotin-anti-Vα11, FITC-anti-IgM, FITC-anti-Igκ, FITC-anti-Igλ, PE/Cy7-anti-CD117, biotin-anti-CD117, biotin-anti-IE^{k}, biotin-anti-IA^{b} were purchased from BD Biosciences (San Jose, CA). Cell Trace CFSE kit was

purchased from Invitrogen (Carlsbad, CA). Thymidine [-methyl-3H-] was from PerkinElmer (Waltham, MA).

Pre/Pro and FO donor B cell harvest. Bone marrow and spleens were harvested from donors and B cells enriched by magnetic cell sorting (MACS). BM samples from wild type or 102S were stained and sorted with a FACS Vantage cell sorter (BD Biosciences, San Jose, CA) for $Ig\kappa^{-}Ig\lambda^{-}CD117^{-}CD19^{+}B220^{lo}$ pre/pro B cells. BM samples from Ars mice were stained and sorted for $CD117^{-}CD19^{+}B220^{lo}$ because there was not a clear $Ig\kappa^{-}$ $Ig\lambda^{-}$ population due to early BCR transgene expression. Spleen samples were stained and sorted for $CD19^{+}CD93^{lo}CD23^{+}CD21^{lo}$ FO B cells. Purified cells were washed and injected IV ($400\text{-}800x10^{3}$ cells per recipient).

AND Tg T cell harvest. Spleen and lymph nodes were harvested from AND $Rag^{-/-}$ mice. A small aliquot was analyzed for percent transgenic T cells, identified as $CD4^{+}$ $V\beta3^{+}$ (FACS Calibur, BD Biosciences, San Jose, CA). The remaining cells were labeled with CFSE, washed, and injected 2 days following BM transfer, or 3 days following FO B cell transfer IV at $1.2\ x10^{6}$ transgenic T cells/recipient.

T cell harvest and in-vitro proliferation assay. 7 days following transfer of AND transgenic T cells, spleens were harvested and CD4+ T cells were purified by MACS. A small aliquot of each recipient spleen was analyzed for percent transgenic T cells following easy separation (ES-MACS), identified as $CD4^{+}V\beta3^{+}Thy1.1^{-}$ by FACS Calibur. A small aliquot of spleens prior to ES were analyzed for CFSE dilution, B cell recovery, and non-B cell APC recovery. 8,000 post-ES transgenic T cells from each recipient were co-cultured with 300,000 (Fig. 2) or 800,000 (Fig. 3) C57BL/6 or

102S transgenic APCs that had been irradiated with 1000rads in complete RPMI. On day 5 (Fig. 2) or day 4 (Fig. 3), 1μCi of ^{3}H-thymidine was added to each well. One day later, plates were harvested and ^{3}H-thymadine uptake was analyzed by scintillation counting (TopCount, Packard Instrument Company, Meriden, CT).

Pre/pro B cell developmental time course assay. BM was harvested from C57BL/6 or Ars transgenic mice and B cell enriched by MACS. BM samples from wild type or 102S transgenic were stained and sorted with FACS Vantage (BD Biosciences, San Jose, CA) for $Ig\kappa^{-}Ig\lambda^{-}CD117^{-}CD19^{+}B220^{lo}$ pre/pro B cells. Purified cells were washed and injected IV into CD45.1 congenic mice (400-800k cells per recipient). On day 2, 4, 6, and 11, spleens were harvested and analyzed for CD19, CD93, CD21, and IgM to determine the developmental stage of transferred cells.

Results

Purified pre/pro B cells develop through transitional stages in-vivo after adoptive transfer. To study the role of transitional B cells in peripheral T cell tolerance induction, we developed a model in which small numbers of transgenic B cells could be followed through development. To this end, we sought to confirm that adoptively transferred pre/pro B cells would develop through transitional stages to mature B cells similar to endogenous cells. Purified $CD19^{+}B220^{lo}Ig\kappa/\lambda^{-}CD117^{-}$ pre/pro B cells from the BM were adoptively transferred to wild type recipients (Fig. 1A). Two, four, and six days following transfer, recipient spleens were harvested and analyzed. At two days post-transfer, the majority of transferred cells were $CD93^{hi}$ T1 ($IgM^{hi}CD23^{lo}$) phenotype (Fig. 1B). Over the course of seven days, the transferred cells clearly developed, and by day six the majority were mature $CD93^{lo}$. That said, the percentage donor cells that were $CD93^{hi}$ at day six remained higher than the recipient B cell population. From this preliminary data we conclude that purified pre/pro B cells develop through transitional stages and into mature B cells in a physiological manner.

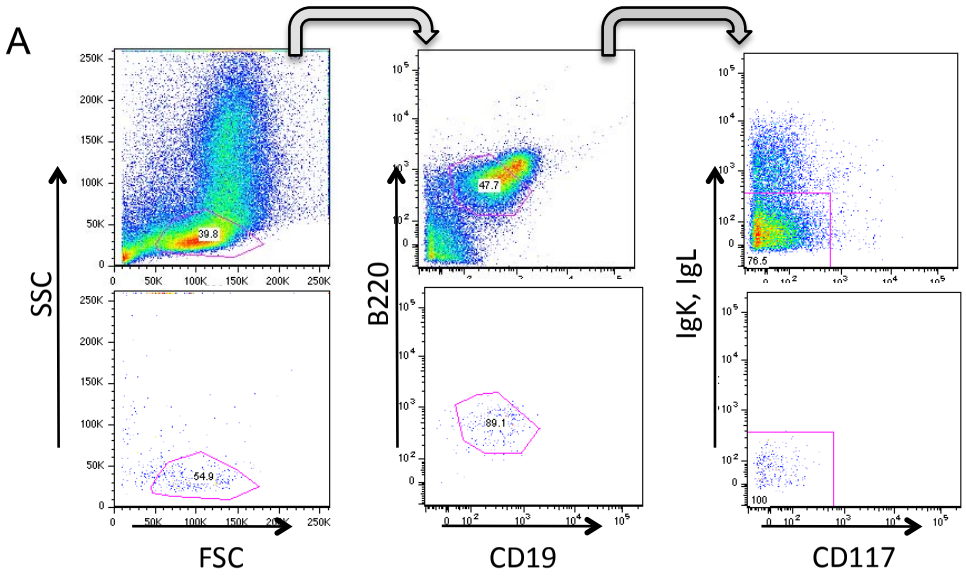
Follicular and Transitional B cells induce antigen-specific T cell division and tolerance to secondary stimulation. We next utilized 102S transgenic mice that express IE^{k}-CytC as donors. Previously, our lab showed that 102S FO B cells can induce anergy in a similar model (unpublished data). Therefore, we adoptively transferred purified 102S FO B cells as a positive control group on day zero. On day one, we transferred 5-800,000 purified 102S or wild type pre/pro B cells to separate groups. Two days

later, all animals received $1.3x10^6$ CFSE-labeled AND T cells that express a TCR specific for IE^k-CytC. Seven days later, spleens were harvested and analyzed for donor AND T cell division (Fig. 2A). Compared to mice that received wild type pre/pro B cells, AND T cells from the FO B cell recipients underwent considerable proliferation (Fig. 2B). The greatest amount of proliferation occurred in AND T cells from recipients of 102S pre/pro B cells that developed into Tr B cells. 58.7% of AND T cells from control, 25.7% from FO, and 14.8% from Tr groups remained undivided. From this data, we conclude that although both B cell subsets induce T cell division in an antigen-specific manner, Tr B cells are superior to FO at inducing division.

To determine the tolerance inducing capabilities of Tr B cells, we enriched the AND T cells after seven days *in vivo* with the B cells for an *in vitro* proliferation assay. 300,000 irradiated splenic APCs from 102S or wild type mice were co-cultured with 100,000 AND T cells. ^{3}H-thymidine was added at day four of *in vitro* culture, and incorporation was analyzed at day five. AND T cells from control mice underwent more proliferation in response to 102S APCs than wild type APCs, as evidenced by their stimulation index of eight (Stimulation index is defined as 102S CPM divided by wild type CPM) (Fig. 2C). In contrast, 102S APCs did not induce more proliferation among AND T cells from both FO and Tr groups, as indicated by their stimulation indexes near one. Figure 1 shows that greater than 50% of cells on days 4 and 6 are no longer $CD93^{hi}$ transitional. From this, we conclude that tolerance could be due to Tr B cells or induced solely by FO B cells in the pre/pro B cell

transfer. Therefore, we went on to utilize the Ars transgenic model, which should yield few FO B cells following pre/pro B cell transfer.

Ars transgenic model confirms Tr B cell capability to induce T cell tolerance. To confirm that Tr B cells can induce T cell tolerance, we next utilized the Ars transgenic model, in which more than 75% of B cells in the periphery in steady state are blocked at the Tr B cell stage (Merrell, 2006). We purified 675,000 pre/pro B cells from 102S, 102S/Ars, or Ars mice and transferred them to wild type recipients. Two days later, 1.2 x 10^6 purified AND T cells were adoptively transferred, and seven days later the spleens were harvested. More than 60 percent of AND T cells from control mice that received Ars cells remained undivided (Fig. 3B). Similar to our previous data, AND T cells from the102S only group underwent more division. Likewise, 102S/Ars donor cells induced AND T cell division comparable to the 102S group. We saw clear differences between the groups following co-culture of 100,000 AND T cells with 800,000 irradiated 102S or wild type APCs for five days. Compared to the Ars control group, both 102S groups induced tolerance (Fig. 3c). Interestingly, the 102S cells, which develop through Tr stages and into mature B cells, had a higher stimulation index than the 102S/Ars group that should only have Tr B cells. Although these results represent a single experiment that needs to be repeated, they indicate that Tr B cells are at least as effective as FO B cells at inducing T cell tolerance.



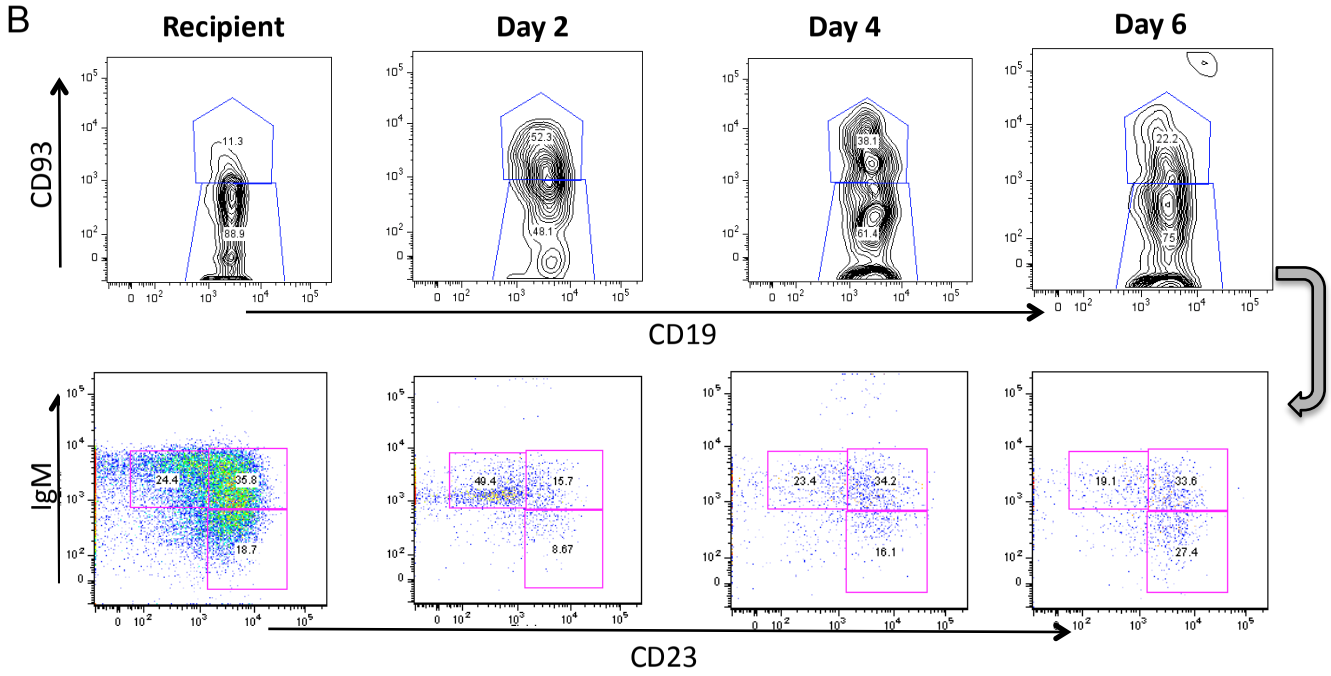


Figure 2-1. Purified pre/pro B cells develop through transitional stages after adoptive transfer. (A) Gating scheme (top) and post-sort purity (bottom) for purification of pre/pro B cells from BM. Lymphocytes were gated using fsc and ssc (left), then sorted for $CD19^{+}B220^{lo}$ (middle), and $Ig\kappa^{-}Ig\lambda^{-}CD117^{-}$ (right). 500-750 thousand congenically marked pre/pro B cells were adoptively transferred into syngenic recipients. On day 2, 4, and 6, recipient spleens were analyzed for donor cell phenotypes (B). Immature B cells ($CD19^{+}CD93^{hi}$) (top) were analyzed for T1 ($IgM^{hi}CD23^{lo}$), T2 ($IgM^{hi}CD23^{hi}$) and T3 ($IgM^{lo}CD23^{hi}$) (bottom). Plots representative of at least two animals per group, 2 independent experiments.

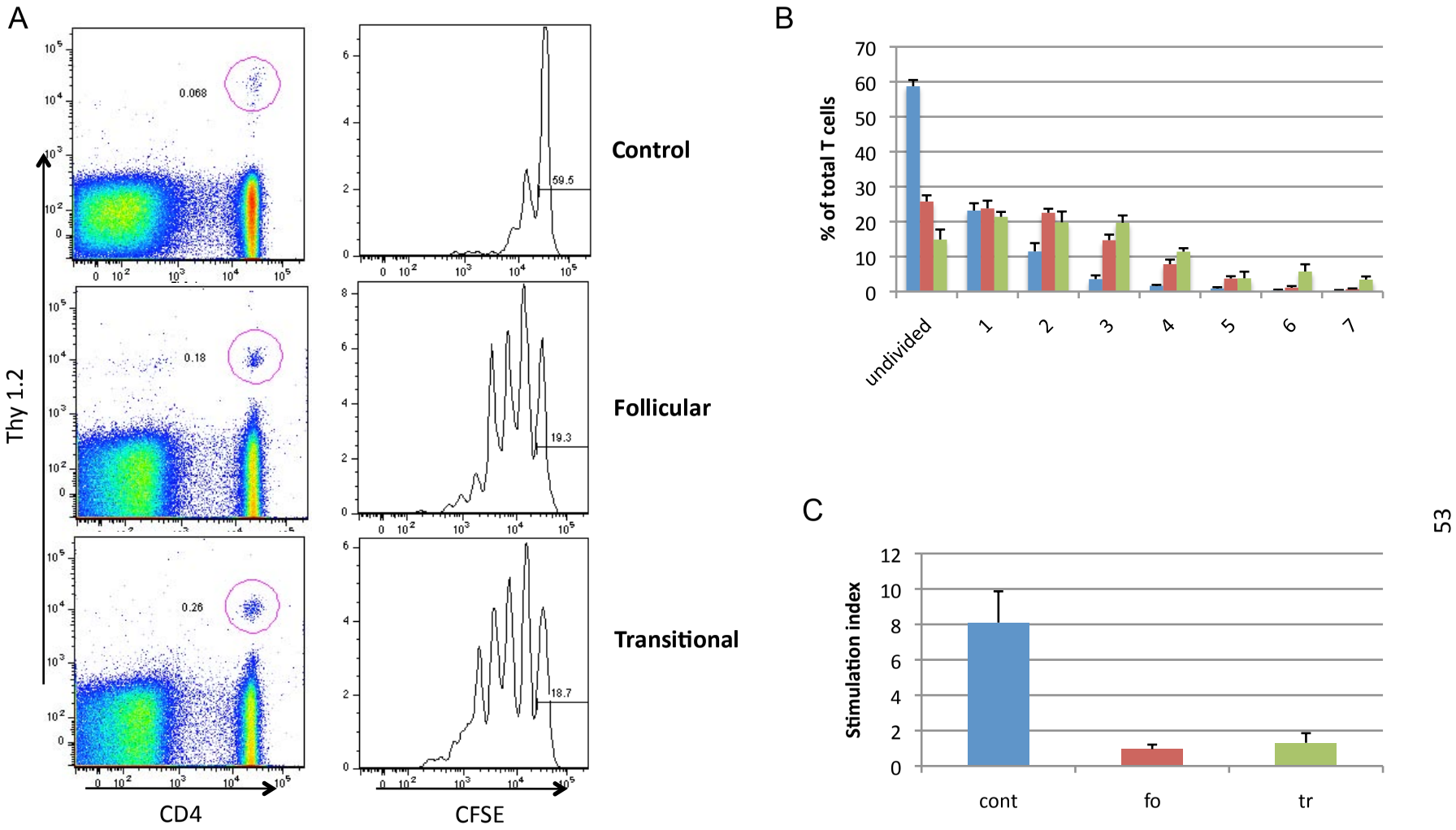


Figure 2-2. Follicular and transitional B cells induce antigen-specific T cell division and tolerance to secondary stimulation. (A &B) 1.2 x 10^6 naïve $CD4^+$ AND Tg T cells were adoptively transferred at day 2 following transfer of 500-800,000 102S follicular B (middle), 102S pre/pro B (bottom) or wild type pre/pro B control (top). (A) 7 days after transfer, T cells were analyzed for T cell division by CFSE dilution. Spleen cell recovery, left, gated on lymphocytes. CFSE dilution, right, gated on $CD4^+Thy1.2^+V\beta3^+$ population. (B) Percent transgenic T cells that were undivided or underwent 1-7 division from control (blue), FO (red) or TR (green) groups. (C) 7 days after transfer, transgenic T cells were enriched and co-cultured with irradiated 102S or wild type splenocytes. At 5 days of culture, tritiated thymidine was added, and incorporation was analyzed on day 6. Stimulation index, defined by 102S cpm divided by control cpm, for each group. Data are representative of two independent experiments, at least 3 animals per group. C represents at least three wells per condition. All data are the mean ±SEM

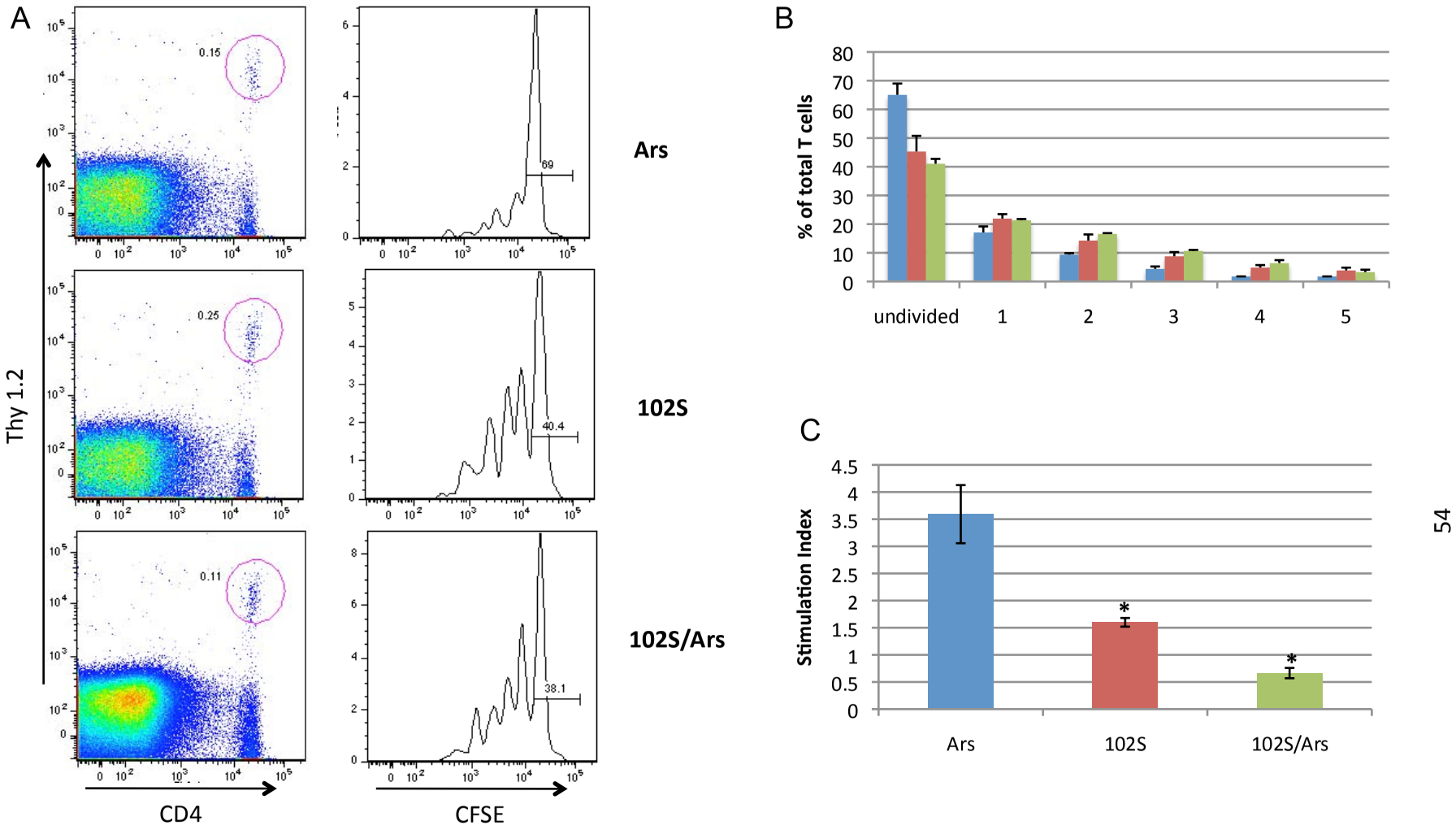
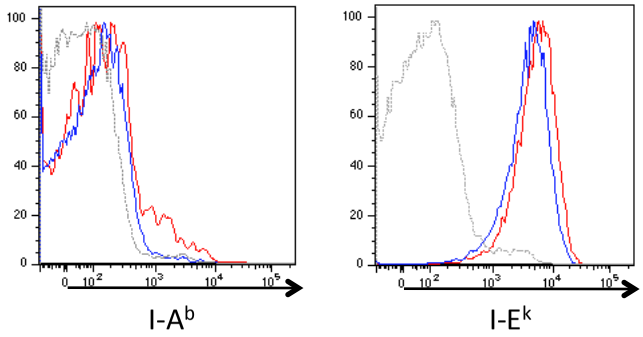


Figure 2-3. Ars trangenic model confirms Tr B cell capability to induce T cell tolerance. (A &B) 1.2 x10^6 naïve $CD4^+$ AND T cells were adoptively transferred at day 2 following transfer of 735,000 Ars (top), 102S (middle) or 102S/Ars cells (bottom). (A) 7 days after transfer, T cells were analyzed for T cell division by CFSE dilution. Spleen cell recovery, left, gated on lymphocytes. CFSE dilution, right, gated on $CD4^+Thy1.2^+V\beta3^+$ population. (B) Percent transgenic T cells that were undivided or underwent 1-7 division from Ars (blue), 102S (red) or 102S/Ars (green) groups. (C) 7 days after transfer, T cells were enriched and co-cultured with irradiated 102S or wild type splenocytes. At 4 days of culture, tritiated thymadine was added, and incorporation was analyzed on day 5. Stimulation index, defined by 102S cpm divided by control cpm, for each group. Data are representative of one experiment with at least three animals per group. C represents at least three wells per condition. All data are the mean ±SEM. *, $P<0.01$ by student's *t* test between 102S and 102S/Ars.



Supplemental Figure 2-1. Expression of IEk and IAb on transitional and mature B cells. B cells were isolated from the spleen and analyzed by flow cytometry for I-A^b (left) and I-E^k (right) on $CD93^{hi}$ transitional (red) and $CD93^{lo}$ mature (blue) B cell subsets. Black shows staining on $CD19^-$ lymphocytes. Data representative of one experiment, with four animals.

Discussion

Tolerance mechanisms that occur in the BM to purge the repertoire of auto-reactive B cells are incomplete (Allman, 2004). Therefore, potentially pathogenic B cells continuously exit the BM, and must compete with other B cells for survival factors such as BAFF (Thein, 2004). Indeed, the level of BAFF that is required for survival is different for each Tr B cell, determined at least in part by BCR reactivity (Hodgkin, 1995). This is illustrated by the Ars mouse model, in which B cells with specificity for single stranded DNA escape deletion in the BM, develop into Tr B cells, and encounter a developmental block at T3 due to the presence of auto-antigen in the periphery. Despite being at a developmental dead end that cannot be rescued by excess BAFF, T3 cells remain alive in the periphery for up to 4-5 days (Teague, 2007).

Tolerant B cells, such as DNA-specific Ars cells, accumulate along the T/B border due to follicular exclusion (Cyster, 1994). Consequently, tolerant Tr B cells are afforded the opportunity to interact with T cells. Although we did not determine the homing of our transferred B cells, our CFSE dilution data indicates division among our AND T cells, supporting the notion that T cells do indeed encounter and recognize antigen on rare Tr B cells (Figures 2A and 3A). Our data correlates with a study published by Townsend et al., in which B cells from MD4xML5 or wild type mice were loaded with HEL and co-transferred with HEL-specific T cells to wild type recipients (Townsend, 1998). In that study, T cells that were co-transferred with tolerant Tr B cells divided at nearly the same rate as those transferred with antigen-

loaded naïve B cells. Similarly, in our system, T cells that were incubated with 102S B cells proliferated, regardless of ArsA1 BCR expression (Fig. 3A). In contrast, the control groups without 102S expression failed to induce significant T cell division in our system. Although cell division can be an indication of T cell activation, we found that the cells in our system were tolerant to re-stimulation *in vitro* (Fig. 2B, 3B). Our result is indicative of tolerance owing to an abortive immune response, in which most proliferating cells die and the surviving cells are tolerant (Kearney, 1994; Lathrop, 2004; Townsend 1998)

Previous studies indicate that the AND T cell encounter with tolerant 102S Tr B cells should not result in productive T-B collaboration leading to T cell activation or an antibody response. Rather, FasL presented by the T cell should induce tolerant Tr B cell death (Rathmell, 1995). Indeed, in the previously mentioned Townsend et al. study, when transferred with naïve TCR transgenic T cells, tolerant B cells disappeared within 60 hours while *lpr* (Fas- deficient) tolerant B cells remained intact (Townsend, 1998). Although we were able to find transferred 102S B cells at day six post-transfer of pre/pro B cells, the Ars B cells disappeared by this time point and no cells were recovered from either group at day nine (day seven post-T cell transfer) (data not shown). Because the majority of 102S cells had differentiated into CD93lo mature B cells by day six, it is probable that most auto-reactive T3 cells had died. It follows that by day six, the majority of Ars B cells should be T3 and encounter the same fate, perhaps due to contact with the co-transferred AND T cells. Further experiments with and without T cells are required to determine this.

This suggests a possible role for the FO B cells that develop from 102S donor cells and out-live the Tr B cells. Our experiment with Ars/102S donors indicates that Tr B cells have an equal or increased capacity induce tolerance in T cells over FO B cells. That is, the T cells from the Ars/102S group had a lower stimulation index than those from the 102S only group, the difference between groups being the developmental block at T3 encountered by Ars cells and not wild type. In our system, it is possible that the acutely transferred FO B cells are partially activated, leading to increased T cell division. This is supported by previous studies from our lab, in which FO B cells transferred seven days prior to T cell transfer induced minimal T cell division but still induced tolerance as measured by *in vitro* stimulation assay (unpublished data). Further experiments utilizing the Ars/102S transgenic mice as well as MD4xML5 are necessary to confirm our hypothesis. Additionally, it may be interesting to utilize Ars *lpr/lpr* mice, in which the T3 cells would not succumb to FasL-induced cytotoxicity upon initial T cell contact. In this system, increased survival of T3 cells may lead to enhanced T cell tolerance and eliminate any possible role played by dying Tr cells.

Transgenic mice that express the Ars BCR that cross-reacts with single stranded DNA are relevant to systemic lupus erythrematosus (SLE), which is characterized by auto-antibodies against nuclear antigens (ANA) and DNA. The role of B cells in SLE is implicated by a handful of studies utilizing mouse-anti-human CD20 antibody Rituximab. This antibody effectively depletes naïve and memory B cells from the peripheral blood, but is less effective in depleting tissue resident MZ and germinal center B cells. Nonetheless, five studies of Rituximab treated SLE patients reported

decreased ANA titers as well as decreased numbers of activated $CD4^+CD40L^+$ T cells (Lund, 2010). Of interest to our study, lupus-prone mouse models, including MRL/*lpr*, have significantly reduced numbers of T3 B cells. This, paired with the decreased autoantibody titers in Rituximab treated patients, indicates that auto-reactive Tr B cells in lupus-prone mice bypass tolerance and mature into functional FO B cells. If this is the case, in healthy individuals the pathogenic Tr B cells do not mature into FO B cells, but become tolerant T3 cells that have the opportunity to induce T cell tolerance.

In this study, we provide evidence that Tr B cells can effectively induce T cell anergy in an antigen-specific manner. Although further experiments will be required to confirm our findings, they are scientifically intriguing and relevant.

CHAPTER 4. SUMMARY AND CONCLUSIONS

The work herein supports the hypothesis that potentially pathogenic B cells are capable of inducing tolerance to auto-antigens through interactions with T cells. Whether tolerance is induced by B-1 B cells presenting tissue-specific antigens or auto-reactive Tr B cells in the spleen, co-stimulation or lack thereof appears to be the primary dictator of T cell fate. Our results indicate B7 and PD-L1 expression on B cell subsets are responsible for their different Treg induction capabilities. Similarly, anergic Ars Tr B cells, which have decreased levels of B7 relative to wild type, appear to be better than FO B cells at induction of T cell tolerance (Benschop, 2001).

Recent studies have identified two subsets of regulatory B cells. The first, termed B10, are $CD5^{+}CD1d^{hi}$ and represent around 1-2% of splenocytes and 7-8% of PC B cells (Mauri, 2010). A series of reports from Tedder and colleagues have shown B10 cells have the capacity to ameliorate experimental autoimmune encephalomyelitis (EAE) and murine lupus (Watanabe, 2010; Haas, 2010; Yanaba, 2008; Matsushita, 2008). Stimulation of newborn spleen or adult blood and LN cells with TLR2 and TLR4 can induce B10 cell development (Yanaba, 2009). The second regulatory B cell population is termed T2-MZB, based on their phenotypic resemblance to T2 and MZ subsets (Mauri, 2010). These cells are $CD19^{+}CD21^{hi}CD23^{+}CD93^{+}$ and express high levels of CD1d, similar to B10 cells. In contrast to B10 cells, IL-10 production is triggered in T2-MZB cells by CD40 engagement. T2-MZB cells have been shown to suppress the development of chronic colitis, experimental arthritis and EAE (Evans,

2007; Mauri, 2010). Interestingly, Blair et al. found that treatment of mice with agonistic anti-CD40 enriches the pool of Bregs, which are derived from T2 cells (Blair, 2009). Transfer of the anti-CD40-generated T2-MZB cells ameliorates renal disease and prolongs survival of MRL/*lpr* mice.

The existence of a Tr cell-derived regulatory population of B cells that produces IL-10 in response to CD40 ligation indicates a secondary fate for auto-reactive Tr B cells. Fillatreu et al. have proposed a model in which TLR signaling of Tr B cells leads to IL-10 induction, which must be followed by CD40 engagement and BCR ligation in order to establish the Breg phenotype (Fillatreau, 2008). Interestingly, when splenic $CD19^+$ B cells are cultured in the presence of excess BAFF for 72 hours, there are increased numbers of IL-10-producing $CD1d^{hi}CD5^+$ B cells (Yang, 2010). This finding has been extended to *in vivo* models, in which BAFF treatment induced regulatory B10 cells that accumulated in the MZ. This result is paradoxical in relation to previous studies that show excess BAFF can rescue autoreactive B cells from anergy, as well as the pathogenic phenotype of mice engineered to over-express BAFF (Thein, 2004; Mackay, 1999). Although it remains unclear exactly how B cells are selected into the Breg compartment, these studies indicate that the Tr B cells that induce tolerance in our system may be precursors to T2-MZ cells and may potentially go on to regulate the immune response.

Although the bulk of recent Breg studies focus on B-2 cells, $CD5^+$ B-1 B cells are known to have regulatory function as well (Shimumura, 2008). The subset has been shown to produce particularly large amounts of IL-10 following LPS stimulation

(Fillatreau, 2008). This fact, together with data that reveals a protective role for $CD1d^+$ IL-10 producing B cells in colitis, supports our data that implicates B-1 B cells in Treg induction. Indeed, B cells have been found to be important for the maintenance of a protective cytokine environment in the GALT (Wei, 2005). In addition to the GALT, experimental evidence indicates that tissue-specific B cells play a role in Treg generation and maintenance. Mann et al. found that B cell deficient mice have delayed production of $FoxP3^+$ Tregs and IL-10 in the CNS, but not in the peripheral lymphoid organs, in response to EAE (Mann, 2007). Similarly, IL-10 producing $CD1d^+$ regulatory B cells have been implicated in the $FoxP3^+$ T cell response to allergic airway inflammation (Amu, 2010).

Together, these studies indicate that the role of B cells in T cell tolerance depends on several factors. Our *in vitro* Treg induction experiments highlight differences in the innate ability of B cell subsets to induce FoxP3 expression, based on expression levels of B7 and PD-L1. Our *in vivo* tolerance induction studies implicate Tr B cells as effective inducers of T cell anergy, likely due in part to low levels of co-stimulation (Benschop, 2001). Additionally, factors such as efficiency of T/B collaboration and location owing to follicular exclusion may play a role in the efficiency of T cell tolerance induction by Tr B cells (Cyster, 1994; Ho, 1994; Eckland, 2004) It is clear from the plethora of previous work that T cell tolerance induction by B cell subsets is determined not only by co-stimulation, but other factors such as homing, antigen specificity, and availability of secondary stimulation. As further studies work to unravel the complexities of T cell tolerance, it will be interesting to see how the work described herein will fit into the overall picture.

CHAPTER 5. REFERENCES

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