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Research paper

A novel and rapid method to quantify Treg mediated suppression of CD4 T cells

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ABSTRACT

Measuring regulatory T cell suppression provides important insight into T cell dysfunction in autoimmune disease. However, to date, suppression assays are limited by the requirement for freshly isolated cells, and significant cell numbers. Here, we present a novel and rapid *in vitro* assay using effector T cell surface expression of both CD25 and CD134 as a surrogate marker of regulatory T cell-mediated suppression. This surface marker-based suppression assay works for frozen samples and for samples with limited cell numbers. It is also shorter taking two days to complete compared to the four days required for proliferation-based assays. Furthermore, this assay works with both *in vitro* expanded and natural Tregs, as well as anti-CD3/anti-CD28 bead-based and APC stimulation conditions. In conclusion, we have developed and validated a new suppression assay for cryopreserved samples with limited cell numbers that may be helpful to investigate T cell regulation in the context of infection or autoimmune diseases.

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1. Introduction

Quantification of immune cell function is paramount to our understanding of the pathogenesis of immunological diseases and to identifying successful therapies. In patients with autoimmune disease, the regulation of the immune system is of particular interest and CD4+ regulatory T cells (Tregs) have been an important research area. CD4+CD25+FOXP3+ Tregs suppress CD4 T cells *via* multiple mechanisms, leading to decreased cell proliferation, changes in expression of cell surface proteins, and diminished production of cytokines. In the study of human disease, measurement of suppression *in vitro* is vital to research and has been achieved through co-culture of Tregs and T effector cells (Teff). Methods include measuring cell proliferation (thymidine incorporation or CFSE dye dilution); cytokine production (typically IFNγ and TNFα) (McMurchy and Levings, 2012); and activation markers (CD69 and CD154) on the cell surface (Canavan et al., 2012). Although *in vitro* assays may not fully reflect *in vivo* suppression, co-culture assays do identify defective Treg function (Bacchetta et al., 2006) and demonstrate resistance of effector cells (CD4+CD25−) to suppression by Treg in the setting of several autoimmune diseases (Lawson et al., 2008; Schneider et al., 2008; Goodman et al., 2009; Schneider and Buckner, 2011; Schneider et al., 2013).

T cell proliferation is dependent on signals *via* the TCR and co-stimulatory molecule CD28. Among the proteins that are expressed in response to T cell activation is IL2Rα (CD25), which is very tightly controlled through transcriptional regulation. CD25 is minimally expressed in resting CD4 T cells, but is rapidly upregulated upon stimulation of the TCR (Kim et al., 2006). A number of other cell surface molecules are rapidly upregulated following TCR signaling including OX40 (CD134), ICAM-1 (CD54), CD69, and CD40L (CD154), which have a variety of functions such as aiding proliferation, survival, cytokine production, and cell adhesion (Tohma et al., 1992; Mardiney et al., 1996; Chatzigeorgiou et al., 2009; Croft et al., 2009).

Overall, the assessment of Treg mediated suppression of effector T cells has been limited due to the number of cells required, the need for long culture times (4–6 days), and the complexity of experimental set up of Treg assays. CSFE based co-culture assays have become the gold standard of *in vitro* assays, but consume larger numbers of cells, due to cell loss during the CSFE staining process (approx. 50% cells lost), and require 4 days of culture. Measurement of proliferation by uptake of tritiated thymidine can be achieved with a limited number of cells, but does not allow concurrent assessment of number of cell divisions and the characteristics of dividing cells available with flow cytometry techniques. Early activation markers CD69 and CD154 have been used to assess suppression (Canavan et al., 2012; Ruitenberg et al., 2011), an approach that decreases the time of co-culture to as few as 7 h and has been used successfully to assess the function of Tregs, which have been enriched

Abbreviations: eTreg, expanded regulatory CD4+ T cell; nTreg, *ex vivo* 'natural' CD4+ regulatory T cell; Teff, effector CD4+ T cell; d1, day 1; d2, day 2; d4, day 4; CFSE, carboxyfluorescein succinimidyl ester; aCD3/aCD28, anti-CD3/anti-CD28 Dynabeads; PBMC, peripheral blood mononuclear cells.

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or expanded *in vitro* or the target of IL-2 immunotherapy (Berglund et al., 2013; Hannon et al., 2014; Ito et al., 2014; Landwehr-Kenzel et al., 2014). However, this assay has barriers due to the need to perform the assay within a single extended workday and high reagent costs. In other studies, CD25 and CD134 have been used to identify antigen specific responses and their regulation in the context of viral infection (Endl et al., 2006; Zaunders et al., 2009; Shaw et al., 2011; Keoshkerian et al., 2012).

Our goal was to develop a method to measure events close to the start of cell division, but prior to extensive proliferation. To achieve this, we examined the cell surface molecules known to be upregulated in activated/proliferating cells at 48 h (the time in which initial T cell division occurs after activation) in order to identify an alternative marker that could be used to measure suppression. This study showed that the combined expression of CD25 and CD134 on effector cells is a good surrogate for, or additional measure to proliferation that is suppressed by Tregs to a similar degree.

2. Materials and methods

2.1. Subjects

This study was approved by the Benaroya Research Institute's Institutional Review Board, and all subjects signed written informed consent prior to inclusion in the study. Frozen PBMCs and fresh peripheral blood samples were obtained from the Benaroya Research Institute Immune Mediated Disease Registry and Repository. In total, cryopreserved samples from 50 healthy adult volunteers were used for suppression assays. In addition, six volunteers provided fresh blood samples for expanded Tregs, CD4 + CD25 + nTregs, or large quantities of Teff from a single source as assay controls.

2.2. Autoantibodies and reagents

Flow cytometry data were acquired on a FACS Canto (BD Biosciences). Teff (CD4 + CD25 −) were stained with CFSE (Invitrogen) and Tregs with eFluor670 (EF670) (eBiosciences) as previously published (Schneider and Buckner, 2011). In addition, the following were used: FITC anti-CD69; PE CD25, CD54, CD134, CD137, CD152, CD154, CD183 or CD278; PE-Cy5 CD4 or CD25; PE-Cy7 CD25; BV421 CD279 or CD134; APC-Cy7 CD4; and Live/Dead Aqua (Invitrogen). Analysis of cell cycle progression was carried out according to manufacturer's instructions using a FITC BrdU kit (BD Biosciences). Cells were cultured in RPMI1640 HEPES media containing 10% pooled human serum, additional L-glutamine, penicillin/streptomycin, and sodium pyruvate (Sigma).

2.3. Cell sources

Unless otherwise specified, experiments used an expanded source of regulatory cells (eTregs) from a single healthy control, and effector cells (Teff) were isolated from frozen PBMC. The eTregs were generated by isolation of CD4 + CD25^{HI}CD127 − using a FACS Aria (BD) and expansion over 14 days with IL-2 (Chiron) and anti-CD3/anti-CD28 (aCD3/aCD28)–coated Dynabeads (Invitrogen) (Putnam et al., 2009). The percentage of FOXP3 + cells was verified to be >90%. Effector cells (CD4 + CD25 −) were isolated using MACs kits through negative selection for CD4 and positive selection for CD25 (Miltenyi Biotec) (Schneider and Buckner, 2011). In experiments using *ex vivo* Tregs (nTregs), the highest 2% of CD25 stained CD4 + cells were isolated from PBMC using a FACs Aria. In these experiments, freshly sorted Teffs were the lowest 50% of CD25 stained CD4 + cells. The marker CD127 was not included in this isolation strategy, for simplicity, based on previous data showing high (90%) FOXP3 expression in the cells with the highest 3–5% of CD25 expression (Mikacenic et al., 2014).

2.4. Suppression assays

Analysis was carried out at 7 h, 1 day (20–24 h, d1), 2 days (44–48 h, d2), and/or 4 days (90–96 h, d4) as indicated. For all time points Teffs (number of cells varied between assays) were co-cultured with several ratios of Tregs cells in 96-well plates with anti-CD3/anti-CD28 Dynabeads (Invitrogen). Tregs and Teff were cultured at ratios between 0:1, 1:2 to 1:64 in doubling dilutions. Where insufficient cells were available ratios of 1:8 > 1:4 > 1:2 > 1:16 were prioritized. For analysis, Teff cultured in media alone were used to set gates for the various activation markers used or proliferation. EF670 was used to identify Treg; Teff cells were in the EF670-gate. Representative gating for 7 h, 2 days, and 4 days is shown in Figs. S1–S3. Flow cytometry was standardized between experiments using eight peak beads (Spherotech).

For the 7-hour assay, five wells of 50,000 Teff were prepared per condition. These were stimulated with Dynabeads (Invitrogen) at a bead:Teff ratio of 1:5 (high) or 1:28 (low). Anti-CD154 PE was added at start of assay all other markers were cell surface stained at the end of assay. Total cell requirement for this method was approximately 1.8 million Teff and 250,000 eTregs per person.

For the 1 day, 2 day, and 4 day experiments 100,000 Teff were plated three times for each condition, and Dynabeads were added at a ratio of 1:28 (beads:Teff). Adaptations of this method included the use of a 1:35 (beads:Teff) ratio for nTreg experiments, and a 1:4 ratio (Teff:irradiated CD4 − APC) with additional aCD3 (UCHT1, 5 μg/ml) and aCD28 (CD28.2, 1 μg/ml) for APC experiments. When added, Pam3CSK4 (1 μg/ml; Invivogen) was included at the start of co-culture. Analysis of proliferation by CFSE and activation by cell surface marker were carried out on the same cells. For these experiments approximately 2.5 million CD4 + CD25 − and 300,000 Tregs were used for each day of analysis. An adaptation of this method used 5000 Teff plated in triplicate, this required 90,000 Teff and 15,000 nTregs per 'miniature' assay; In these experiments flow cytometry based sorting of 60 million frozen PBMC yielded between 20 and 70,000 nTregs after staining with EF670.

Percentage suppression (S) was calculated as follows:

$$S = \frac{a-b}{a} \times 100$$

where a is the percentage proliferation or marker + (*e.g.* CD25 + CD134 +) in the absence of Tregs and b is the percentage proliferation or marker + in the presence of Tregs.

2.5. Statistical analysis

Spearman's rank correlation was used to analyze the relationship between different measures of activation/proliferation and suppression. Bland-Altman analysis was used to interrogate bias between different measures for activation/proliferation and suppression. Wilcoxon matched pairs test was used to compare suppression in the presence/absence of Pam3CSK4. Kruskal-Wallis Test was used to compare suppression based on source of Teff and Treg in 'miniature' assays. FlowJo software (Tree Star) was used for all analysis of flow cytometry and Prism (GraphPad Software) was used for all statistical analysis.

3. Results

3.1. CD25 cell surface expression correlates with CFSE-based proliferation at day 4

CD25 is upregulated in response to T cell stimulation and by IL-2 within 48 h of initial activation, and persists for at least 4 days (Wang et al., 2008). To determine whether CD25 expression on Teff correlated with number of proliferating cells, co-culture experiments were performed with Teffs (CD4 + CD25 −) from 14 healthy volunteers. Teffs were labeled with CFSE, and then placed in culture with *in vitro* expanded Tregs

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