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A novel method for assaying human regulatory T cell direct suppression of B cell effector function

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Abstract

We have established a highly reproducible and reliable protocol for testing human regulatory T cell function in suppressing IgM production from an immature human B cell line. The autoreactive Ramos B cell line provides a stable reporter of B cell effector function that can be tested by a straight-forward IgM ELISA. Tregs from healthy volunteers display a range of ability for suppressing baseline IgM production in a contact- and death-independent manner. Having established the normal range for human Treg direct suppression of B cell effector function, it will now be possible to efficiently test Tregs from various autoimmune conditions in which B cell hyperactivity and secretion of auto-antibodies are a hallmark of disease.

Keywords

Treg; Autoimmunity; B cell; Antibody; Suppression assay; Human

1. Introduction

The regulatory T (Treg) cell suppression assay, as originally described in the study by Thornton et al., has become a mainstay of immunology (Thornton and Shevach, 1998). It has been used successfully to test the ability of both human and animal Tregs to inhibit the IL-2-dependent proliferation of conventional (Tconv) CD4 T cells in a contact-(or at least close proximity-) dependent fashion. Variables which have been shown to influence suppression include the ratio of Treg to Tconv in co-culture, the nature of stimulation (ranging from soluble to plate-bound to bead-conjugated antiCD3 or anti-CD28 as well as inclusion of various inert antigen presenting cells providing co-stimulation), and the addition of various soluble factors (e.g., inflammatory cytokines or antibodies to suppressive cytokines IL-10 or TFG β) (McMurchy and Levings, 2012). Using less pure Treg

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Appendix A. Supplementary data

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preparations, the earlier report of decreased function of Tregs in certain disease states, such as Systemic Lupus Erythematosus (SLE), was brought into question because of the possibility that contaminating cells were directly opposing suppression (Horwitz, 2008). This has been especially problematic for human studies, where Treg markers like CD25 were found to be less useful for discriminating Tregs from activated Tconv cells (Gavin et al., 2006).

As methods for purifying or enriching Tregs have improved, it has become possible to achieve a cleaner system with less concern for contaminating Tconv cells. In addition, the inclusion of post-enrichment confirmation of Treg purity with costaining for intracellular markers (such as FoxP3 and Helios), has improved the analysis of Treg function (Tran, 2013). While earlier studies suggested that Treg function is diminished in autoimmune disease such as SLE (Valencia et al., 2007), more recent studies utilizing more highly purified Tregs have actually indicated that SLE derived Tconv cells are resistant to Treg suppression even by Tregs isolated from healthy individuals (Long and Buckner, 2011). Similarly, whereas earlier studies using less specific Treg markers concluded that Treg numbers were reduced in clinically active SLE patients, the combination of markers (Helios + and FoxP3+or CD25high together with CD127neg) to identify bona fide thymically-derived Tregs (tTregs) (Thornton et al., 2010) has shown that Treg numbers are for the most part maintained even in active SLE patients (Golding et al., 2013; Alexander et al., 2013).

In an attempt to clarify the mechanism behind a lack of or resistance to suppression in autoimmunity, a limited number of studies have looked beyond Tconv proliferation to other read-outs for Treg suppression. Some of these include cytokine release by Tconv cells (Oberle et al., 2007) as well as early up-regulation of Tconv activation markers (Hill et al., 2012; Canavan et al., 2012). However, these studies have increased complexity that make evaluating the results less straightforward. Analyzing cytokine secretion by reporter T cells requires additional manipulation, such as addition of brief calcium flux (PMA/ionomycin) and golgi inhibitors, or the use of more complicated cytokine-capture antibodies for flow cytometry. Testing secretion of cytokines in co-culture supernatants does not discriminate between Tconv and Tregs, because in some cases it has been shown that Tregs can also secrete some inflammatory cytokines such as IFN γ (Pandiyan and Zhu, 2015).

Additional effector mechanisms that have been reported to be subjected to Treg suppression, but have not been well studied in relation to autoimmunity, include CD8 (Zhang et al., 2014) and NK cell (Smyth et al., 2006) cytotoxicity as well as B cell antibody secretion (Zhao et al., 2006a; Iikuni et al., 2009). The latter is particularly relevant to many autoimmune disorders in which a critical component of disease and end-organ damage is auto-antibody production and immune complex formation. The limited number of studies that have been done, while informative, were still susceptible to the influence of intrinsic resistance to suppression on the part of effector cells (Ohl and Tenbrock, 2015). The ratio of Tregs:B cells used in a human study was quite high—5:1—and therefore not reflective of typical Treg:B cell ratios found in peripheral blood (Szymczak-Workman et al., 2011). Furthermore, in this study, the primary mechanism invoked for Treg suppression of B cells was direct B cell killing. It is not yet clear whether B cell death is the only relevant mechanism for direct Treg

suppression, and the unusually high Treg:B cell ratio may have been a driver of B cell death (Szymczak-Workman et al., 2011).

Ultimately, it has yet to be established that Tregs are part of the underlying pathophysiology of SLE or any other human autoimmune disease, as opposed to just being innocent bystanders. This distinction has important implications for the therapeutic use of expanded autologous Tregs or therapeutic manipulation of Tregs (rapamycin or low-dose IL-2) (Trzonkowski et al., 2015). For the advancement of scientific knowledge and treatment, it is critical that we elucidate the suppressive functionality of Tregs from patients with autoimmune disease in a way that is straightforward, focused on intrinsic Treg properties, and relevant to an important effector function in disease. A simple, reliable, easily reproducible functional assay of Treg suppressive function that can be correlated with clinical activity in SLE across multiple donors and centers of SLE research will improve our understanding of autoimmunity and allow for a sustained, widespread impact in the field of autoimmune treatment.

We sought to develop a novel, affordable, reliable suppression assay of B cell effector function that is entirely dependent on intrinsic Treg properties. We also sought to remove the potential influence of reporter cells from affected individuals having resistance to suppression. In our assay, the Ramos B cell line serves as a homogeneous, reliable reporter cell that secretes a product that can only come from the reporter cell and not from Tregs, namely IgM (Benjamin et al., 1982). Assaying for de novo IgM production by cultured Ramos cells is straight-forward using a standard ELISA technique and reagents that are readily available and affordable. Additional advantages include the avoidance of using radioactive nucleotide (³HThymidine) or fluorescent label (CFSE) uptake as well as the ability to perform batch analysis with stored supernatants from multiple suppression assays.

2. Methods

2.1. Regulatory T cell isolation

Human buffy coats (New York Blood Center) were first incubated with RosetteSep™ Human CD4⁺CD127^{low} T Cell Enrichment Cocktail (Stemcell Technologies, Catalog #15361). The cells isolated from the Ficoll interface were then incubated with CD25 MicroBeads II (Miltenyi Biotec, Catalog #130-092-983) and underwent positive selection by the autoMACS Pro Separator. The purity of the CD4⁺CD127⁻CD25⁺ and CD25⁻ populations was confirmed by flow cytometry staining for the extracellular markers CD3 (Biolegend, Catalog #317306, CloneOKT3), CD4 (Invitrogen, Catalog #MHCD0412, Clone S3.5), and CD25 (Miltenyi Biotec, Catalog #130-0920-858, Clone 4E3) and the intracellular markers Foxp3 (eBioscience, Catalog #48-4777-42, Clone 236A/E7) and Helios (Biolegend, Catalog #137,216, Clone 22F6).

2.2. Ramos B cell culture

Ramos B cells (ATCC® CRL-1596) were cultured in complete RPMI at 37 °C with a concentration of between 2×10^5 and 2×10^6 cells per mL. The Ramos B cell line used in

these experiments was authenticated and found to share 15 alleles of 15 alleles (100%) with the ATCC reference.

2.3. In vitro Treg:Ramos suppression assay

Both the CD4⁺CD127⁻CD25⁺ and CD25⁻ primary cell populations were co-cultured with 20,000 Ramos B cells in decreasing ratios on a standard 96-well plate (Thermo Fisher Scientific, Carlsbad, CA) or a trans well 96-well culture plate (Corning, Corning, NY). Co-culture proceeded in the presence of 250 μ L of complete RPMI with 100 U/mL IL-2 alone (Gemini Bio-Products, Catalog #300–146P), or also in the presence of 16 ng/mL recombinant human CD40L (Biolegend, Catalog #591702) and 16 ng/mL recombinant human IL-21 (Biolegend, Catalog #571202). Beyond IL-2, no additional T cell stimulation was included in the co-cultures and the T cells were not pre-activated before co-culture. The co-cultures were incubated at 37 °C for 4 days. Each co-culture was carried out in replicate when the yield of T cells allowed. Supernatants were harvested from the co-cultures and stored at –20 °C. Each suppression assay is designated by “SA#”.

2.4. IgM secretion by ELISA

To determine the concentration of IgM, the supernatants from the co-cultures were diluted by 50 fold and tested with anti-human IgM ELISA developed in our lab from standard, commercially available reagents. Plates were coated overnight at 4 °C with 100 μ L of 10–20 μ g/mL of donkey anti-human IgM (Jackson ImmunoResearch, Catalog #709–005-073) diluted in 1 $\times$ PBS. To block unspecific interactions, plates were incubated with 250 μ L of blocking buffer (1 $\times$ PBS with 1% BSA and 0.05% TWEEN) for 2 h at room temperature. Plates were washed (1 $\times$ PBS with 0.05% TWEEN) four times after a 2 hour incubation with diluted supernatants at room temperature. Goat anti-human IgM conjugated to HRP (Life Technologies, Catalog #A18835) was incubated on the plate for 1 h at room temperature to detect the amount of IgM in the co-culture supernatant. Again plates were washed four times and then 100 μ L of TMB solution (eBioscience, Catalog #00–4201-56) was added followed by 100 μ L of Stop Solution (Biolegend, Catalog #423001). Absorbance at 450 nm was measured on a standard ELISA reader. A standard curve using human IgM (Sigma, Darmstadt, Catalog #I8260) was included for every ELISA and the concentration of IgM for each sample was calculated based on the standard curve. ELISAs were performed in triplicate and the average OD was reported. Results were calculated and analyzed using GraphPad Prism version 6.0 g for Macintosh.

2.5. Cell death/proliferation analysis

Following co-culture, cells were harvested, washed with flow cytometry buffer, and stained with anti-CD4 for 1 h. Cells were then fixed and permeabilized overnight and then stained with Ki67 (ThermoFisher Scientific, Catalog #MHKI6701, Clone 7B11) and 7AAD (BD Biosciences, Catalog #559925).

2.6. Calculation of percent suppression

Suppression of IgM secretion was calculated using the following formula:

$$([\text{IgM}] \text{ in absence of Treg} - [\text{IgM}] \text{ in presence of Treg} / [\text{IgM}] \text{ in absence of Treg}) \times 100].$$

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 for Macintosh. The statistical test used was a paired, two-tailed *t*-test. *P* values in individual figures/experiments are indicated as follows: ns indicates $p > 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0005$.

3. Results

3.1. Regulatory T cells purified by surface markers are mostly CD4+CD127–CD25+Foxp3+Helios+

Purified CD4+CD127–CD25+ and CD25– T cells, as well as a portion of ficoll-enriched, total peripheral blood mononuclear cells (PBMCs), were stained with flow cytometry antibodies for CD3, CD4, CD25, Foxp3, and Helios. The results demonstrated that, in total PBMCs, the frequency of Foxp3+Helios+ cells in the CD4+ population ranged from 4 to 6% as previously published (Thornton et al., 2010; Golding et al., 2013). Buffy coat enriched CD4+CD127–CD25+ cells contained a frequency of Foxp3+Helios+ tTregs ranging from 70 to 87% of CD4 T cells. A representative example of PBMC and post-Treg purification flow cytometry histograms is shown in Fig. 1A and B. As we developed our assay, it was evident that having a pure population of Tregs, CD4+CD127–CD25+ cells, was important to a successful outcome. In cases of Treg purifications yielding <85% FoxP3+ cells and <70% Helios+Foxp3+ cells, minimal to no suppression was observed (data not shown), presumably due to contaminating Tconv cells. A summary of purification yields for the Tregs used in suppression assays that are published herein is shown in Fig. 1C.

3.2. Ramos B cells secrete IgM but not IgG

Ramos B cells are an undifferentiated, lymphoma cell line that is EBV negative. Others have shown that Ramos express autoreactive IgM on their surface, as well as secrete IgM, but they do not secrete any other Ig isotype (Benjamin et al., 1982; Pugh-Bernard et al., 2001). To better characterize how the Ramos would behave in our suppression assays, we cultured 20,000 Ramos cells at a concentration of 8×10^4 cells/mL for 4 days in the presence of 1L-2. We chose this concentration of cells because Tregs are only ~1% of leukocytes in the periphery and are the limiting factor in our assay. Therefore, it was realistic to design and characterize the assay so that a relatively small number of cells can be used per individual suppression assay well. In addition, 1L-2 was included to promote Treg function and homeostasis. Fig. 2A shows that the cultured Ramos B cells secrete IgM without stimulation and that this baseline level of secretion is not altered by the addition of IL-2 alone. However, the level of secretion does increase when recombinant CD40L and IL-21 are added both at a concentration of 16 ng/mL. In contrast, Ramos do not secrete IgG, even when stimulated, as shown in Fig. 2B.

3.3. CD4+CD127–CD25+ Tregs from healthy donors reliably suppress unstimulated IgM secretion by Ramos B cells

We developed a modified suppression assay because we desired a reliable methodology for testing the suppressive capacity of Treg cells from healthy and diseased donors. The only variable in the assay is the source of the Tregs, which, for the development of this assay, were isolated from healthy donor buffy coats. As a representative example, Fig. 3A shows that Tregs from a single healthy donor suppress the secretion of IgM by Ramos B cells by a significant amount when co-cultured at a ratio of 1:1 Tregs:Ramos in the presence of IL-2 compared to Ramos cells alone. This suppression was not simply a result of co-culturing Ramos B cells with any CD4 T cells, which is shown in Fig. 3B. Fig. 3C shows the average suppression of IgM secretion by Tregs to be 43%, which is based on data from 12 healthy donors. Information on the healthy donors is shown in Supplemental Table 1. We did not see significant differences in levels of suppression based on either age or gender (Supplemental Fig. 1).

Tregs are only 1% of leukocytes in the periphery whereas effector cells, such as CD4+ T cells and B cells, range from 10 to 40% of leukocytes. Therefore, when demonstrating Treg suppression, it is important to do so at multiple ratios of Tregs:effector cells. In our modified suppression assay, Ramos B cells are co-cultured with decreasing ratios of Tregs ranging from 1:1 to 1:16, as shown in Fig. 3A–D. We observe significant differences in suppression between each Treg:Ramos ratio such that mean suppression increases from 8.5 to 15.7 to 22.5 to 33.8 to 42.6%, respectively going from 1:16 up to 1:1 Treg:Ramos. Fig. 3E shows that there is a significant decrease in the concentration of IgM in the supernatant between the 0:1 and 1:1 Treg:Ramos co-cultures. In contrast, there is an increase in the concentration of IgM in the supernatant between the 0:1 and 1:1 Tconv:Ramos co-cultures (Fig. 3B, D, and F). This confirms that suppression in our assay is a unique feature of Tregs and not a general feature of CD4 T cells.

As discussed in Section 3.1, a reasonably pure population of Treg cells was critical for suppression. Controlling the growth rate and passage number of the Ramos cells is also important in generating a successful assay in which IgM secretion is suppressed. Ramos B cells constitutively mutate their V regions and these mutations can result in a loss of IgM expression (Zhang et al., 2001; Upton and Unniraman, 2011). This mutation rate is low (2×10^{-5} mutations/bp/generation). Therefore, we found that using Ramos cells that were passaged (split) <10 times ensured that the basal IgM secretion was still high enough to observe suppression. Also, the Ramos culture was maintained at a fairly constant, high cell density ranging from 1.5–2.0 million/mL. Ramos cells were observed to proliferate more rapidly in the first 24–48 h after dilution in the assay if they were maintained in culture at a cell density below 1.5 million/mL. This resulted in less reliable ratios of Tregs:Ramos B cells and diminished suppression (data not shown).

Inflammatory/autoimmune conditions likely involve not only baseline but also stimulated levels of immunoglobulin secretion from B cells. We sought to determine if Tregs from healthy donor buffy coats could suppress stimulated (CD40L and IL-21) IgM secretion. In the limited number of experiments we have done thus far, there is greater variability in the ability of the Tregs to suppress stimulated IgM secretion by Ramos cells (data not shown).

3.4. Mechanism of suppression is not death-dependent and may be partially contact-independent

Tregs are known to exert their regulatory effects via contact dependent mechanisms, such as CTLA-4, and contact independent mechanisms, such as the secretion of anti-inflammatory cytokines (Shevach, 2009). In addition, it has been suggested that Tregs may suppress other immune cells by inducing cell death (Zhao et al., 2006b). To assess whether the mechanism by which Tregs may be suppressing IgM secretion by Ramos B cells is contact-dependent, we performed our modified suppression assay using a transwell culture plate, which allows cells to share media but blocks direct contact. As shown in Fig. 4A, the suppression of IgM secretion is equally efficient even when cells are separated by a transwell membrane, suggesting the suppression is not dependent on direct physical contact. Therefore, the suppression healthy Tregs are exerting on Ramos IgM secretion is mostly contact-independent and likely dependent on a secreted factor.

In order to determine if Tregs are actively inducing cell death in Ramos B cells, we evaluated the percentage of dividing and rapidly proliferating cells (using intracellular 7AAD and Ki67) versus apoptotic/dead cells (sub-normal 7AAD DNA staining) at the end of co-culture as previously described (Vignon et al., 2013). Fig. 4B is a representative example of this staining protocol, where cells in Quadrant 2 are presumed to be proliferating and cells in Quadrant 4 are presumed dead. As shown in Fig. 4C, there was no significant difference in the percentage of dead cells when the Ramos were cultured with or without Tregs. These results were also confirmed using standard extracellular 7AAD/Annexin staining, which did not demonstrate increased apoptosis or total cell death of Ramos cells in the presence of Tregs (data not shown). Tregs also did not consistently influence the percent of rapidly proliferating (Ki67+) Ramos B cells, as shown in Fig. 4D. This suggests that mechanism/s of Treg B cell suppression in this assay are due to a direct regulation of Ramos IgM production/secretion rather than due to an indirect inhibition of Ramos viability or proliferative capacity.

4. Discussion

We established and optimized a modified suppression assay that we will use, in the future, to determine if functional discrepancies exist between Tregs from healthy controls and individuals with active rheumatologic disease. This assay measures the suppression Tregs exert on the baseline secretion of IgM by Ramos B cells, a human cell line that constitutively secretes IgM. We designed this assay so that Tregs are the only variable because we seek to compare their suppressive capacity without concern for additional variables that are present in disease, such as effector cells resisting suppression, which have been described for conditions such as systemic lupus erythematosus (SLE) (Long and Buckner, 2011).

Another important consideration is the limitation of absolute Treg number in peripheral blood, where they are only ~1% of leukocytes in a healthy adult (Thornton et al., 2010; Golding et al., 2013). Many of the patients we hope to study often have significant lymphopenia and their blood donation usually coincides with a blood draw for lab tests, which limits the amount of blood we can safely collect. Therefore, we developed the

modified suppression assay to use the smallest number of Tregs possible without compromising the reliability and reproducibility of the results.

Our preferred method for isolation of enriched CD4⁺CD127⁺CD25⁺ Tregs, like most published methods for purifying human Tregs (Tran, 2013), favors purity at the expense of yield. We estimated a final yield of approximately 250,000 Tregs based on 50mL of blood. Because a fraction of these cells isolated are set aside for confirmatory flow cytometry, we developed the assay using 20,000 Tregs for the 1:1 ratio of Tregs:Ramos. We confirmed that 20,000 Ramos B cells per well provides adequate sensitivity for detecting IgM production by ELISA. In order to perform in triplicate our 1:1 Treg:Ramos and 1:2–1:16 Treg:Ramos co-culture conditions, the modified suppression assay requires 120,000 Tregs. It is reasonable to expect to be able to purify this number of Tregs from 50 mL of blood, which offers a significant advantage over standard Treg:Tconv assays that routinely require 200,000 Treg cells per donor (Tran, 2013).

Prior studies have shown that, in investigating Treg suppression, it is critical to have a highly purified population of Tregs. While fluorescence-activated cell sorting (FACS) may garner the most pure population, it is expensive and time consuming. Therefore, we utilized a magnetic bead-based Treg isolation procedure that is relatively inexpensive and very effective. Using this procedure, we showed that if the population of Tregs is >85% Foxp3⁺ cells in the CD4⁺ population and >70% Helios⁺ cells in the Foxp3⁺CD4⁺ population, and the suppression assay is performed according to our protocol, Tregs from healthy volunteers will reliably exert suppression of baseline IgM secretion by the Ramos B cell line. We assume that FACS-sorted Tregs should perform just as well in this assay. Also, the use of Helios and FoxP3 in combination to confirm the identity of human Tregs is preferred, but other makers of bona fide human Tregs are also valid (Santegoets et al., 2015).

Using this modified suppression assay, we demonstrate that healthy Tregs reliably suppress basal IgM secretion by Ramos B cells. By repeating the assay with multiple healthy donors, we have also begun to establish a range of suppression that can be expected from healthy Tregs and we plan to compare suppression by Tregs from patients with rheumatologic disease to this healthy range. To further investigate the mechanism and extent of this suppression, we experimented with increasing the B cell stimulation, blocking direct contact of the two cell populations, and measuring cell death.

We conclude that, for the purposes of comparing Treg functional capacity with this assay, it is best to co-culture the cells in complete RPMI with 100 U/mL IL-2. This condition offers IL-2 for the maintenance of the Tregs and the complete RPMI sustains the Ramos so that they are secreting detectable levels of IgM. We find that activation of the Tregs with anti-CD3/CD28 is not necessary to observe suppression of IgM secretion, perhaps because the IL-2 alone induces a suppressive phenotype and/or because a portion of the Tregs are already activated when isolated from the peripheral blood. In addition, when we add CD40L and IL-21 to stimulate the Ramos cells to secrete higher levels of IgM, the percent suppression is more variable among healthy donors. Therefore, we need to carry out a greater number of experiments in order to determine what conditions reliably favor Treg suppression of stimulated Ramos.

In addition, we conclude that the suppression enacted by the Tregs is contact-independent. The suppression in co-cultures that proceeded on transwell plates showed equivalent suppression to that of the co-cultures that proceeded on a standard 96-well plate, suggesting there is a/are secreted factor(s) that are necessary for suppression of IgM secretion. This observation suggests that Treg suppression of B cells is qualitatively/mechanistically distinct from suppression of effector T cells, which appears to be contact-dependent (Thornton and Shevach 1998). Future experiments will be directed at determining what secreted factors, such as TGF- β , are necessary for Treg suppression of B cells. This also suggests that different subsets of Tregs (e.g., thymically-derived vs peripherally induced Tregs) may have similar potential for suppressing B cell effector function (Xu et al., 2016).

Finally, by measuring the amount of dead/dying Ramos cells at the end of the co-culture, we concluded that the mechanism of suppression is not dependent on direct Treg killing of Ramos cells. This is in contrast to previously reported data on Treg suppression of B cells which invoked various mechanisms of direct killing, including both granzyme B mediated and FAS/FASL mediated cell death (Zhao et al., 2006a; Iikuni et al., 2009). This difference may be explained by our use of a B cell line as the target of suppression, which may be relatively resistant to cell death as compared to primary B cells. Future experiments will be aimed at elucidating the mechanism of Treg suppression both of Ramos B cells and primary B cells.

Testing Treg suppression of IgM secretion by the Ramos B cell line is intended to be an innovative platform for testing clinically relevant defects in Tregs in disease. It may also serve as a useful tool for the investigation of how Tregs may directly suppress primary B cell development, activation, antibody secretion or class-switching (Kinnunen et al., 2013). Our primary goal was to establish a highly reliable Treg B cell suppression assay that can be easily reproduced in other laboratories and used to efficiently determine intrinsic Treg suppressive defects, particularly when patient blood cell quantities are limiting. We are currently applying this assay to the functional study of Tregs from SLE patients and we are also developing variations on the assay that look at Treg suppression of primary naïve or class-switched human B cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Purification of CD4+CD127–CD25+ cells enriches for FoxP3 + Helios + tTregs. Regulatory T cells were purified from human buffy coats based on surface marker expression. After isolation, enriched CD4+CD127–CD25+ cells (B), as well as the total peripheral blood mononuclear cells (A), were stained with CD3 FITC, CD4 PeCy7, CD25 APC, CD127 PerCP, Foxp3 Pacific Blue, and Helios PE. Foxp3+ staining indicates Treg identity and Helios+FoxP3+indicates thymically-derived Tregs (tTregs). The purity of Tregs is critical for the subsequent suppression assay. A summary of purification yields for the CD4+CD127–CD25+ Tregs used in suppression assays is.

Fig. 2.

Ramos secrete IgM and stimulation increases secretion. 20,000 Ramos B cells were cultured at a density of 8×10^4 cells/mL in complete RPMI $\pm$ 100 U/mL IL-2, 16 ng/mL recombinant human CD40L, and 16 ng/mL recombinant human IL-21. After 4 days, supernatant was harvested and Ig concentration was determined by ELISA. The Ramos cells secrete a basal amount of IgM and IL-2 does not affect this level of secretion. The addition of CD40L and IL-21 increases the secretion of IgM but does not induce class switch and secretion of IgG. (ns indicates $p > 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0005$ using a paired, two-tailed *t*-test).

Fig. 3.

Tregs, but not Tconv, suppress basal IgM secretion. Purified healthy, human, peripheral Tregs, as well as Tconv (CD4+CD127-CD25-), were co-cultured with Ramos B cells for 4 days at various ratios in the presence of 100 U/mL IL-2. As a representative example, (A) shows the suppression observed when Ramos were co-cultured Treg from a healthy donor (n=1) and (B) shows a lack of suppression when Ramos were cocultured with Tconv from the same healthy donor (n=1). Error bars represent the average of three replicate suppression assays with cells from one donor. (C) shows the average suppression of IgM secretion by Tregs from 10 healthy donors, whereas (D) shows the lack of suppression by Tconv from 6 healthy donors. Each dot represents one donor and is the average of replicates. (E) and (F) show the concentration of IgM (ng/mL) in the supernatant of the 0:1 and 1:1 Treg:Ramos (E) or Tconv:Ramos (F) co-cultures. In (E), X, Y, and Z represent the 3 donors whose Tregs were also used in a suppression assay when the Ramos were stimulated (see Fig. 4). Again, each dot represents one donor and is the average of replicates. (ns indicates $p > 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0005$ using a paired, two-tailed ttest.)

Fig. 4. Mechanism of suppression is not death-dependent and may be contact-independent. To assess the mechanism of suppression, the contact dependence of the assay was tested, and cell death was measured. (A) Purified healthy, human, peripheral Tregs, were co-cultured with Ramos B cells as described in the presence of 100 U/mL IL-2. These co-cultures proceeded either on a standard culture plate (black symbols) or on a transwell culture plate (gray symbols). The concentration of IgM in the supernatant was determined by ELISA. (n=2) (B–D) Cells were harvested after 4-day co-culture on a standard plate with IL-2 and the percent of dead Ramos cells was determined by Flow Cytometry. Total cells were stained with CD4, fixed and permeabilized, and then stained with Ki67 and 7AAD (B). Cells that were CD4–Ki67–7AAD– were presumed to be dead Ramos cells. (C) shows the percent dead Ramos at the 0:1 and 1:1 ratio of Tregs:Ramos B cells in 5 separate experiments and (D) shows the percent proliferating Ramos at the 0:1 and 1:1 ratio of Tregs:Ramos B cells in 4 separate

experiments. No significant difference was observed between Ramos cultured without or without Tregs.