Characterization of differentially activated human B cells and effects of their soluble products on regulatory T cell suppressive function: assay development and design.

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1. Abstract

B cell depletion therapy with rituximab significantly decreases new disease activity in multiple sclerosis (MS) patients; however, these benefits do not correlate with a reduction in circulating or cerebrospinal fluid antibody levels. These findings implicate antibody-independent, pro-inflammatory roles of B cells in MS. Furthermore, in other autoimmune diseases, such as systemic lupus erythematosus (SLE), B cell depletion reportedly resulted in increased function of circulating regulatory T (Treg) cells. Since MS patients have been found to exhibit deficient Treg function, we hypothesized that activated B cells in MS patients can abnormally suppress the function of Treg cells. Therefore, B cell depletion with rituximab allows for restored Treg function, and the prevention of new autoimmune disease activity. In particular, we postulated that the abnormal pro-inflammatory cytokine profile secreted by MS B cells was responsible for defective Treg function. To begin studying the potential relationship between B and Treg cells, I optimized and validated an in vitro human B cell activation assay, as well as a human Treg suppression assay in healthy controls, to subsequently determine the effects of supernatants from differentially activated B cells on Treg suppressive function.

Human B cells were isolated using magnetic-activated cell sorting (MACS), then stimulated with B cell crosslinking antibody (X), CD40 ligand (40), a combination of the two (X40), or CpG-nucleotides. Their supernatants were collected and responses found to support previously published findings. Treg and T responder (Tresp) cells were isolated using both MACS and fluorescence-activated cell sorting (FACS) techniques; then stimulated in coculture with and without B cell supernatants; and proliferation was determined by either standard beta scintillation counting (3H-TdR) or carboxyl-fluorescein succinimidyl ester (CFSE) dilution of Tresp cells. We found that Treg cells isolated using the MACS technique contain high numbers of contaminating CD4+CD25neg Tresp cells; are non-proliferative when stimulated alone; and do not robustly suppress Tresp cell proliferation. In contrast, FACS-isolated Treg cells have higher purity and are suppressive of Tresp cell proliferation and cytokine secretion. Suppression could be modulated by treating cells with either IL-10 to promote suppression, or Pam3Cys to decrease suppression, establishing the dynamic range of the

suppression assay. When supernatants from B cells activated with CpG-nucleotides or CD40 ligation were added to the suppression assay, we did not find any significant changes. As such, this may reflect a more subtle biology than can be captured within this assay and still requires further investigation.

2. Résumé

La thérapie au rituximab visant la déplétion des lymphocytes B diminue la sévérité de la sclérose en plaques (SP) de façon significative. Cependant, ces avantages ne sont pas accompagnés d'une réduction du niveau d'anticorps présents dans le sérum ou dans le liquide céphalo-rachidien. Ces résultats suggèrent certains rôles des lymphocytes B dans la SP indépendants de la production d'anticorps et qui seraient pro-inflammatoires. Dans le lupus érythémateux disséminé (LED), la déplétion des lymphocytes B entraine une hausse des fonctions des lymphocytes T régulateurs (Treg) circulants. Puisque les patients atteints de la SP démontrent un fonctionnement déficient de leurs Treg, nous émettons l'hypothèse que l'activation des lymphocytes B chez ces patients pourrait avoir un impact négatif sur le fonctionnement des lymphocytes T régulateurs. Ainsi, la déplétion des lymphocytes B grâce au rituximab permettrait le rétablissement du fonctionnement des Treg et préviendrait de nouvelles maladies auto-immunes. Notamment, nous avons postulé que les lymphocytes B des patients atteints de la SP produisent un profil anormal de cytokines pro-inflammatoires, et que ceci serait responsable de la dysfonction des Treg. Pour commencer l'étude de la relation entre les lymphocytes B et Treg, j'ai optimisé et validé un test d'activation de lymphocytes B humains in vitro, ainsi qu'un test de suppression des Treg humain chez des contrôles sains afin de déterminer les effets des surnageants provenant de lymphocytes B activés différemment sur la suppression de fonctions chez les Treg.

Des lymphocytes B humans ont été isolés à l'aide d'un tri cellulaire magnétique (MACS), et ont été stimulés avec un anticorps lié aux récepteurs des lymphocytes B (X), CD40 ligand (40), une combinaison des deux (X40), ou avec des nucléotides CpG. Les surnageants recueillis démontrent des réponses cellulaires similaires aux résultats publiés auparavant. Les lymphocytes T régulateurs et lymphocytes T répondeurs ont été isolés en combinant MACS et un tri cellulaire fluorescent (FACS), puis ont été stimulés en culture ensemble avec ou sans surnageants de lymphocytes B. La prolifération a été déterminée par soit l'incorporation de la thymidine tritiée, ou par d'ester de 5,6-carboxyfluorescéine diacétate succinimidyl (CFSE). Nous avons trouvé que les lymphocytes T régulateurs isolés en utilisant la technique MACS contiennent un grand nombre de lymphocytes T répondeurs CD4+CD25neg, ne prolifèrent pas lorsqu'ils sont

stimulés seuls, et n'étouffent pas la prolifération des lymphocytes T répondeurs. En revanche, les cellules Treg isolées par FACS sont d'une plus grande pureté et inhibent de la prolifération des lymphocytes T répondeurs ainsi que leur sécrétion de cytokines. Cette suppression peut être modulée en traitant les lymphocytes avec IL-10 afin de promouvoir la suppression, ou avec Pam3Cys afin d'atténuer la suppression, établissant une gamme dynamique au test de suppression. Lorsque les surnageants des lymphocytes B activés à l'aide de nucléotides CpG ou de CD40 ligand ont été ajoutés au test de suppression, nous n'avons trouvé aucun changement significatif. À ce titre, ceci pourrait refléter une biologie plus subtile qui ne peut être captée au sein de ce test, exigeant une enquête plus approfondie.

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4. Abbreviations

3H-TdR: standard beta-scintillation counting

40: bystander B cell activation condition, where B cells are stimulated with L cells

7-AAD: 7-aminoactinomycin D

ab: antibody

ACC: accessory cells

ag: antigen

APC: antigen presenting cell

B+40L: bystander B cell activation condition, where B cells are stimulated with soluble CD40 ligand

B+CpG: TLR9 ligated B cell activation condition, where B cells are stimulated with CpG

BAFF: B cell activation factor

BCR: B cell receptor

Be1: B effector cell 1

Be2: B effector cell 2

Breg: regulatory B cell

CFSE: carboxyl-fluorescein succinimidyl ester

CSF: cerebrospinal fluid

CpG: CpG-DNA, TLR9 ligand

EAE: experimental autoimmune encephalomyelitis

ELISA: enzyme-linked immunosorbent assay

FACS: fluorescence-activated cell sorting

FCS: fetal calf serum

FoxP3: forkhead box P3

ICS: intracellular staining

IFNg: interferon gamma

Ig: immunogloblin

IgD: immunogloblin D

IgG: immunogloblin G

IgM: immunogloblin M

IL-2: interleukin 2

IL-6: interleukin 6

IL-10: interleukin 10

IL-12: interleukin 12

IL-17: interleukin 17

IPEX: immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome

L cells: CD40 ligand-expressing cell line

L-glu: L-glutamine

LT: lymphotoxin

MACS: magnetic-activated cell sorting

MBP: myelin basic protein

MHC: major histocompatibility complex

mRNA: messenger RNA

MS: multiple sclerosis

Pam3Cys: TLR2 ligand

PBMCs: Peripheral blood mononuclear cells

PHA: phytohaemagglutinin

RRMS: relapsing-remitting multiple sclerosis

sup: supernatant

SLE: systemic lupus erythematosus

Th1: T helper cell 1

Th2: T helper cell 2

Th17: T helper cell 17

TLR: toll-like receptor

TNFa: tumor necrosis factor-alpha

Treg: regulatory T cell

Tresp: T responder cells

unstim: unstimulated

X: B cell receptor cross-linking antibody

X40: dual B cell activation condition, where B cells are stimulated with X and L cells

5. Introduction

Multiple studies over the years have implicated exaggerated pro-inflammatory T cell responses and insufficient function of regulatory T (Treg) cells in the pathogenesis of multiple sclerosis (MS) (Viglietta, Baecher-Allan et al. 2004; Frisullo, Nociti et al. 2009). The traditional concepts of immune system involvement in MS were in large part shaped by observations in animal models, such as the commonly used experimental autoimmune encephalomyelitis (EAE). EAE can be induced by direct immunization of an animal with myelin-associated autoantigens in combination with different types of adjuvant or passively by adoptive transfer of activated pro-inflammatory T cells that recognize myelin-associated autoantigens (Mokhtarian, McFarlin et al. 1984; Schluesener, Sobel et al. 1987). While EAE continues to provide important insights into molecular cascades involved in peripheral immune regulation and immune-neural interaction, it has not fully captured the complex genetic and environmental factors implicated in the human disease, nor the multiplicity of immune mediated injury mechanisms that appear to contribute in MS. Furthermore, testing of experimental therapeutics in EAE has not always predicted success or failure in patients with MS. As such, studies of the human disease in patients are imperative, which also requires development of assay systems that utilize human samples.

While MS has largely been viewed as a T cell mediated disease and, as a consequence, most MS therapies have been considered in terms of how they may impact pathologic T cell responses, the recent studies of B cell depletion in patients with MS have been very revealing (Cross, Stark et al. 2006; Bar-Or, Calabresi et al. 2008; Hauser, Waubant et al. 2008; Bar-Or, Fawaz et al. 2010; Piccio, Naismith et al. 2010; Barun and Bar-Or 2012). Results from these clinical trials, together with studies of the impact of B cell depletion on immune responses, emphasize the importance of B cells to relapsing MS disease biology and have implicated a number of novel B cell mediated pathogenic mechanisms that have only recently been recapitulated in animal studies. As further described below, one emerging concept is that soluble factors released by activated B cells can modulate other immune cell functions, such as the suppressive capacity of Treg cells, which is another immune cell subset widely appreciated for its contribution towards the maintenance of self-tolerance and immune homeostasis.

5.1. B cell depletion therapy with rituximab in MS patients

Clinical trials with the anti-CD20 antibody rituximab in patients with relapsing-remitting MS (RRMS) have indicated that selectively depleting peripheral B cells can substantially reduce new MS disease activity (Cross, Stark et al. 2006; Bar-Or, Calabresi et al. 2008; Hauser, Waubant et al. 2008; Naismith, Piccio et al. 2010; Barun and Bar-Or 2012). CD20 is expressed on human B lineage cells from pre-B cells through memory cells, but not on either pro-B cells or plasma cells (Tedder and Engel 1994; Eisenberg and Looney 2005; Levesque and St Clair 2008). Rituximab induces B cell depletion by complement- and antibody-mediated cytotoxicity, although there is some evidence to suggest that some non-circulating, tissue B cells are not depleted (Di Gaetano, Cittera et al. 2003; Martin and Chan 2006). Detailed pharmacokinetic and pharmacodynamic studies carried out in the context of the two clinical trials on rituximab in RRMS confirm a near-complete (>98%) depletion of circulating CD19+ B cells within two weeks of initial infusion (Bar-Or, Calabresi et al. 2008; Bar-Or, Fawaz et al. 2010). Since rituximab does not directly target plasma cells or circulating antibodies (DiLillo, Hamaguchi et al. 2008), this treatment can only deplete short-lived plasma cells via their precursors and would not be expected to deplete long-lived plasma cells (Manz, Thiel et al. 1997; Maruyama, Lam et al. 2000; Sze, Toellner et al. 2000).

While highly effective in decreasing new clinical relapses (Bar-Or, Calabresi et al. 2008; Hauser, Waubant et al. 2008), treatment with rituximab was found to have no impact on abnormal levels of cerebrospinal fluid (CSF) IgG or oligoclonal IgG bands, typically found in patients with MS (Monson, Cravens et al. 2005; Cross, Stark et al. 2006; Petereit, Moeller-Hartmann et al. 2008; Piccio, Naismith et al. 2010). This provides strong evidence that antibody-independent functions of B cells must contribute to relapsing MS biology, which until recently have not been well appreciated and as such still need to be further investigated.

5.2. B cells and their possible antibody-independent functions

In addition to their potential to produce antibodies, accumulating evidence from human and animal model studies points to several B cell functions that are likely relevant to both normal and pathologic immune responses. B cells have the capacity to internalize antigen bound to the B cell receptor (BCR), process it, and present it efficiently to T cells in a MHC-restricted manner. Memory B cells can accomplish this at concentrations approximately 100-fold lower than required for presentation by naïve B cells or monocytes (Lanzavecchia 1985; Janeway, Ron et al. 1987; Ron and Sprent 1987; Bar-Or, Oliveira et al. 2001). In addition to antigen uptake and MHC Class loading, activation through the BCR prepares the B cell to interact with T cells of the same antigenic specificity (so called 'cognate' B cell: T cell interaction) by upregulating various costimulatory molecules, such as CD80 and CD86. This dual activation of the B cell where it receives two signals, initially through its BCR and then engagement of costimulatory molecules, such as CD40 via T cells that express CD40L (or CD154), is traditionally considered as the requirements to activate a naïve B cell (Cambier, Pleiman et al. 1994; Bar-Or, Oliveira et al. 2001; Shilling, Bandukwala et al. 2006).

In addition to their APC function, B cells have been identified as contributors to modulating immune responses through production of distinct effector cytokine profiles. Early studies in mice described two distinct effector B cell subsets (Be1 and Be2) that functioned as polarized cytokine-producing cells, capable in turn of influencing Th1 and Th2 T cell differentiation (Harris, Haynes et al. 2000). Since then, the concept of regulatory B cells (or Bregs) has evolved based on a series of observations that particular B cell subsets can normally function to down regulate immune responses and that abnormalities in such functions can contribute to autoimmune disease (Mizoguchi and Bhan 2006; Evans, Chavez-Rueda et al. 2007; Blair, Chavez-Rueda et al. 2009; Mauri and Blair 2010; Iwata, Matsushita et al. 2011).

Several groups have described B cell subsets that can downregulate T cell–mediated inflammatory responses through the production of IL-10 (Evans, Chavez-Rueda et al. 2007; Yanaba, Bouaziz et al. 2008; Blair, Chavez-Rueda et al. 2009; Rafei, Hsieh et al. 2009; Carter, Vasconcellos et al. 2011; Iwata, Matsushita et al. 2011). Bregs were originally identified in mice, when B cell-deficient mice immunized with myelin basic protein (MBP) in complete Freund's adjuvant were unable to recover from EAE (Wolf,

Dittel et al. 1996). Recent investigations have shown that mice do not recover from EAE if they lack B cells or the ability to produce IL-10, thus indicating an immunoregulatory role for IL-10-producing B cells (Fillatreau, Sweenie et al. 2002; Mauri, Gray et al. 2003). Naturally occurring (Yanaba, Bouaziz et al. 2008) or inducible (Blair, Chavez-Rueda et al. 2009; Rafei, Hsieh et al. 2009) subsets of Bregs have been described in mouse, but there remains a lack of overall consensus about the combinations of markers that identify these cells, which may reflect the potential for existence of different subsets of Bregs and/or plasticity of B cells to acquire immune regulatory capacities, likely in a context dependent way.

Besides their capacity to secrete cytokines that may be pro- or anti-inflammatory, B cells also bridge adaptive and innate responses. Multiple reports have shown that human B cells express a wide-variety of innate receptors, such as toll-like receptors (TLRs), including TLR1, 2, 3, 4, 5, 6, 7, 8 and 10 (reviewed by Crampton, Voynova et al. 2010). Most TLRs are dependent on the downstream adaptor molecule MyD88, except TLR3 and 4, which can use an alternative pathway mediated by another adaptor molecule called TRIF. B cell activation, proliferation and receptor class-switching, have all been shown to be influenced by TLR ligation, even in the absence of BCR cross-linking (first reported by Krieg, Yi et al. 1995, and reviewed by Crampton, Voynova et al. 2010). In animal studies, it has been previously shown that MyD88 knockout mice are EAE resistant (Prinz, Garbe et al. 2006; Marta, Andersson et al. 2008). It has also been demonstrated in one model of EAE that recovery required B cell-restricted expression of MyD88, specifically in a TLR2/4 manner (Lampropoulou, Hoehlig et al. 2008). In this case, B cells stimulated by TLR2/4 led to a regulatory phenotype, preventing T cells from adopting a pro-inflammatory Th17 phenotype that has previously been implicated as pathogenic in MS (Lampropoulou, Hoehlig et al. 2008).

We recently reported that MS peripheral B cells exhibit aberrant pro-inflammatory cytokine responses when stimulated with the combination of BCR cross-linking, CD40 engagement, and TLR9 ligation with CpG-DNA as a third signal (Bar-Or, Fawaz et al. 2010). Under this 3-signal activation paradigm, MS B cells secreted significantly higher levels of the pro-inflammatory cytokines TNFa and LT, the latter a critical cytokine

involved in generation and maintenance of germinal center formation and lymphoid architecture. Using the Th1 cytokine IFNg as the third signal, stimulated MS B cells also secreted significantly lower levels of the regulatory cytokine IL-10. This propensity of MS B cells to respond to activation with exaggerated pro-inflammatory cytokine profiles may be relevant to the observed benefit of peripheral B cell depletion in MS.

How then might the beneficial effects of rituximab be explained, in the absence of an effect on antibody titres? It has been observed in MS patients undergoing rituximab treatment, both B cell and T cell numbers are decreased in CSF collected approximately 6 months after peripheral B cell depletion (Cross, Stark et al. 2006). This suggests that B cells contribute to abnormal presence of T cells in the MS CNS, which may reflect CNS B cells attracting T cells into the CNS, and/or the capacity for B cells to activate T cells. Activation of T cells by B cells could occur within the CNS, resulting in local T cell expansion, and/or in the periphery, resulting in activated T cells that can then more efficiently traffic into the CNS. In keeping with the latter mechanism, studies in MS patients participating in the clinical trials with rituximab have demonstrated that in vivo B cell depletion significantly diminished proliferative and cytokine responses of both CD4 and CD8 T cells in the periphery, including Th1 (IFNg) and Th17 (IL-17) T cell responses (Bar-Or, Fawaz et al. 2010). In absence of B cells, T cell responses were reduced, in response to a range of stimuli including PHA and anti-CD3 stimulation, as well as glatiramer acetate, a copolymer comprised of many antigenic epitopes that are presented to T cells in context of MHC molecules (Bar-Or, Fawaz et al. 2010).

Together, these observations support a role of B cells as relevant activators of a range of effector CD4 and CD8 T cell responses, in part through a process that may be considered ‘bystander activation’. The decreased T cell responses seen following B cell depletion with rituximab could be restored when T cell activation was carried out in presence of soluble products of activated B cells obtained from the same patients prior to B cell depletion. However, the restored T cell response was abrogated when TNFa and LT within the B cell products were selectively neutralized (Bar-Or, Fawaz et al. 2010). Together, our observations suggest that abnormal B cell cytokine responses, triggered by signals that may include pathogen associated molecules, contribute to bystander

activation of T cells, and results in further promotion of MS disease activity. These findings, which highlight the potential role of B cells at the innate-adaptive interface, provide a plausible mechanism for the long-recognized observation of an association between infection and triggering of MS relapse (Gilden 2005; Correale, Fiol et al. 2006), and a possible explanation for how B cell depletion effectively reduces new relapses in patients with MS. In disease contexts other than MS, such as systemic lupus erythematosus (SLE), patients treated with rituximab were reported to have increased mRNA expression of the Treg marker Forkhead box P3 (FoxP3) (Sfikakis, Souliotis et al. 2007), as well as suggested increased Treg suppressive function post treatment (Vigna-Perez, Hernandez-Castro et al. 2006). Whether B cells, though one or more of the above described mechanisms, can modulate T effector cells directly, or indirectly through other immune cells such as Tregs, which have also been implicated as important regulators of pathogenic T cell responses, still requires investigation.

5.3. Regulatory T cells and their function

Tregs were first described in mice as CD4+ T cells that expressed the IL-2 receptor alpha chain (CD25), and the functional consequence of their removal, which was a phenotype of severe autoimmunity (Sakaguchi, Sakaguchi et al. 1995). Soon afterwards, several groups described similar "natural" Tregs in humans that also expressed high levels of CD25, such as previously observed in mice, as well as their functional capacity to suppress the pro-inflammatory responses of other immune cells by numerous contact and cytokine dependent mechanisms (Thornton and Shevach 1998; Baecher-Allan, Brown et al. 2001; Dieckmann, Plottner et al. 2001; Jonuleit, Schmitt et al. 2001; Baecher-Allan, Viglietta et al. 2002; McHugh and Shevach 2002; Sakaguchi, Miyara et al. 2010). However, it is well-recognized that CD25 expression is not restricted to Tregs in human peripheral blood, where functionally suppressive Tregs are most enriched within the highest 1-2% of CD25 expressing CD4 cells (Baecher-Allan, Brown et al. 2001; Baecher-Allan, Viglietta et al. 2002).

The transcription factor FoxP3 has been attributed as the master control switch for the Treg phenotype. FoxP3 mutations were first described in scurfy mice, which

demonstrate a similar phenotype of autoimmunity when compared to Treg-depleted mice (Brunkow, Jeffery et al. 2001). In humans, FoxP3 mutations result in the rare human disease known as immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, characterized by refractory enteritis as well as autoimmune endocrinopathies, including type 1 diabetes, thyroiditis and allergy (Bennett, Christie et al. 2001; Brunkow, Jeffery et al. 2001; Wildin, Ramsdell et al. 2001).

It has more recently been appreciated that FoxP3 expression is also not necessarily indicative of Treg function. Similar to CD25, FoxP3 is expressed by T cells in response to cell activation (Walker, Kasprowicz et al. 2003; Allan, Crome et al. 2007), and these 'induced FoxP3 positive cells' maintain their Th1 cytokine profiles (Gavin, Torgerson et al. 2006). Indeed, when these induced FoxP3 cells were directly compared to naturally occurring Tregs, they were found to be neither anergic nor suppressive (Tran, Ramsey et al. 2007). Recently, it has been further demonstrated that there is a broad range of functional heterogeneity of suppression within the natural FoxP3+ Treg pool (d'Hennezel and Piccirillo 2011). Furthermore, as an intracellular marker, FoxP3 cannot be used to isolate viable human Tregs (needed to actually assess suppressive function), and as such can only be used to phenotype and assess the purity of cells post-isolation. Therefore, as it currently stands, the only way to validly assess regulatory T cell functional status is not through phenotyping frequencies of presumed Tregs, but rather based on their actual capacity to suppress. As such, an important goal has been to establish well-defined assays that would reliably and validly quantify Treg suppressive function.

Presently, there are a number of basic protocols published that aim to assess Treg function. Typically, Treg function is determined by examining the proliferation of CD4+CD25neg T responder (Tresp) cells when activated in the presence of Tregs at varying Tresp:Treg ratios. Current Protocols in Immunology has a comprehensive protocol of human Treg isolation using both flow cytometry and magnetic bead isolation approaches with a final readout using standard beta-scintillation counting (3H-TdR) (Baecher-Allan and Hafler 2006). Other protocols have highlighted potential advantages of a flow cytometry readout using carboxyl-fluorescein succinimidyl ester (CFSE) dilution to be able to more specifically analyze Tresp

proliferation, as Treg suppression assays often include not only Treg and Tresp cells in coculture, but also irradiated antigen presenting cells or accessory cells (ACC) to provide additional stimulation (Brusko, Hulme et al. 2007; Venken, Thewissen et al. 2007; d'Hennezel and Piccirillo 2011; Schneider and Buckner 2011). A recently published protocol describes a "rapid" Treg suppression assay, which assesses Tresp suppression in a 7-20hr coculture (Ruitenberg, Boyce et al. 2011). However, this protocol only works on expanded Tregs and not on freshly isolated subsets, and therefore may not represent the suppressive capacity of freshly isolated natural Tregs. Indeed this approach underscores how expansion protocols may affect Treg function, in this case resulting in Tregs that are more highly suppressive, which may be beneficial for generating large Treg numbers for therapeutic applications (Tang, Henriksen et al. 2004; Tarbell, Yamazaki et al. 2004) though their quantification of suppression will not necessarily be reflective of the true functional suppressive capacity of the circulating natural Treg compartment.

5.4. The potential interaction between Treg and B cells

The potential of B cells to influence Treg function has been suggested in various human autoimmune diseases. For example, B cell depletion in patients with SLE has been associated with increased FoxP3 mRNA expression levels (Sfikakis, Souliotis et al. 2007) and increased frequency of CD4+CD25high cells (Vigna-Perez, Hernandez-Castro et al. 2006) as well as the suggestion of increased Treg suppressive function post treatment (Vigna-Perez, Hernandez-Castro et al. 2006). In chronic idiopathic thrombocytopenic purpura patents, treatment with rituximab increased FoxP3-expressing cells by both percentage and absolute counts and these cells were found to be functionally more suppressive compared to those taken before treatment (Stasi, Cooper et al. 2008; Li, Mou et al. 2011). Although dysregulation in both cell types has been demonstrated in MS, direct effects of human B cells on Treg suppressive function have not been described to date.

We do know based on immunohistochemistry techniques of human tonsilar tissue that FoxP3+ Treg and IgD+ B cells are in close contact within the T-B border areas

(Lim, Hillsamer et al. 2005). Furthermore, the same group showed that Treg can suppress B cell immunoglobulin (Ig) secretion and inhibit Ig class switch recombination in a contact dependent manner (Lim, Hillsamer et al. 2005). It has also been implicated that malignant B cells from patients with B-cell non-Hodgkin's lymphoma may induce FoxP3 expression in CD4+CD25neg Tresp cells and that these induced Tregs are functionally suppressive (Han, Wu et al. 2011). Although not B cell dependent, it is also interesting that human plasmacytoid dendritic cells, which can function like B cells in their capacity to present antigen to T cells, when activated with CpG-nucleotides, induce the generation of CD4+CD25+FoxP3+ Treg cells that are functionally suppressive (Moseman, Liang et al. 2004).

In animal studies, several reports have suggested B:Treg interactions. It has been noted early on in Treg characterization studies and reproduced by several groups that B cell deficient mice have a lower absolute number of splenic Tregs compared to wildtype controls (Suto, Nakajima et al. 2002; Shah and Qiao 2008; Ray, Basu et al. 2012). Furthermore, it has been shown that B cell activation factor (BAFF) transgenic mice have an increased number of peripheral CD4+Foxp3+ T cells that is via a B cell dependent mechanism (Walters, Webster et al. 2009). It has been implicated before in mice that B cells can induce FoxP3 expression in CD4 T cells (Shah and Qiao 2008). Furthermore, in other animal models where B cells present self-antigen (Morlacchi, Soldani et al. 2011) and those of oral tolerance (Sun, Flach et al. 2008), peripheral FoxP3+ Treg cells that are functionally suppressive can be induced by B cells. B cell depletion using an anti-CD20 monoclonal antibody in a mouse model of arthritis resulted in an increase in FoxP3-expressing cells by both percentage and absolute counts. These cells were found to be significantly more suppressive than those from control ab-treated mice (Hamel, Cao et al. 2011). In another arthritis mouse model, it was found that IL-10+ B cells were required to polarize T cells towards Treg in lieu of Th17/1 (Carter, Vasconcellos et al. 2011).

However, in studies of EAE there have been conflicting observations regarding the necessity of B:Treg interactions. A recent study found that resolution of EAE by IL-10 secreting B cells was independent of Treg contribution. They attributed this to the fact that B cells in their model of active EAE had an early initial impact on disease,

whereas Tregs inhibited late-stage immunopathogenesis (Matsushita, Horikawa et al. 2010). However, another group found in their model of passive EAE, where EAE is induced by adoptive transfer MBP-specific encephalitogenic T cells into naïve mice, B cell deficiency resulted in a delay in the induction of Foxp3+ Treg cells and IL-10 in the CNS, but not in the periphery (Mann, Maresz et al. 2007). In this case, reconstitution of mice with wild-type B cells resulted in disease recovery and normalized IL-10 and FoxP3 expression levels, and was found to be a contact-dependent mechanism of B7 expressing B cells (Mann, Maresz et al. 2007). More recently, it has been suggested that IL-10-independent mechanisms, such as GITR ligation of Tregs by B cells may be necessary to maintain the Treg pool (Ray, Basu et al. 2012). However, as it currently stands, whether and how human B cells may influence the function of Tregs still needs to be investigated. This may be particularly relevant in the context of disease, and in MS in particular, it may represent one of the therapeutic modes of action of B cell depletion.

6. Hypotheses and Objectives

Our overall hypothesis is that activated B cells in patients with MS can abnormally suppress the function of Treg cells. The corollary would be that B cell depletion with rituximab allows for restored Treg function, and the prevention of new autoimmune disease activity. To begin studying the potential relationship between B and Treg cells, I optimized and validated in healthy controls an in vitro human B cell activation assay, as well as a human Treg suppression assay, to subsequently determine the effects of B cell soluble products on Treg suppression function.

As such, the objectives of this project were as follows: to assess whether, and how, particular contexts of human B cell activation influence their effector cytokine profile; to validate an assay that measures human Treg function with a dynamic range (capable of evaluating increases as well as decreases in suppression); and to examine the effects of supernatants from differentially activated human B cells on Treg suppressive function.

7. Materials and Methods

7.1. PBMC isolation

Venous blood was drawn from healthy volunteers into EDTA-containing (lavender topped) tubes (BD Bioscience). Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation with Ficoll-Paque (GE Lifesciences) and washed in PBS + 2mM EDTA before cell-specific isolation by magnetic-activated cell sorting (MACS, Miltenyi Biotech) or fluorescence-activated cell sorting (FACS, Beckman Coulter MoFlo). All cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS, Sigma), 1% penicillin-streptomycin (P/S, Gibco), and 1% L-glutamine (L-glu, Gibco).

7.2. B cell isolation and activation conditions

B cells were isolated using commercially available CD19 MACS beads (Miltenyi Biotec, cat# 130-050-301). For activated B cell characterization experiments, the stimulation conditions were as follows: unstimulated B cells, plated at 150,000 cells/well in a 96 well round-bottom plate; stimulation with B cell receptor cross-linking antibody (referred to as X; 0.65µg/ml goat anti-human IgG/IgM, available from Jackson); initial stimulation with X for 24 hours before being transferred to onto irradiated CD40L-expressing L cells for 72 hours at a B cell: L cell ratio of 15:2 (referred to as X40); stimulation with only L cells for 72 hours (referred to as 40); stimulated with CpG-DNA (referred to as CpG; 1uM ODN2006, Invivogen); and stimulated with both X and CpG.

To collect supernatants from 40 and X40 activated B cells, media was carefully pipetted from the well as to not disturb the cells pelleted at the bottom, and supernatants were collected from wells containing only L cells (referred to as L cell conditioned media or L alone). To look at the effect of L cell-independent CD40 ligation, B cells were stimulated with 0.5ug/ml CD40L (MegaCD40L, Enzo Life Sciences) for 24 hours, then washed twice with media, and kept in culture for an additional 48 hours before their supernatants were collected (referred to as B+40L). Similarly, for CpG B cell supernatants, B cells were stimulated with 1uM CpG (ODN2006, Invivogen) for 24

hours, then washed twice with media, and kept in culture for an additional 24 hours before supernatants were collected (referred to as B+CpG).

7.3. T cell subset isolation

7.3.1. Magnetic-activated cell sorting (MACS) technique

Treg and Tresp cells were isolated using commercially available MACS kits according to product specifications (CD4+CD25+ Regulatory T Cell Isolation Kit, cat# 130-091-301). In brief, PBMCs were first depleted of non-CD4+ cells using a LD column and the flow through containing CD4+ cells were collected. These CD4 cells were then treated with CD25 magnetic beads and put through two MS columns; Tresp cells were collected from the flow through fraction of the first column and Treg cells were eluted from the second MS column when it was removed from the magnet.

7.3.2. Fluorescence-activated cell sorting (FACS) technique

PBMCs were depleted of non-CD4+ cells using MACS (Mitenyi Biotech, CD4+ T cell isolation kit II, cat# 130-091-155) prior to staining with antibodies. CD4 cells were stained with CD4-FITC (BD Biosciences, cat# 555346) and CD25-PE (BD Biosciences, cat# 555432) as well as with appropriate unstained, single stain and isotype (BD Biosciences; IgG1k-FITC, cat# 555748; IgG1k-PE, cat# 555749) controls. Optimal concentrations of antibodies were 4ul CD4-FITC and 8ul CD25-PE per 1 million cells as determined by dose titration experiments. Staining was done by resuspending CD4 cells 100 million cells per ml in cell sorting buffer (PBS+1% FCS), and staining 20 million cells per tube. Cells were incubated with antibodies on ice in the dark for 30 minutes. Afterwards, stained cells were washed with cell sorting buffer, and after centrifugation, pooled into one 15ml tube and washed a second time. Cells were filtered with a 40µm cell strainer before being sorted. Gating was done as described in results (refer to Fig 8). Treg (CD4+CD25high) and Tresp (CD4+CD25neg) cell fractions were sorted into 15ml falcon tubes coated with 1ml FCS on ice.

7.4. B and T cell purity check

Post MACS isolation, B cells were stained with CD20-APC (BD Bioscience, cat# 559776), CD14-FITC (BD Bioscience, cat# 555397) and CD3-PE (BD Bioscience, cat# 555333), along with unstained and isotype controls (IgG2bk-APC, IgG2ak-FITC, and IgG1k-PE; all from BD Bioscience). B cells were routinely 97% or greater CD20 positive with CD3+ T cells as the predominant contaminating cell type (refer to Fig 1).

MACS and FACS-sorted Treg and Tresp cell purities were determined by intracellular staining for FoxP3, using a commercially available kit from eBioscience (Human Regulatory T cell staining kit#2, clone PCH101, cat#88-8998) with the following modifications to the manufacture's protocol: cell were stained in a 96 well plate, using 200 µl of buffer and 6µl of FoxP3 antibody. To avoid false positives, we used the Tresp cells to set the FoxP3 negative gate rather than the provided isotype (Tran, Ramsey et al. 2007; Pillai and Karandikar 2008; Law, Hirschkorn et al. 2009).

7.5. Treg Suppression Assays

Treg and Tresp cells were cultured with anti-CD3 (ebioscience, clone OKT3, cat# 16-0037) and either irradiated accessory cell (ACC) or anti-CD28 (clone CD28.2, Immunotech, Beckman Coulter) for costimulation. Concentrations of activation reagents, cell seeding numbers, and incubation times were optimized. Initial experiments were done with MACS-isolated T cells and 3H-TdR as the readout, using a minimum of 2,500 cells stimulated with 0.5µg/ml to 5µg/ml anti-CD3 and 10:1 ACC:Tresp or 0.5µg/ml anti-CD28 for 5 to 7 days in culture. For later optimized experiments with FACS-isolated T cells and CFSE staining, Tresp were stained with 1uM CFSE, and 50,000 Tresp cells were plated and stimulated with 30ng/ml anti-CD3 and 4:1, 2:1 and 1:1 ACC:Tresp for 3 days. We treated cells with 25ng/ml IL-10 (Gibco) to promote suppression and 5µg/ml Pam3Cys (EMC Microcollections) to reduce suppression, as well as with the B cell activation reagents, 0.5ug/ml CD40L (MegaCD40L, Enzo Life Sciences) and 1uM CpG-DNA (ODN2006, Invivogen) to account for any possible carryover effects not eliminated by washing. All B cell supernatants were added as 25% of total culture media. Treatments were added to the plate at the same time as the T cell stimulation agents, while cells were being sorted.

8. Results

8.1. Human B cell isolation and differential activation

Human CD19+ B cells were isolated using the MACS technique and their purity was assessed by flow cytometry using the B cell specific marker CD20. Furthermore, the isolated cells were stained with CD3 and CD14 to determine whether contaminating T cells and monocytes were present. B cells were routinely 97% or greater in purity and supernatants from B cells were only used if purity was greater than 95% CD20+ (refer to Fig 1 for representative B cell purity check data). These isolated CD19+ B cells were then stimulated under various activation conditions. Using the previously established two signal activation paradigm, B cells received either a bystander activation by through CD40 with CD40L-expressing L cells (defined as condition "40") or were dually activated by receiving BCR ag engagement for 24 hours before being stimulated via CD40:CD40L (condition "X40"). As previously published, B cells stimulated using these two modes of activation express different cytokine profiles, where B cell receiving bystander CD40 signaling express significantly lower proinflammatory cytokines such as IL-6 and LT and higher amounts of anti-inflammatory IL-10 compared to the X40 condition where B cells have seen their antigen of interest (Duddy, Niino et al. 2007).

Replicating these previously published findings, and as summarized in Figure 2, we found that 40 stimulated B cells proliferate (4310±3480 (mean±std dev) CPM, n=7, $p<0.01$ compared to unstimulated B cells by student t-test); express high amounts of IL-10 (560±540 pg/ml, n=12, $p<0.003$); and relatively low amounts of LTa (170±120 pg/ml, n=12, $p<0.001$) and IL-6 (2650±2250pg/ml, n=12, $p=0.001$). X40 stimulated B cells also proliferate (2870±1890 CPM, n=7, $p<0.005$), but express lower amounts of IL-10 (340±350 pg/ml, n=12, $p=0.005$) and higher amounts of LTa (700±440 pg/ml, n=12, $p<0.0005$) and IL-6 (5370±3790 pg/ml, n=12, $p<0.001$). Although the standard deviations of these values are large, this is due to inherent differences between human healthy controls, and these results were still found to be highly statistically significant by student t-test. Furthermore, we replicated the differences in cytokine secretion between

the differential activations, 40 and X40, and they were found to be highly significant ($p<0.01$ for IL-6 and LT; $p<0.05$ for IL-10; refer to Fig 2). Also, since B cells proliferate to similar extents under either the 40 or X40 condition, these cytokine differences are not the result of differential impact of B cell proliferation (refer to Fig 2).

B cells can also be stimulated with TLR ligands. Of particular interest in recent years has been the effect of TLR9 ligation on B cells, which is bound by CpG nucleotides, known to be components of certain viruses and bacteria. Though it has been reported that addition of CpG-DNA can increase B cell IL-10 production (Iwata, Matsushita et al. 2011), we did not see a significant increase in IL-10 in the supernatants of CpG-stimulated B cells. This may be because the study looked at IL-10 production by flow cytometry methods (intracellular staining, ICS), and we looked at IL-10 levels in the supernatants by ELISA. We would expect ELISA to be more sensitive than ICS since it reflects the total secretion of cytokine in the supernatant as opposed to a brief snapshot in time that ICS reflects; however, it is possible that IL-10 is being significantly consumed after it is secreted by the cells. Compared to unstimulated B cells, CpG stimulated B cells exhibited increased proliferation (11500±4390 (mean ± std dev) CPM compared to unstimulated, $n=4$, $p<0.01$; student t-test) and increased IL-6 production (450±150 pg/ml compared to unstimulated, $n=4$, $p<0.01$). The addition of BCR crosslinking antibody (X) did not significantly modulate this affect (refer to Fig 3). B cell viability was also assessed by 7-AAD, and B cells stimulated with both X and CpG were found to be significantly increased when compared to either unstimulated B cells or B cells stimulated with X (refer to Fig 4). Furthermore, B cells upregulated costimulatory molecules CD80 and CD86 when stimulated with CpG or X+CpG (refer to Fig 5). However, it seemed that CpG was driving the effects in this system and that X was not modulating cytokine expression as was previously observed within the X40/40 paradigm.

8.2. Approaches to Treg cell subset isolation

In order to examine the effects of supernatants from differentially activated B cells on Treg suppressive function, we first established and validated an assay to isolate human Tregs. To do this, we isolated Tregs using two techniques: magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS).

8.2.1. Magnetic-activated cell sorting (MACS) technique

Our experience has been that when Treg and Tresp cell fractions are isolated using the MACS technique and their purity assessed based on intracellular staining for FoxP3, Treg cell fractions consistently contain contaminating Tresp cells (on average 30-45% CD4+CD25negFoxP3neg, refer to Fig 6 for representative data). Furthermore, Tresp cell fractions can also contain contaminating CD25low and CD25mid cells, which though typically are thought to represent activated T cells, can also be FoxP3+ Tregs (refer to Fig 6). Several modifications to the suggested manufacturer's protocol may improve the purity of cell subsets isolated by MACS, including titrating the recommended amount of CD25 beads to a lower concentration (e.g. one quarter), as we and others (Baecher-Allan and Hafler 2006) have found. The manufacturer recommends eluting CD25-labeled cells into a second MS column in order to improve purity. Collecting the initial flow through of cells before washing the column and using this fraction as the Tresp cells may also result in lower contamination of the Tresp population by CD25high, potentially regulatory, T cells.

Even when using the above strategies, we found that using MACS for cell subset isolation resulted in Tregs that lacked robust suppressive capacity. As would be expected for true Tregs, we found MACS-isolated Treg cells cultured alone are consistently non-proliferative in response to adequate stimulus. A representative data set is shown where 2,500 Tresp/well are stimulated with 5.0ug/ml anti-CD3 and 10:1 ACC:Tresp cells with varying ratios of MACS-isolated Tregs (refer to Fig 7). This lack of proliferative response, but also limited suppressive capacity, was seen across a wide range of assay conditions, including varying cell densities, titrated strength of signal of the anti-CD3 and ACC/anti-CD28, and time points ranging from 3 to 7 days. Furthermore, these important results are consistent with those described by other groups (Baecher-Allan, Brown et al. 2001; Baecher-Allan, Viglietta et al. 2002; Baecher-Allan and Hafler 2006). Thus, while MACS-isolation of 'Treg' and Tresp' cell subsets has its attractions, at issue is how well

will such subsets perform in suppression assays and consequently how reliable will results be with respect to quantifying the true suppressive capacity of Tregs.

8.2.2. Fluorescence-activated cell sorting (FACS) technique

FACS cell sorting can be used to isolate human Treg cells and their subsets using antibodies for cell surface markers of interest. Total PBMCs can be stained with antibodies or depleted of non-CD4 cells using MACS prior to staining. We have found that enriching for CD4 cells in this way shortens the required length of the cell sort, and generally results in higher yields of Tregs of greater viability. We then stain our enriched CD4 cells with optimized concentrations of anti-human CD4-FITC and CD25-PE antibodies, as well as prepare appropriate unstained, single stained and isotype control stained samples.

Using the FACS technique we find that Treg and Tresp cell fractions consistently contain less contaminating cells compared to the MACS technique. In part this is because flow cytometry sorting enables more selective gating of the cells of interest, focusing on the highest percentage (approximately 1.5%) of CD25high in the case of Tregs (refer to Fig 8), yielding a functionally highly suppressive population (refer to Fig 9). Furthermore, since CD25 expression on adult human peripheral CD4 T cells is a smear, the concentration of antibody and incubation time will need to be optimized to determine the ideal conditions to capture the 1.5% CD25high Treg population. For the Tresp cell population, gating on the CD4+CD25neg population typically captures 40-50% of the total CD4 cells (refer to Fig 8). With this approach, Treg fractions on average exhibit minimal contamination (3-5%) by CD4+CD25neg cells, and Tresp fractions are also highly pure, with less than 1% contaminating CD25+ cells (refer to Fig 8 for representative data). Consequently, the percentage of CD25+ FoxP3+ cells within in FACS sorted Treg fractions is considerably higher than when isolated with the MACS method (over 85% compared to 23-60%; refer to Fig's 8 and 6 respectively for representative data).

In cocultures with Tresp stained with CFSE, Tregs were significantly suppressive when stimulated with anti-CD3 and either 4:1 ACC:Tresp or 2:1 ACC:Tresp (refer to Fig 9). FACS-isolated Tregs were found to be significantly suppressive particularly when

using suboptimal stimulation (2:1 ACC:Tresp), in all Tresp:Treg coculture conditions in a dose-dependent manner (refer to Fig 9). However, at very low stimulation (1:1 ACC:Tresp), Tresp proliferation alone was very low and as such suppression could not be detected or assessed (refer to Fig 9). A detailed explanation on how 'percent suppression' was calculated using the division index of the proliferating CFSE-labeled Tresp cells (as shown in Fig 9) with the flow cytometry analysis software Flowjo, is outlined in Appendix 1 (as well as described in Brusko, Hulme et al. 2007). LT and IL-2 levels in the coculture supernatants were measured by ELISA and cytokine suppression followed similar trends to what was seen in the proliferation assays (refer to Fig 10).

A major disadvantage to the FACS method compared to the MACS is lower Treg cell yields; however, this is associated with greater purity of the FACS-isolated Tregs. In our hands, 120 to 140 ml of blood typically yields 500,000 to 1 million FACS-sorted Tregs, depending on the donor. This is why Treg expansion protocols are commonly used after FACS cell sorting; however, as previously mentioned, they can impact Treg suppressive function (Tang, Henriksen et al. 2004; Tarbell, Yamazaki et al. 2004) and hence be less indicative of the true suppressive capacity of Tregs ex vivo.

8.3. Establishing dynamic range of Treg suppression assay

Once an optimal approach to isolating and stimulating Treg and Tresp subsets was defined, we established that the suppression assay has a dynamic range, such that it is possible to examine whether Treg suppression can be either up- or down-modulated. To accomplish this, we used agents previously described to directly influence natural Treg functionality. We used either recombinant IL-10, reported to promote Treg suppression (Chaudhry, Samstein et al. 2011) or Pam3Cys, reported to reduce Treg suppression (Nyirenda, Sanvito et al. 2011). When we added these conditions to our optimized assay, we found that Treg suppression was amenable to either enhancing or reducing Treg function (refer to Fig 11), consistent with what has been previously published, and demonstrating that our assay has a dynamic range of Treg suppression. This is particularly important when seeking to address questions of changes to Treg functionality.

8.4. Effect of differentially activated B cell products on regulatory T cell suppression

Initial experiments done with MACS-isolated Tregs showed that L cell conditioned control media was highly suppressive of Tresp proliferation in the absence of Tregs (refer to Fig 12). Consistent with previous findings (refer to Fig 7), MACS-isolated Tregs in this experiment were not robustly suppressive, and were non-proliferative when stimulated alone (refer to Fig 12). As such, B cells were stimulated with a commercially available soluble recombinant CD40 ligand and then the supernatants were collected in order to generate soluble products in the absence of L cells (referred to as B+40L sup). B cells were extensively washed and compared to a control where the CD40L was added directly to the culture in order to control for possible carryover effects. When used in the optimized FACS-isolated Treg suppression assay, using CFSE as the final readout, the addition of B+40L sup were not highly suppressive of Tresp alone proliferation as seen with the supernatant generated from 40 stimulated B cells in Fig 12, and in fact did not exhibit consistent effects on Treg:Treg coculture (refer to Fig 13). At the higher stimulation 4:1 ACC:Tresp condition, B+40L sup treated Treg had increased suppression; however, at the 2:1 ACC condition, the same supernatant caused a decrease in percent suppression (refer to Fig 13B). The inconsistent effects of the B+40L sup were also seen at additional ratios of Tresp:Treg (data not shown).

For the supernatants generated from B cells stimulated with CpG (B+CpG sup), at low stimulatory conditions (2:1 ACC:Tresp), it was found that Tresp alone proliferation was significantly increased when the supernatant was added (refer to Fig 14C and D). As such, any effects of the supernatant in this condition cannot be considered Treg-specific since we cannot account for whether the supernatant has a direct effect on the Tresp cells. However, at higher stimulatory conditions (4:1 ACC:Tresp) where Tresp proliferation was less modulated by the addition of B+CpG supernatant, there was not a significant change in percent suppression (refer to Fig 14A and B). When FACS-isolated Treg yields were high enough to allow for additional conditions, CpG, recombinant IL-10, and Pam3Cys were included as additional controls and demonstrated

that suppression could be modulated, as previously summarized in Fig 11 (data not shown).

9. Discussion

It has been previously published by our group that healthy human peripheral blood contains both naïve (CD27neg) and memory (CD27+) B cells, whose activation in culture ex vivo can result in secretion of anti-inflammatory (IL-10) or pro-inflammatory (TNFa, LT, IL-12 and IL-6) cytokines in a context- and subset-dependent fashion (refer to Fig 2; Duddy, Alter et al. 2004; Duddy, Niino et al. 2007; Bar-Or, Fawaz et al. 2010). It has been demonstrated that differential cytokine expression is obtained depending on the stimulus used to activate either B cell subset. When normal human memory B cells are stimulated by crosslinking the B cell receptor and CD40 engagement, they proliferate and secrete the proinflammatory cytokines LT and IL-6 that can function as autocrine signals for growth and differentiation, and can also serve to promote germinal center development, as well as amplify T cell immune responses. In contrast, normal human naïve B cells that undergo stimulation through CD40 engagement alone, which models a B cell receiving bystander T cell help in the absence of their specific antigen recognition, express negligible amounts of the proinflammatory cytokines, but secrete a significant amount of IL-10 that could suppress inappropriate local immune responses (Duddy, Alter et al. 2004). Interestingly, our lab has demonstrated that this cytokine network appears to be dysregulated in MS patients in several ways.

MS peripheral B cells exhibit aberrant pro-inflammatory cytokine responses when stimulated with the combination of BCR cross-linking, CD40 engagement, and TLR9 ligation with CpG-DNA as an additional third signal (Bar-Or, Fawaz et al. 2010). Under this 3-signal activation paradigm, MS B cells secreted significantly higher levels of the pro-inflammatory cytokines TNFa and LT, compared to healthy controls (Bar-Or, Fawaz et al. 2010). Furthermore, we have demonstrated on its own, CpG is a potent activator of B cells. Only the addition of CpG is required in order to promote B cell survival (refer to Fig 4) and increase expression of costimulatory molecules CD80 and CD86 in healthy controls (refer to Fig 5). These findings highlight the potential role of B cells at the innate-adaptive interface, and provide a plausible mechanism for the long-recognized observation of an association between infection and triggering of MS relapse (Gilden 2005; Correale, Fiol et al. 2006), and as such a possible explanation for how B cell depletion effectively reduces new relapses in patients with MS.

Having replicated our group's previous findings of differential cytokine regulation in activated B cells and characterized their responses, I optimized an assay capable of assessing Treg suppression. We have shown that how Treg cells are isolated is particularly important, and that the FACS method is superior over the MACS in regards to isolating a Treg population that has high FoxP3 expression (refer to Fig 6 for MACS and to Fig 8 for FACS Treg purity) and a functional capacity to suppress Tresp proliferation compared to those isolated by MACS (refer to Fig 7 for MACS and to Fig 9 for FACS Treg suppression data). In fact, the observation that MACS Tregs are not robustly suppressive has been recognized previously by other groups (Baecher-Allan and Hafler 2006). The MACS method can also be used to deplete PBMCs of Treg cells; however, it must be kept in mind that CD25 is not solely expressed by Tregs, but also activated T cells, B cells and some myeloid cells, which will also be depleted.

Our optimized suppression assay uses FACS-sorted cells that are not expanded, since it has been well-established that expansion protocols alter Treg function (Tang, Henriksen et al. 2004; Tarbell, Yamazaki et al. 2004). Tresp cells are stained with CFSE in order to more specifically analyze Tresp proliferation, as this assay system has not only Tresp and Treg cells, but also irradiated antigen presenting cells or accessory cells (ACC) to provide additional stimulation. The additional benefit of using CFSE as a readout is that it reflects the proliferative state of the cell for the entire duration of the culture period, in contrast standard beta-scintillation counting (3H-TdR), which only reflects the time period when it is added to culture. We found that titrating the strength of activation using multiple ACC:Treg ratios was required in order to best capture the suppressive function of the Tregs, since it can vary between different healthy control donors.

Using these optimized conditions, I confirmed whether suppression could be modulated by agents previously described to directly influence natural Treg functionality. As such, we treated cells with recombinant IL-10 to promote suppression (Chaudhry, Samstein et al. 2011) and TLR2 ligand Pam3Cys to reduce suppression (Nyirenda, Sanvito et al. 2011) and found that we could replicate what has previously been published, in our system (refer to Fig 11). This is presumably by acting through Treg cells directly; however, a caveat in this assay system is that potential direct effects of the

reagents of interest on the Tresp cells (or indirect effects via the accessory cells) cannot be excluded. However, most notably, these results demonstrate that our assay has a dynamic range of assessing suppression. This is particularly important when seeking to address questions of changes to Treg functionality.

My initial experiments were confounded by the effects of the L cell conditioned media (refer to Fig 12). As such, an L cell independent method of stimulating B cells was optimized. I found that when the supernatants generated from B cells stimulated with soluble CD40L were added to the suppression assay, the effect on suppression was not consistent, and addition of the same supernatant could be associated with either increases or decreases in Treg suppression with no discernible pattern (refer to Fig 13). While we cannot conclude a possible effect, we conclude that the biology cannot be so robust that it is consistently detectable. When supernatants from previously characterized CpG-activated B cells were added to the suppression assay, there were also no observed changes in percent suppression (refer to Fig 14). However, here conclusions on the impact of the supernatants on Treg functionality are complicated by a significant impact of the supernatants on Tresp proliferation at the strength of signal previously established to be optimal for suppression. At the higher 'strength of signal' where Tresp proliferation was not significantly impacted by the addition of B cell supernatants, Treg suppression was similarly not effected. Reproducing the expected changes in suppression with side-by-side addition of IL-10 or Pam3Cys as controls, allows us to more confidently interpret the above results as 'true-negative' results. This also suggests that the IL-10 secretion by CpG-stimulated B cells (as documented recently by groups, such as Iwata, Matsushita et al. 2011) is not enough to modulate suppression by Tregs or possibly countered by something else the B cells secrete.

Although modulation of Treg suppression could not be observed with the addition of activated B cell supernatants, this does not eliminate the possibility of relevant cell-contact interactions of B cells and Tregs. This could be potentially explored in future experiments by looking at coculture systems where differentially activated B cells are used instead of PBMCs as the source of APC in these suppression assays. Furthermore, although differences in Treg suppression of Tresp proliferation were not observed, this does not eliminate the possibility that other responses, such as cytokine secretion, could

be modulated by soluble B cell products. Furthermore, it is tempting to speculate that products secreted by MS B cells, which are lab has previous implicated as producing more pro-inflammatory cytokines and less regulatory IL-10, may have a differential impact in our system, when compared to the negligible effects seen within healthy controls. However, since no overt differences were seen with these experiments in healthy controls, this suggests that any effects of B cells on Treg suppression would likely be sufficiently subtle that they cannot be reproducibly and robustly captured using this dynamic Treg suppression system.

10. Conclusion

Recent B cell depletion studies have implicated the potential contributions of B cells to MS that extend well beyond classical antibody responses, and have suggested previously under-appreciated antibody-independent functions of B cells. Normal B cells are now recognized for having the capacity to present antigen to T cells; secrete distinct effector cytokine profiles that can be pro-inflammatory or regulatory in nature; contribute to lymphogeneis through elaboration of critical cytokines and chemokines; and function as important sentinels at the innate-adaptive immune interface. As such, after characterizing various contexts in which B cells can be activated, we developed an assay to examine whether soluble products from these activated B cells have the capacity to impact regulatory T cell function.

Currently Tregs can only be assessed by their suppressive function of activated immune cells; this is commonly assessed by their impact on activated Tresp proliferation when in coculture with Tregs at varying ratios. Isolation of Tregs for these assays is complicated by the fact currently there are no extracellular markers for human Tregs, and the intracellular marker for Tregs, FoxP3, can be induced by activation. As such, we have developed a methodology for the isolation and to assess the functional suppression of Tregs derived from human peripheral blood monocytes, and have highlighted what we consider to be important optimization steps for this assay. Having validated our assay, we then examined whether supernatants from differentially activated B cells could modulate Treg suppression in our assay. We conclude that there were no obvious effects of soluble products of differentially activated B cells on Treg are appreciated. As such, if such effects exist, they are likely to be subtle, given that our established assay exhibits a good dynamic range. Nonetheless, we would not preclude possible effects of B cells on Treg cells, although detecting a subtle biology may require different modes of B cell activation and/or studies of co-culture, given the possible importance of contact dependent interactions.

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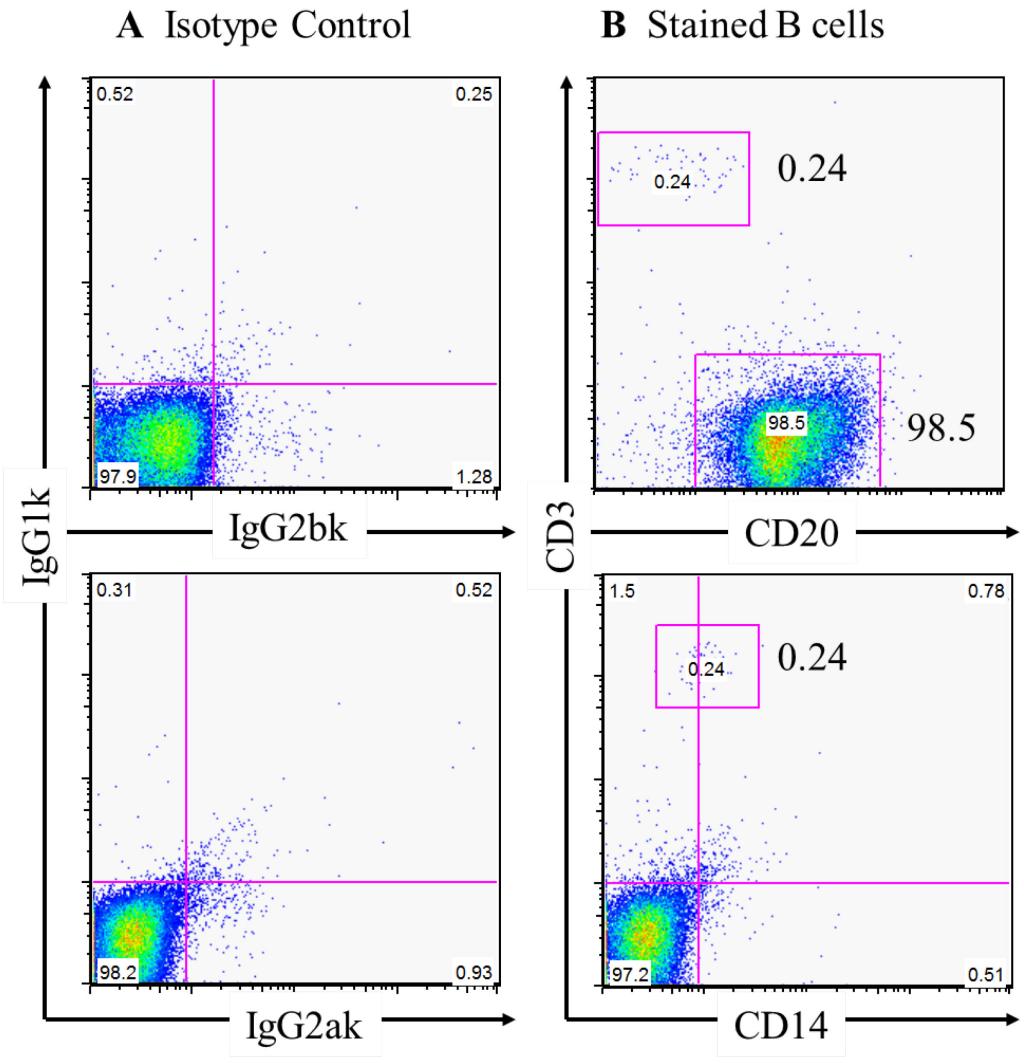
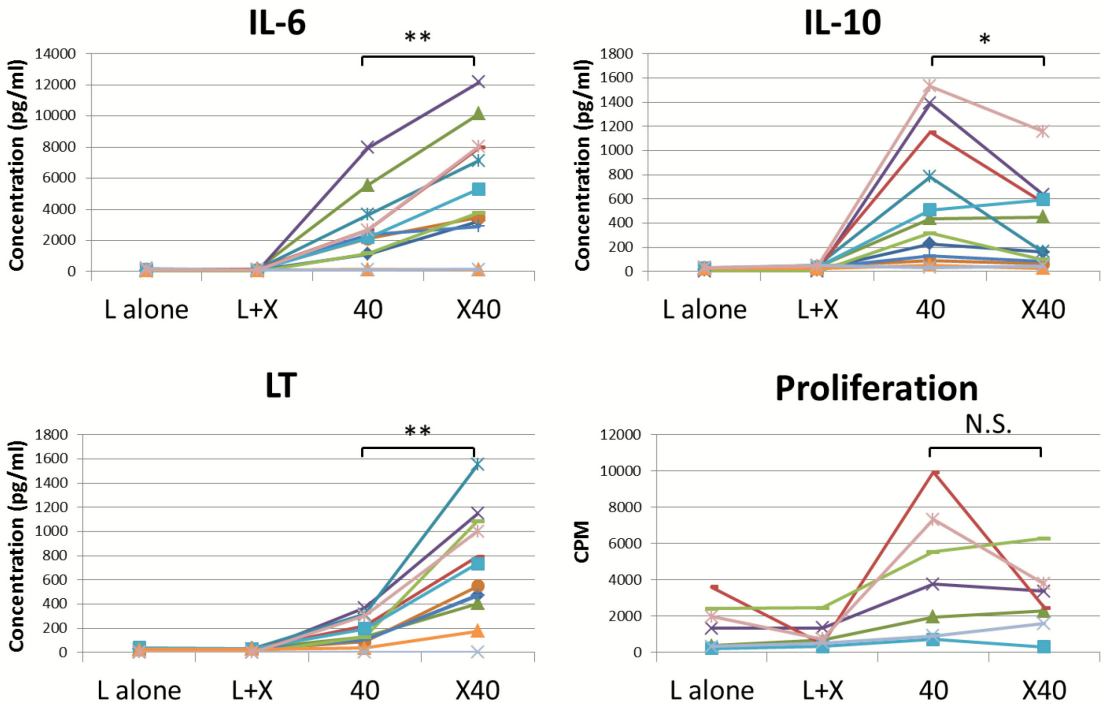
12. Figures

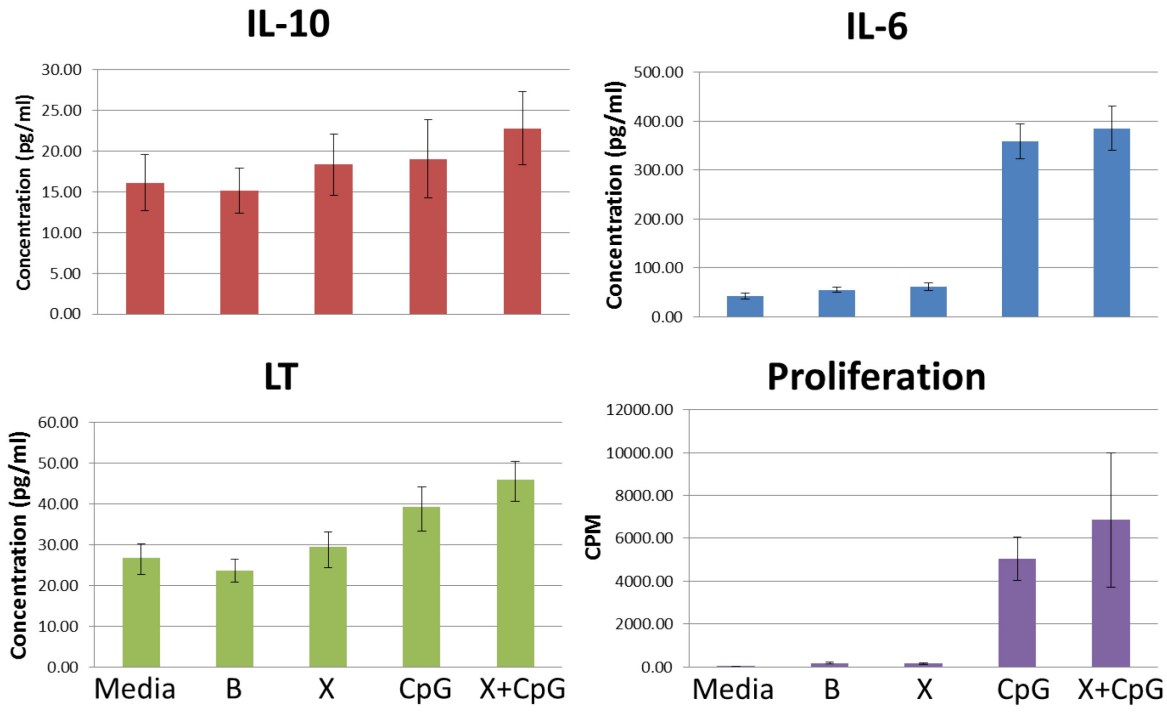
12.1. **Figure 1. Freshly isolated B cell purity is routinely 97% or greater, as determined by flow cytometry.** B cells were stained with CD20-APC (B cell marker), CD14-FITC (monocyte marker) and CD3-PE (T cell marker), along with separate unstained (not shown) and IgG2bk-APC, IgG2ak-FITC, and IgG1k-PE isotype controls (A, upper and bottom left panels). B cells were routinely 97% or greater CD20 positive (B, upper right panel), with CD3+ T cells as the predominant contaminating cell type (B, upper and bottom right panel).



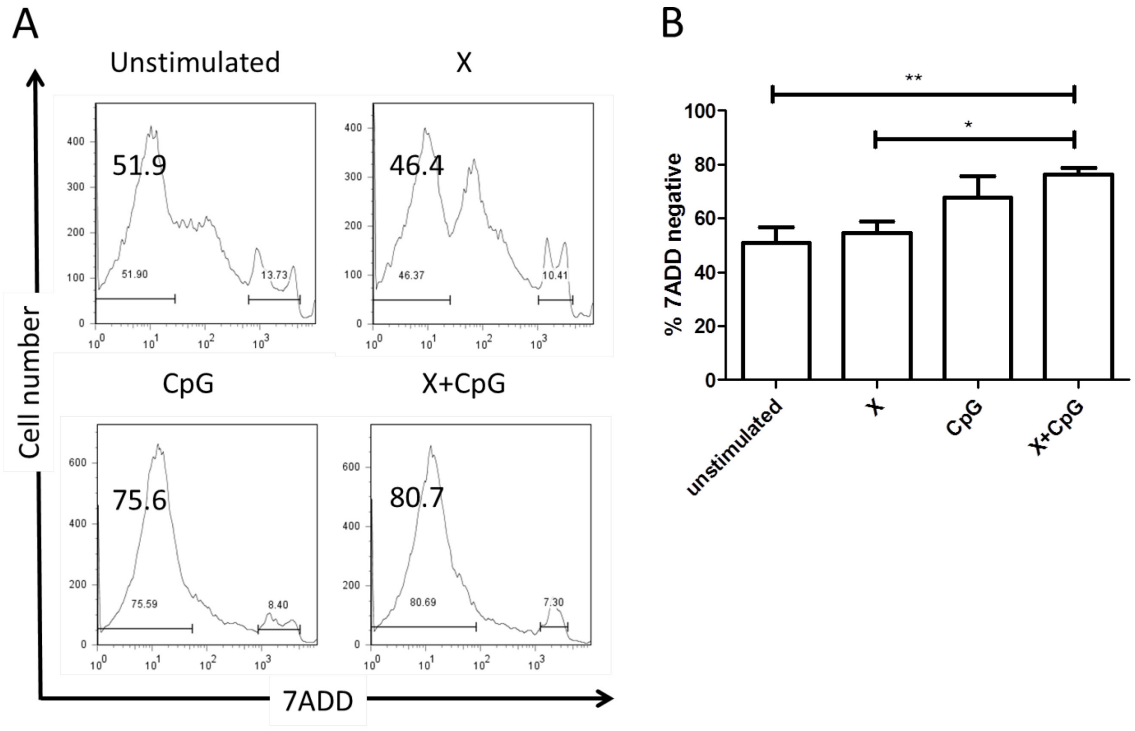
12.2. **Figure 2. Cytokine expression and proliferation of B cells stimulated with CD40L or CD40L and BCR cross-linking antibody.** B cells were stimulated through their BCR (using 0.65µg/mL goat anti-human IgG/IgM, Jackson, referred to as "X") for 24 hours, and then transferred onto irradiated CD40L-expressing L cells for 72 hours (X40) or only stimulated with the L cells for 72 hours (40). IL-6, IL-10, and LT concentrations in B cell supernatants and L cell (L alone) and L cell with BCR crosslinker (L+X) control media were assayed using commercially available ELISA kits (BD Pharmingen) following the manufacturer's protocols. Cells were pulsed for 18-hours with [3H] thymidine and proliferation was assessed by standard beta scintillation counting. (**p<0.01; *p<0.05 by student t-test; n=12 for IL-6, IL-10 and LT; n=7 for proliferation).



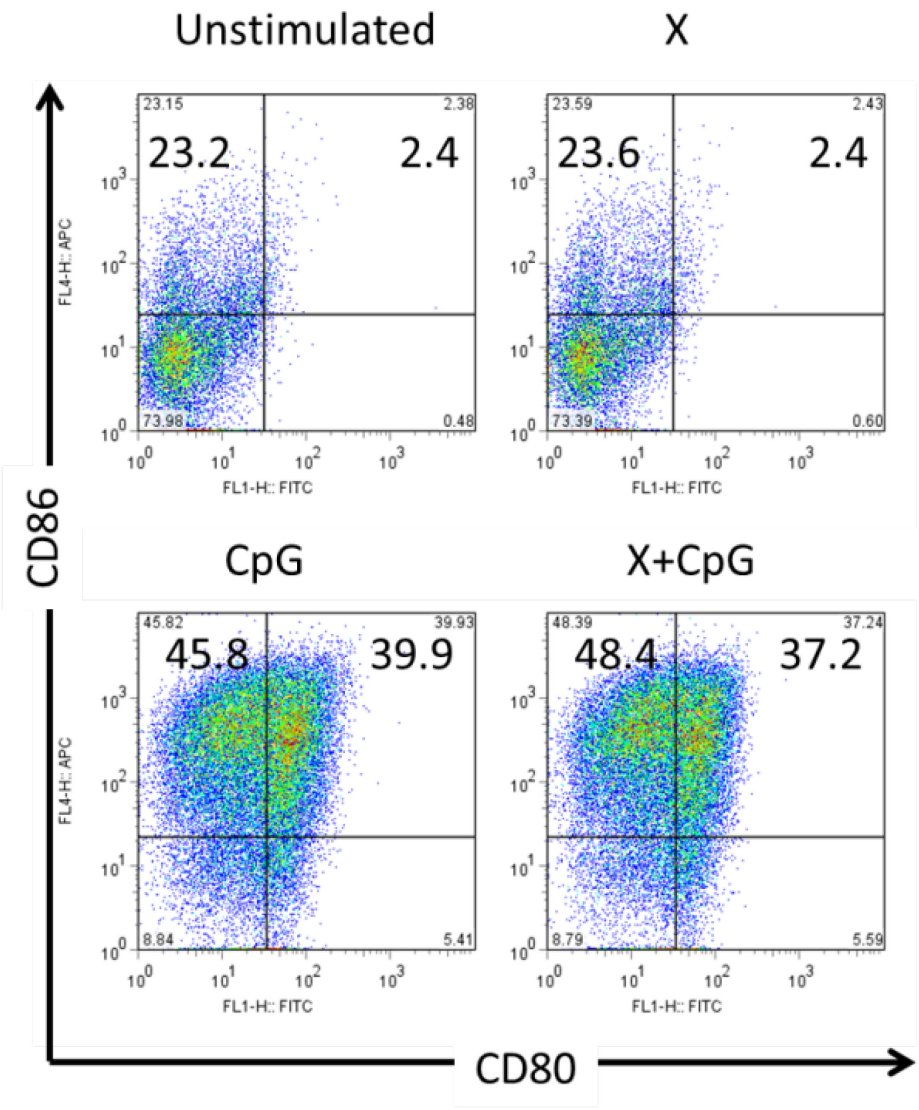
12.3. **Figure 3. Human B cells stimulated with BCR crosslinker and CpG proliferate and secrete IL-6.** Media control; unstimulated B cells (B); B cells stimulated with BCR antibody crosslinker (X); B cells stimulated via TLR9 ligation with CpG (CpG); and B cells activated with both X and CpG (X+CpG) were collected after 48 hours in culture. IL-6, IL-10, and LT concentrations in B cell supernatants were assayed using commercially available ELISA kits (BD Pharmingen) following the manufacturer's protocols. Cells were pulsed for 18-hours with [3H] thymidine and proliferation was assessed by beta scintillation counting (representative data of n=4).



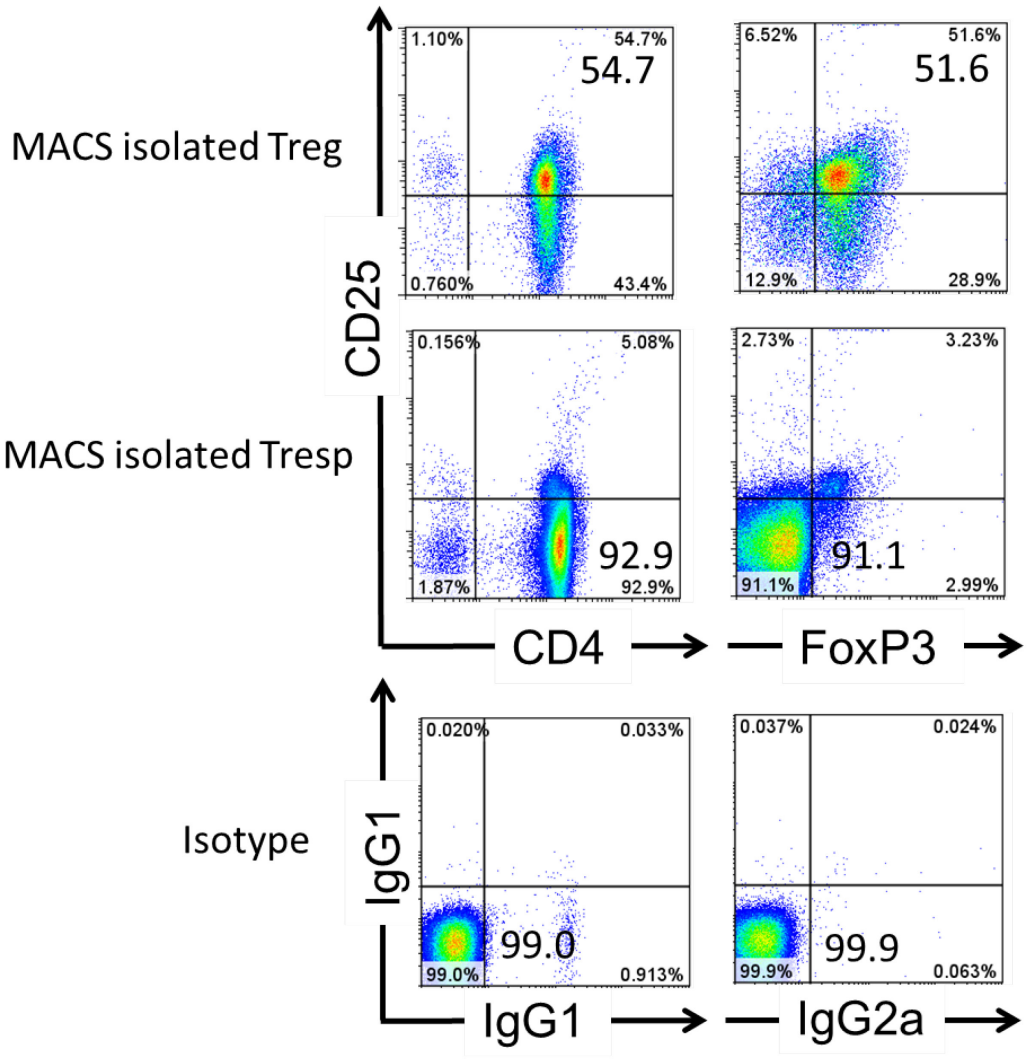
12.4. **Figure 4. B cells stimulated with BCR crosslinker and CpG are viable after 48 hours in culture.** Unstimulated B cells (labeled unstimulated); B cells stimulated with BCR antibody crosslinker (X); B cells stimulated via TLR9 ligation with CpG (CpG); and B cells activated with both X and CpG (X+CpG) were collected after 48 hours in culture and stained with 7-Aminoactinomycin D (7AAD). Gating is based off of unstained controls. Panel A (left) is a representative data set, and panel B (right) is pooled data from five experiments with three donors (mean ± sem; ** $p < 0.01$; * $p < 0.05$; one way ANOVA).



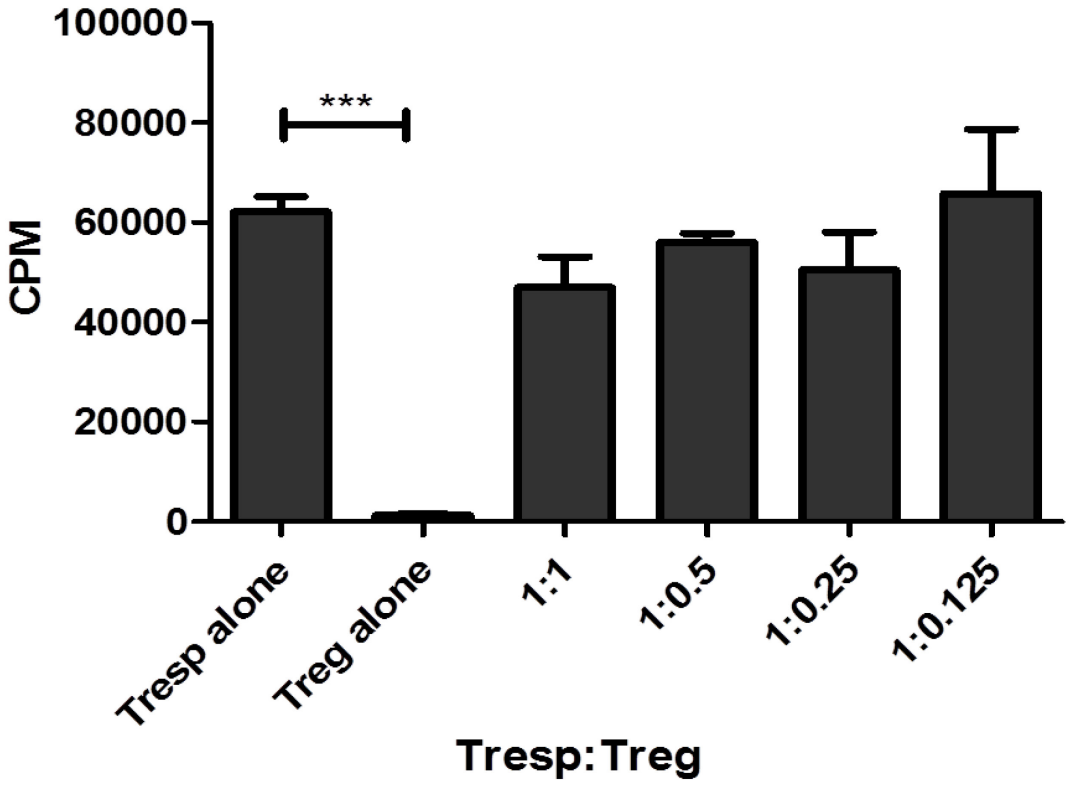
12.5. **Figure 5. B cells stimulated with BCR crosslinker and CpG have increased costimulatory molecule expression after 48 hours in culture.** Unstimulated B cells (labeled unstimulated); B cells stimulated with BCR antibody crosslinker (X); B cells stimulated via TLR9 ligation with CpG (CpG); and B cells activated with both X and CpG (X+CpG) were collected after 48 hours in culture and stained with CD19-PE, CD86-APC and CD80-FITC. Only CD19+ cells are represented, and current gates represent isotype controls.



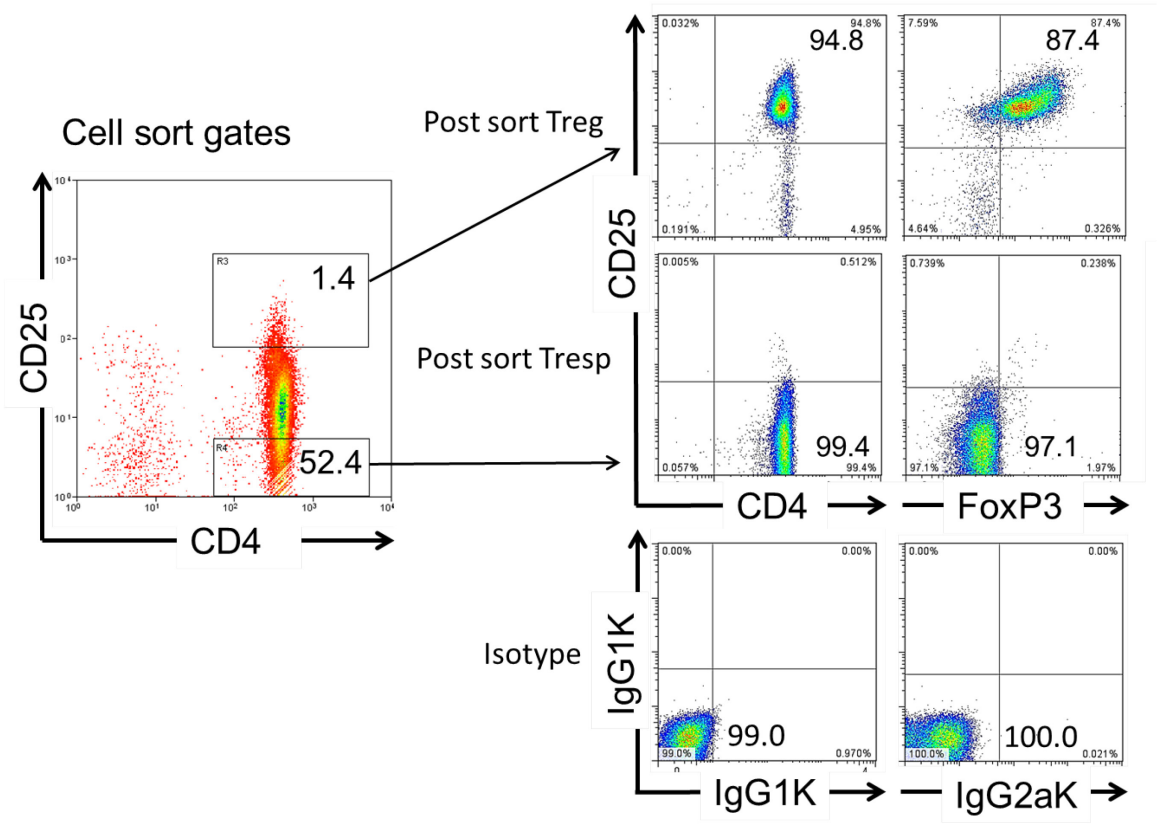
12.6. **Figure 6. Treg cells are enriched based by MACS beads, and purity is confirmed by FoxP3 expression.** Cells were isolated using MACS as outlined in methods. MACS-isolated Treg and Tresp cells were stained immediately after isolation with CD4-FITC, CD25-APC and FoxP3-PE using a commercially available kit (eBioscience). Gating was done using Tresp cells as FoxP3-negative control.



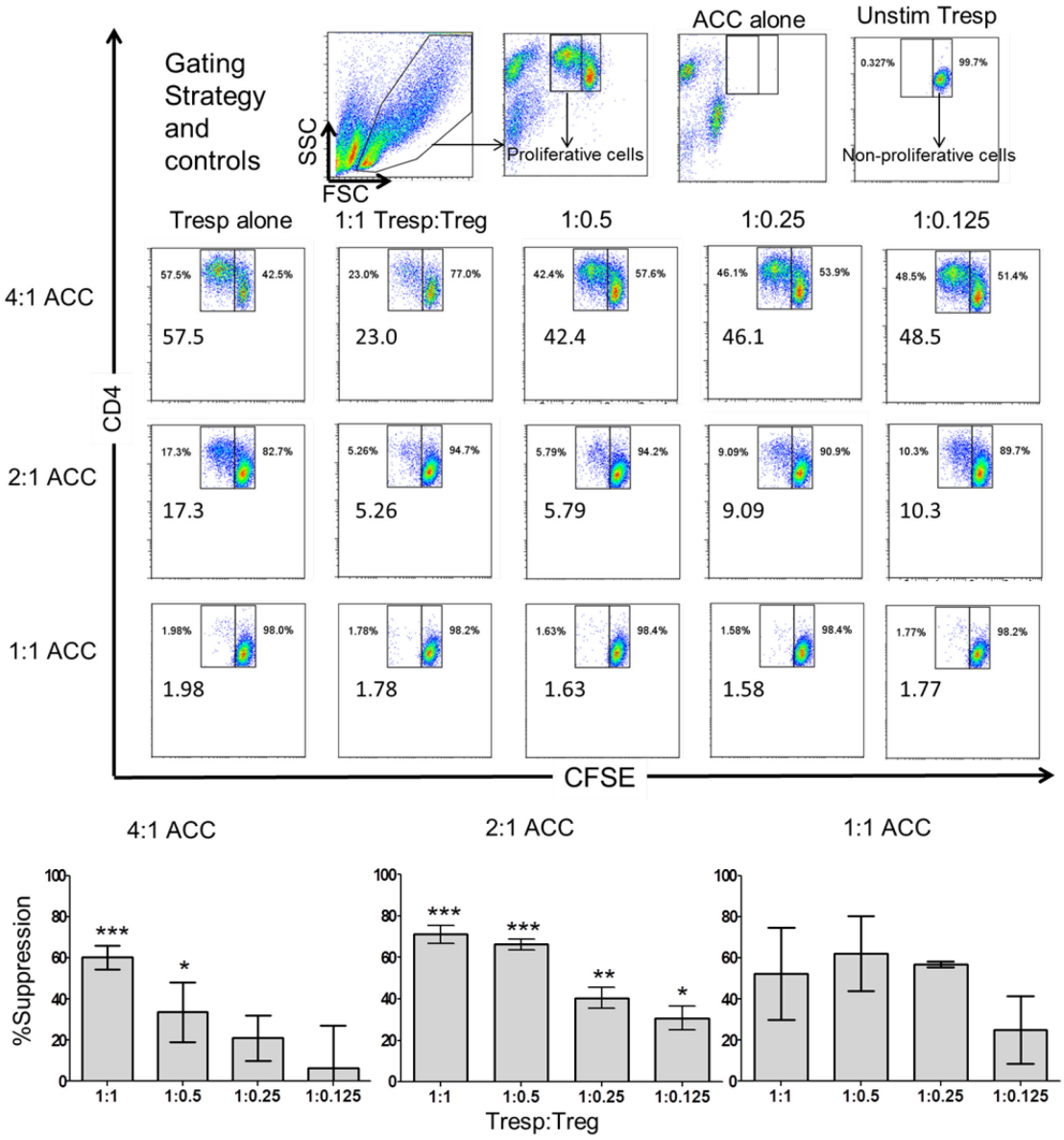
12.7. **Figure 7. MACS-isolated Tregs are non-responsive to stimulation and display non-robust suppressive capacity.** Treg and Tresp cells were isolated using MACS and stimulated with 5.0ug/ml anti-CD3 and 10:1 ACC:Target cells with 2,500 Tresp/well and Tregs that were serial diluted in the ratios indicated. After 7 days in culture, cells were pulsed for 18-hours with [3H] thymidine and proliferation was assessed by standard beta scintillation counting. Data is representative of 3 independent experiments (mean ± sem, calculated from triplicate wells; ***p<0.001; one-way ANOVA).



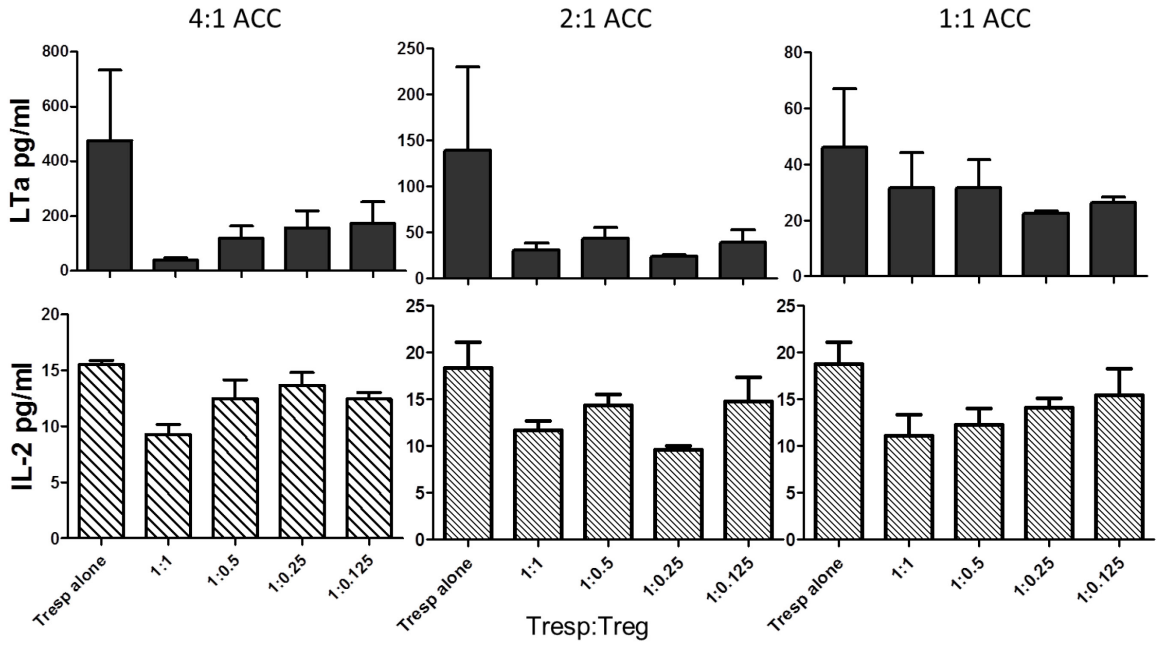
12.8. **Figure 8. Treg cells are sorted based on CD4+CD25high expression, and purity is confirmed by FoxP3 expression.** CD4 cells were negatively selected by MACs, and then stained with CD4-FITC and CD25-PE. Treg and Tresp cell sort gates are as shown. Post-sorted cells were stained with FoxP3-APC using a commercially available kit (eBioscience). Gating was done using Tresp cells as FoxP3-negative control.



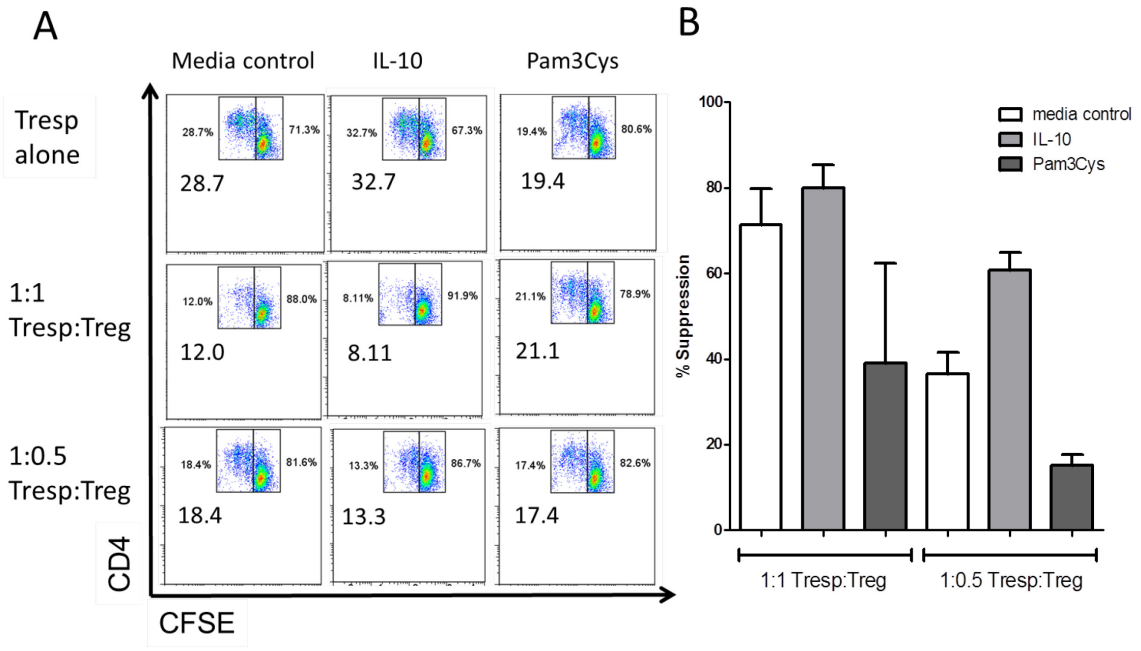
12.9. **Figure 9. Treg-enriched cells are highly suppressive of proliferation in a dose-dependent manner.** Tresp cells were stained with CFSE, and cocultured with Treg-enriched cells in the indicated ratios. Cells were stimulated with 30ng/ml anti-CD3 and either 4:1, 2:1 or 1:1 ACC (Accessory cells:Tresp cells) for 72hrs. Above are representative dot plots of each condition. Below are pooled percent suppression (compared to Tresp alone condition, refer to appendix 1 for example calculation) of 3 independent experiments (mean±std dev.; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ compared to Tresp alone; one-way ANOVA).



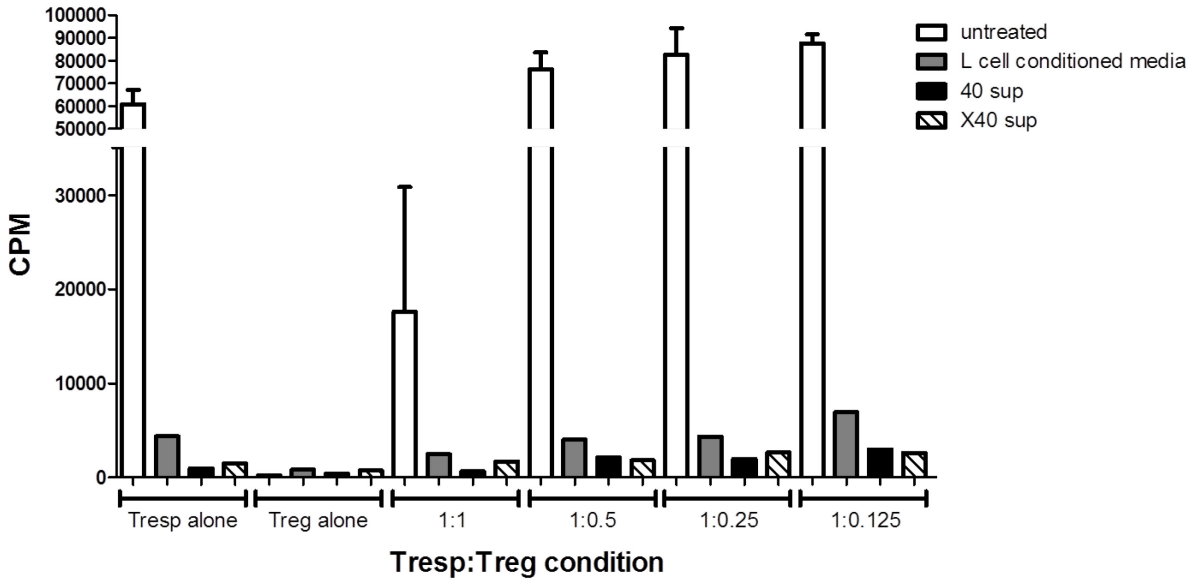
12.10. **Figure 10. Treg-enriched cells suppress LTa and IL-2 in a dose-dependent manner.** Tresp cells were cocultured with Treg-enriched cells in the indicated ratios. Cells were stimulated with 30ng/ml anti-CD3 and either 4:1, 2:1 or 1:1 Accessory cells:Tresp cells (ACC) for 72hrs. LTa and IL-2 cytokine concentrations were assayed using commercially available ELISA kits (BD Pharmingen) following the manufacturer's protocols (n=3; mean±SEM).



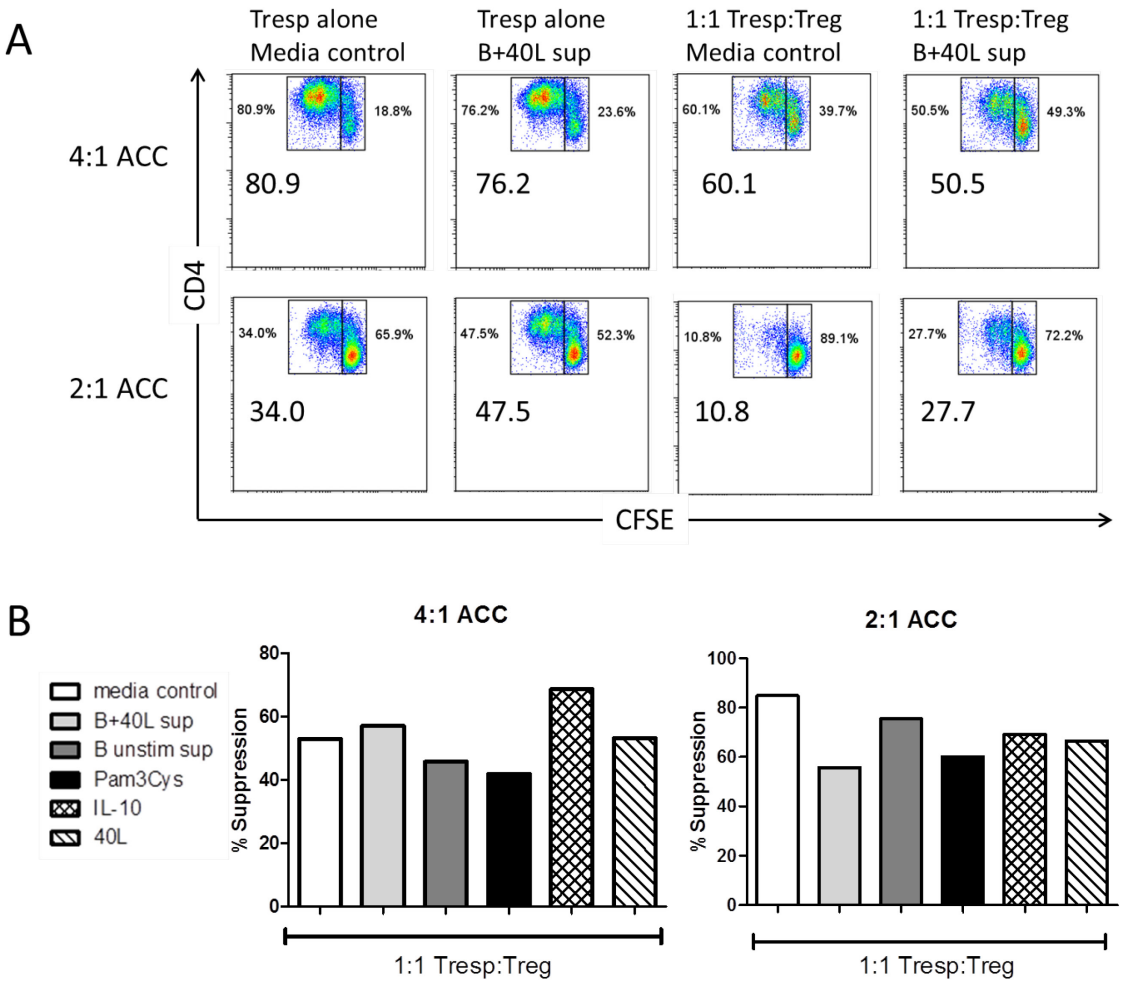
12.11. **Figure 11. Treg suppression can be modulated with the addition of recombinant IL-10 or Pam3Cys, the ligand for TLR2.** Tresp cells were stained with CFSE, and cocultured with Treg-enriched cells in the indicated ratios of 1:1 or 1:0.5 Tresp:Treg. Cells were stimulated with 30ng/ml anti-CD3 and 2:1 Accessory cells:Tresp cells and treated with media control; 25ng/ml IL-10; or 5ug/ml Pam3Cys (TLR2 ligand). After 72 hours, cells were collected and stained with CD4-APC. Dot plots (A, left) are a representative data set and percent suppression (B, right) was pooled from 4 independent experiments.



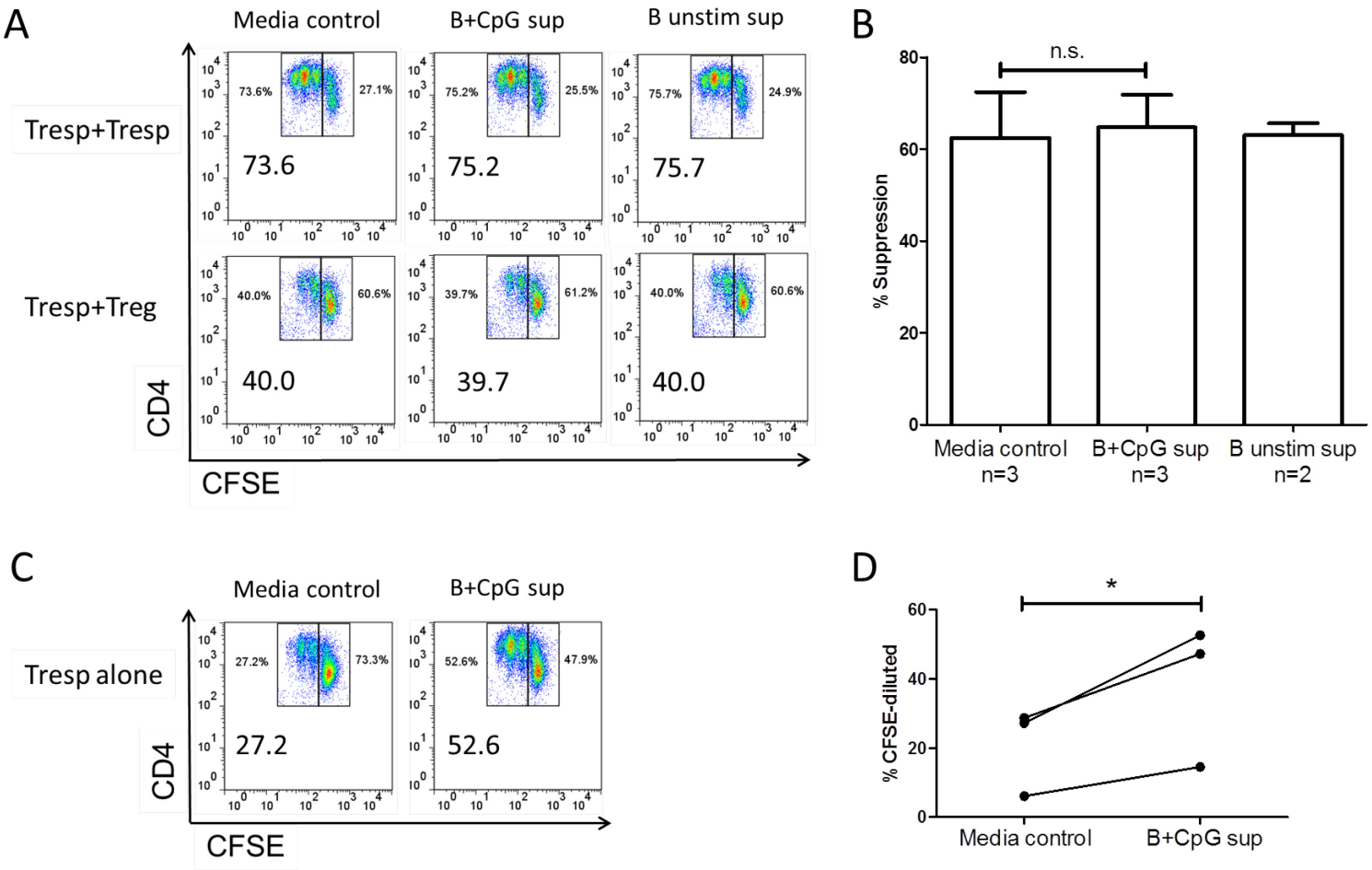
12.12. **Figure 12. L cell conditioned media is highly suppressive of Tresp alone proliferation.** Stimulated B cell supernatants (concentration of 25%) were added to MACS isolated Tresp and Treg cells from the same donor. Cells were stimulated for 5 days with plate-bound anti-CD3 and anti-CD28. Cells were then pulsed for 18-hours with [3H] thymidine and proliferation was assessed by standard beta scintillation counting. This is a representative data set of 3 independent experiments (mean±SEM).



12.13. **Figure 13. CD40L stimulated B cell supernatants did not consistently modulate Treg suppression.** Tresp alone or 1:1 Tresp:Treg in coculture were stimulated with 30ng/ml anti-CD3 and either 4:1 Accessory cells:Tresp cells (4:1 ACC) or 2:1 Accessory cells:Tresp cells (2:1 ACC) and treated with various conditions: media control; 25% supernatant collected from B cells stimulated with CD40L for 24 hours, then washed twice with media, and kept in culture for an additional 48 hours (B+40L sup); 25% supernatant from unstimulated B cells also washed twice with media and kept in culture for an additional 48 hours (B unstim sup); Pam3Cys (TLR2L); IL-10; or CD40L. After 72 hours, cells were collected and stained with CD4-APC. Dot plots are the raw data set from the media control and B+40L sup conditions (panel A, top). Percent suppression was calculated for all conditions and summarized in panel B (bottom left is 4:1 ACC and bottom right is 2:1 ACC).



12.14. **Figure 14. CpG stimulated B cell supernatants did not significantly modulate Treg suppression, but could increase Tresp alone proliferation when activated at a lower strength of signal (2:1 ACC).** Tresp cells were stained with CFSE, and cocultured with CFSE-unlabeled Tresp (Tresp+Tresp) or FACS isolated Treg cells at a 1:1 ratio (Tresp+Treg). Cells were stimulated with 30ng/ml anti-CD3 and 4:1 Accessory cells:Tresp cells (ACC) and treated with media control; 25% supernatant collected from B cells stimulated with CpG for 24 hours, then washed twice with media, and kept in culture for an additional 24 hours (B+CpG sup); or 25% supernatant from unstimulated B cells also washed twice with media and kept in culture for an additional 24 hours (B unstim sup). After 72 hours, cells were collected and stained with CD4-APC. A (top, left) represents an example raw data set. B (top, right) is a summary graph of average percent suppression, calculated as described in Appendix 1. Results were found to be non-significant (n.s.) by one-way ANOVA. For C and D, Tresp cells were stained with CFSE and stimulated with anti-CD3 and 2:1 ACC and treated with media control or B+CpG sup. After 72 hours, cells were collected and stained with CD4-APC. C (bottom, left) represents an example raw data set, and D (bottom, right) is a summary graph showing changes in Tresp alone proliferation (gating on CD4+ CFSE-diluted cells, as shown in C) with B+CpG sup treatment (n=3, *p=0.3 as determined by paired t-test).

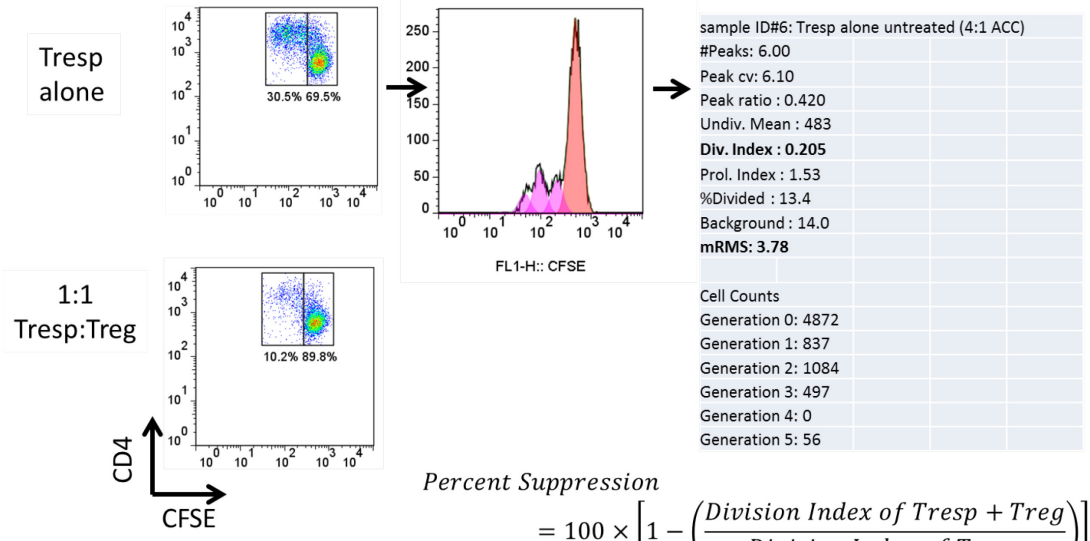

A
Media control
B+CpG sup
B unstim sup
Tresp+Tresp
Tresp+Treg
73.6%
27.1%
73.6
75.2%
25.5%
75.2
75.7%
24.9%
75.7
40.0%
60.6%
40.0
39.7%
61.2%
39.7
40.0%
60.6%
40.0
CD4
CFSE
B
n.s.
% Suppression
Media control
n=3
B+CpG sup
n=3
B unstim sup
n=2
C
Media control
B+CpG sup
Tresp alone
27.2%
73.3%
27.2
52.6%
47.9%
52.6
CD4
CFSE
D
*
% CFSE-diluted
Media control
B+CpG sup

13. Appendices

13.1. Appendix 1

Appendix 1.

Example of Percent Suppression calculation using Flowjo software:



$$Percent\ Suppression = 100 \times \left[1 - \left(\frac{Division\ Index\ of\ Tresp + Treg}{Division\ Index\ of\ Tresp}\right)\right]$$

e.g. for this example, the percent suppression of the 1:1 ratio is:

$$100 \times \left[1 - \left(\frac{0.05}{0.205}\right)\right] = 75.6\%$$