frontiers in Immunology

ORIGINAL RESEARCH
published: 05 February 2019
doi: 10.3389/fimmu.2019.00150

The Value of a Rapid Test of Human Regulatory T Cell Function Needs to be Revised

Desiree J. Wendering[1,2,3], *Leila Amini*[1,2,3], *Stephan Schlickeiser*[1,3], *Petra Reinke*[2,3], *Hans-Dieter Volk*[1,2,3] *and Michael Schmueck-Henneresse*[1,2,3]*

[1] *Institute for Medical Immunology, Charité–Universitätsmedizin Berlin, Berlin, Germany,* [2] *Berlin Center for Advanced Therapies (BeCAT), Charité–Universitätsmedizin Berlin, Berlin, Germany,* [3] *Berlin Institute of Health (BIH) Centrum for Regenerative Therapies (B-CRT), Charité–Universitätsmedizin Berlin, Berlin, Germany*

$CD4^+CD25^+FoxP3^+$ human regulatory T_{CELLS} (T_{REG}) are promising candidates for reshaping undesired immunity/inflammation by adoptive cell transfer, yet their application is strongly dependent on robust assays testing their functionality. Several studies along with first clinical data indicate T_{REG} to be auspicious to use for future cell therapies, e.g., to induce tolerance after solid organ transplantation. To this end, T_{REG} suppressive capacity has to be thoroughly evaluated prior to any therapeutic application. A 7 h-protocol for the assessment of T_{REG} function by suppression of the early activation markers CD154 and CD69 on $CD4^+CD25^-$ responder T_{CELLS} (T_{RESP}) upon polyclonal stimulation via αCD3/28-coated activating microbeads has previously been published. Even though this assay has since been applied by various groups, the protocol comes with a critical pitfall, which is yet not corrected by the journal of its original publication. Our results demonstrate that the observed decrease in activation marker frequency on T_{RESP} is due to competition for αCD3/28-coated microbeads as opposed to a T_{REG}-attributable effect and therefore the protocol cannot further be used as a diagnostic test to assess suppressive T_{REG} function.

Keywords: regulatory T cell functional assay, αCD3/28-coated microbeads, competitive CD3/CD28 binding, nullified Treg-mediated suppression, correlation between T cell-to-αCD3/CD28-coated microbead ratio and activation marker frequency on responder T cells

OPEN ACCESS

Edited by:
Lucienne Chatenoud,
Université Paris Descartes, France

Reviewed by:
Karin Loser,
University of Münster, Germany
Wayne Hancock,
University of Pennsylvania, United States

***Correspondence:**
Michael Schmueck-Henneresse
michael.schmueck-henneresse@charite.de

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *01 November 2018*
Accepted: *17 January 2019*
Published: *05 February 2019*

Citation:
Wendering DJ, Amini L, Schlickeiser S, Reinke P, Volk H-D and Schmueck-Henneresse M (2019) The Value of a Rapid Test of Human Regulatory T Cell Function Needs to be Revised. Front. Immunol. 10:150. doi: 10.3389/fimmu.2019.00150

INTRODUCTION

Regulatory T_{CELLS} (T_{REG}) are key players in maintaining immune homeostasis, resolution of inflammation, and self (1). Exploiting those characteristics, T_{REG} have gained plenty of attention as promising candidates in immunotherapeutic applications for the prevention or reshaping of undesired immune responses such as in autoimmune diseases, chronic inflammation, and allograft rejections. Data from clinical trials identify T_{REG} as an encouraging cell type for use in cellular therapy (2). By the same token, a robust protocol to assess T_{REG} function is of utmost importance to ensure their suppressive function prior to adoptive cell-therapeutic clinical trials, as well as for application in basic research. So far, for assessing T_{REG} functionality, evaluating the suppressive capacity of T_{REG} to inhibit the proliferation of responder T_{CELL} (T_{RESP}) after a 4-day co-cultivation period has been the gold-standard protocol since a decade (3, 4). Recently, Canavan et al. (5) and Ruitenberg et al. (6) described a rapid 7 h assay for the evaluation of T_{REG} functionality by assessing their suppressive capacity using upregulation of the early T_{CELL} activation makers

CD154 (CD40L) and CD69 on conventional $CD4^+CD25^-$ responder T_{CELLS} (T_{RESP}) upon CD3/28 engagement. CD3/28 stimulation is mediated by microbeads coupled with αCD3 and αCD28 antibodies. According to these studies, T_{REG} alleviate CD154 and CD69 expression on T_{RESP} in a dose-dependent manner. Even though this assay has since been frequently applied and cited more than 80 times (7, 8, 10), we observed that the protocol comes with a critical pitfall: T_{RESP} and T_{REG} both express the signaling molecule CD3 and T_{CELL} co-stimulatory receptor CD28 on the plasma membrane, potentially competing for binding αCD3/28 T_{CELL} activating microbeads applied in the rapid 7 h assay. We investigated whether the observed decreased frequencies of activated T_{RESP} can be claimed to be a T_{REG}-attributable effect or if it is rather a result of competition for αCD3/28-coated activating microbeads. We thus explored whether different ratios of αCD3/28 T_{CELL} activation microbeads-to-T_{CELLS} impact the outcome of this functional T_{REG} assay.

MATERIALS AND METHODS

Study Design

The aim of this study was to investigate the influence of αCD3/CD28-coated activating microbeads on the expression of early activation markers CD69 and CD154, used for predicting T_{REG} functionality in basic and translational research. We compared the expression of CD69 and CD154 of T_{RESP} in T_{REG} co-cultures, which were either activated via αCD3/CD28-coated microbeads adjusted to T_{RESP} only or to the total cell number present in one well (T_{RESP} + T_{REG}). To verify the integrity of the T_{REG} used in this study, as well as to demonstrate the T_{REG}-mediated suppressive function in a bead-uncompetitive setting, T_{RESP} proliferation suppression experiments were performed.

Cell Isolation

Peripheral blood mononuclear cells from healthy donors were purified using Ficoll-Paque separation (Biochrom). $CD4^+$ cells were enriched by magnetic-activated cell sorting (Miltenyi) according to manufacturer's instructions (purity>90%). For fluorescence-activated cell sorting (FACS Aria II, BD) of $CD4^+CD25^{high}CD127^{low}$ T_{REG} and $CD4^+CD25^-$ T_{RESP}, cells were stained with CD4 (SK3, Biolegend), CD25 (2A3, BD), and CD127 (R34.34, Beckman Coulter). Post-FACSort analysis by flow cytometry yielded $CD25^+FoxP3^+$ T_{CELL} purity of >95%.

7 h Diagnostic Test for T_{REG} Function and αCD3/28 Microbead Titration

Assays were performed as described by Canavan et al. (5). Briefly, CFSE-labeled T_{RESP} were co-cultured with autologous T_{REG} at T_{RESP}/T_{REG} ratios ranging from 1:1 to 32:1. In two parallel setups, cells were either stimulated with αCD3/28-coated microbeads (Dynabeads® Human T-Activator CD3/CD28, Thermo Fisher Scientific) at a bead/cell ratio of 0.2 adjusted to the T_{RESP} cell number per well (5, 6) or adapting the ratio of 0.2 to the total cell number per well including T_{REG}. Stimulated and unstimulated T_{RESP} without T_{REG} were included as controls. For the microbead titration, T_{RESP} were cultured alone at bead/T_{RESP} ratios ranging from 0.1 to 0.4 (mimicking the presence of T_{REG}). αCD154 (24–31) was added at start of incubation. Cells were incubated at 37°C for 7 h. All cell cultures were performed in *X-Vivo*-15 medium supplemented with 10% FCS (Lonza & Biochrom) and 100 IU/ml Penicillin/Streptomycin. After harvesting, cells were stained with CD3 (OKT3), CD4 (SK3), CD137 (4B4-4), and CD69 (FN50), all Biolegend. Dead cells were excluded (LIFE/DEAD™ Fixable Blue Dead Cell Stain Kit, Thermo Fisher Scientific).

Proliferation Suppression Assay

CFSE-labeled T_{RESP} were cultured alone or with autologous T_{REGS} at T_{RESP}/T_{REG} ratios ranging from 1:1 to 16:1. The cells were stimulated with αCD3/28-coated microbeads (T_{REG} Suppression Inspector, Miltenyi) at a cell/bead ratio of 1:1 and 1:2 adjusted to the total cell number per well and incubated at 37°C for 96 h. Thereafter, cells were stained with CD3 (OKT3), CD4 (SK3), all Biolegend. Dead cells were excluded (Thermo Fisher Scientific). Proliferation was assessed by CFSE dilution and percentage suppression of proliferation was calculated by relating the percentage of proliferating T_{RESP} in the presence and absence of T_{REG}, respectively.

Flow Cytometry Analysis

Data were acquired on a LSR-II Fortessa flow cytometer (BD) and analyzed using FlowJo V10 (TreeStar).

Statistics

Analysis was performed with GraphPad Prism software (version 6, GraphPad, La Jolla, CA) and R (version 3.4.1) (9). We have tested for significant interaction, i.e., non-parallel response profiles of the two bead adjustment methods to the different T_{RESP}:T_{REG} ratios, using a non-parametric rank-based ANOVA-type statistic [as implemented in the *nparLD* package (11)] in a two-way factorial repeated measures design. For bead titration experiments, non-parametric two-tailed Wilcoxon matched-pairs signed rank tests were used to determine significance in pairwise comparison. Data indicate means ± SEMs in all bar graphs. $P < 0.05$ was considered significant.

RESULTS

T_{CELL} Early Activation Marker Expression Is Dependent of TCR Engagement

We first examined T_{REG} functionality according to the protocols published by Canavan et al. (5) and Ruitenberg et al. (6), whereby *ex vivo* FACSorted and CFSE-labeled T_{RESP} were co-cultured in the presence and absence of autologous T_{REG} and stimulated with αCD3/28-coated activating microbeads at a ratio of 0.2 microbeads per T_{RESP} (**Figure 1A**). After 7 h, the mean frequency of $CD154^+$ and $CD69^+$ T_{CELLS} of unstimulated T_{RESP} was 0.14 and 0.45%, respectively and 57.25 and 78.26% on CD3/28-stimulated T_{RESP}, respectively (**Figure 1B**). When T_{RESP} were stimulated in the presence of T_{REG} at ratio 1:1, the mean frequency of $CD154^+$ and $CD69^+$ T_{CELLS} decreased to 47.77 and 69.86%, respectively. With increasing T_{RESP}/T_{REG} ratios both, CD154 and CD69 expression, increased in a linear fashion (**Figure 1C**, quantified in E, F, red columns). We

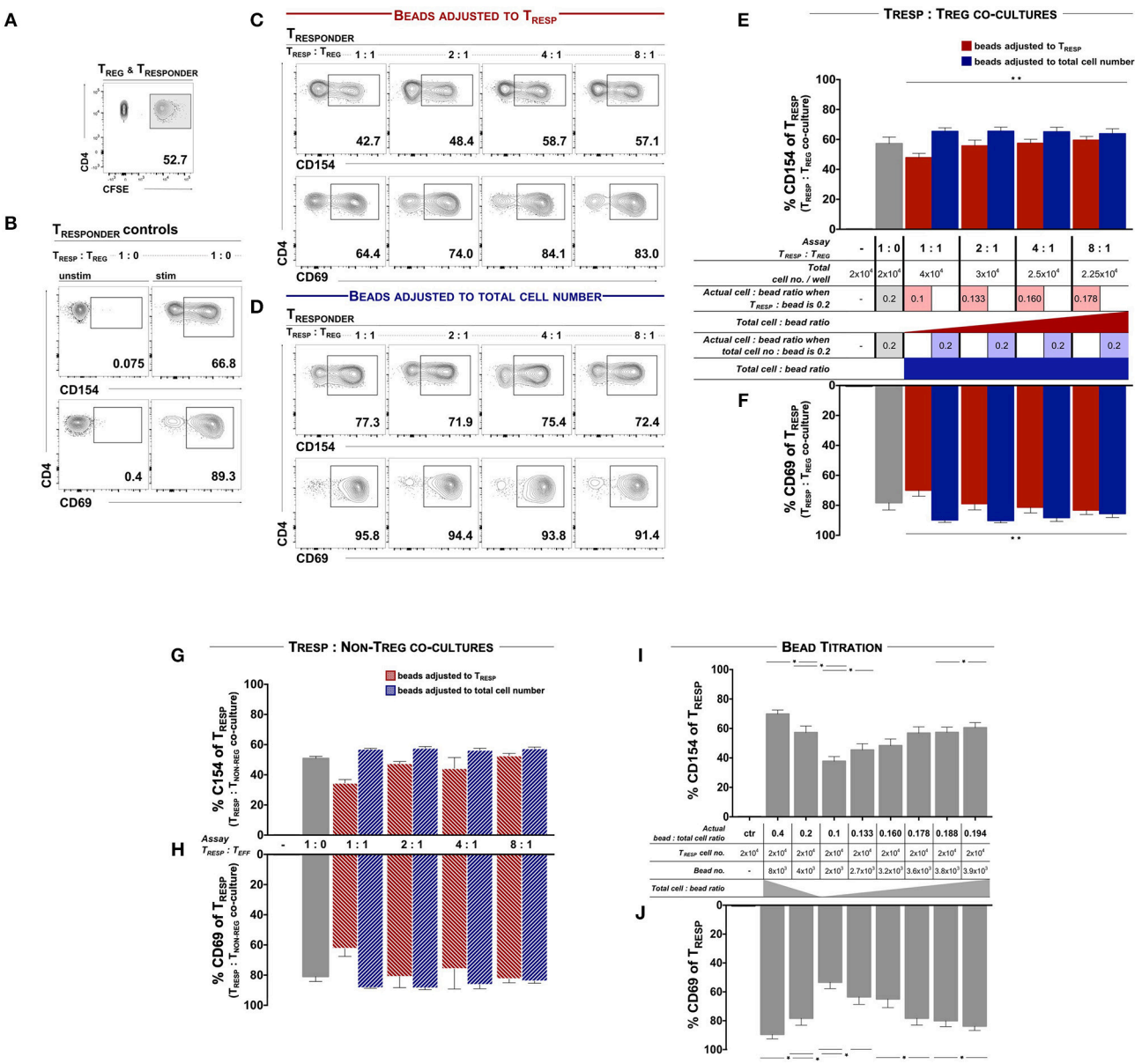

FIGURE 1 | T_{CELL} early activation marker expression is dependent of TCR engagement and cannot be used for T_{REG} functional evaluation. FACSorted $CD4^+CD25^-$ T_{RESP} with and without autologous T_{REG} co-culture were stimulated with anti-CD3/CD28-coated microbeads and analyzed for early activation marker expression. **(A)** For precise T_{RESP}/T_{REG} discrimination, T_{RESP} were labeled with CFDA-SE (CFSE). **(B)** Representative plots of CD154 and CD69 expression on unstimulated and stimulated T_{RESP} cultured without T_{REG}. **(C)** Representative plots of CD154 and CD69 expression of T_{RESP} co-cultured with T_{REG} at different T_{RESP}:T_{REG} ratios and stimulated with anti-CD3/CD28-coated microbeads adjusted to T_{RESP}. **(D)** Representative plots of CD154 and CD69 expression of T_{RESP} co-cultured with T_{REG} at different T_{RESP}:T_{REG} ratios and stimulated with anti-CD3/CD28-coated microbeads adjusted to total cell number. **(E,F)** Quantified data from **(C,D)**, respectively. CD154 and CD69 of $CFSE^+T_{RESP}$ co-cultured with FACSorted T_{REG} at different T_{RESP}:T_{REG} ratios and stimulated with anti-CD3/CD28-coated microbeads adjusted to T_{RESP} (red columns) and to total cell numbers (blue columns). For clarification, the table summarizes the experimental setups. $n = 7$. Non-parametric rank-based ANOVA-type statistic $^{**}p < 0.001$ (CD154: $p = 1.90E\text{-}06$, CD69: $p = 5.527256E\text{-}16$). **(G)** Expression of CD154 and **(H)** expression of CD69 of $CFSE^+T_{RESP}$ co-cultured with FACSorted $T_{NON-TREG}$ at different T_{RESP}:T_{REG} ratios and stimulated with anti-CD3/CD28-coated microbeads adjusted to T_{RESP} (red columns) and to total cell numbers (blue columns). $n = 3$. **(I)** Expression of CD154 and **(J)** expression of CD69 of $CFSE^+T_{RESP}$ after different anti-CD3/CD28-coated microbead:T_{RESP} ratio stimulation. For clarification, the table summarizes the experimental setups. $n = 7$. $^*p < 0.05$, Wilcoxon matched-pairs signed rank test. T_{RESP}:T_{REG}/$T_{NON-TREG}$ co-cultures **(E,F)** and corresponding bead titration **(I,J)** experiments were performed simultaneously using the same donor cells. Median data of independent experiments are shown and error bars represent SEM.

next determined whether the total T_{CELL}/bead ratio influences T_{REG}-induced activation marker suppression. Accordingly, we adjusted the bead numbers to the total cell numbers, including T_{REG}, thereby eluding the bead competition in contrast to Canavan et al. (5) and Ruitenberg et al. (6). In that case, T_{RESP} activation in the presence of T_{REG} equaled control T_{RESP}

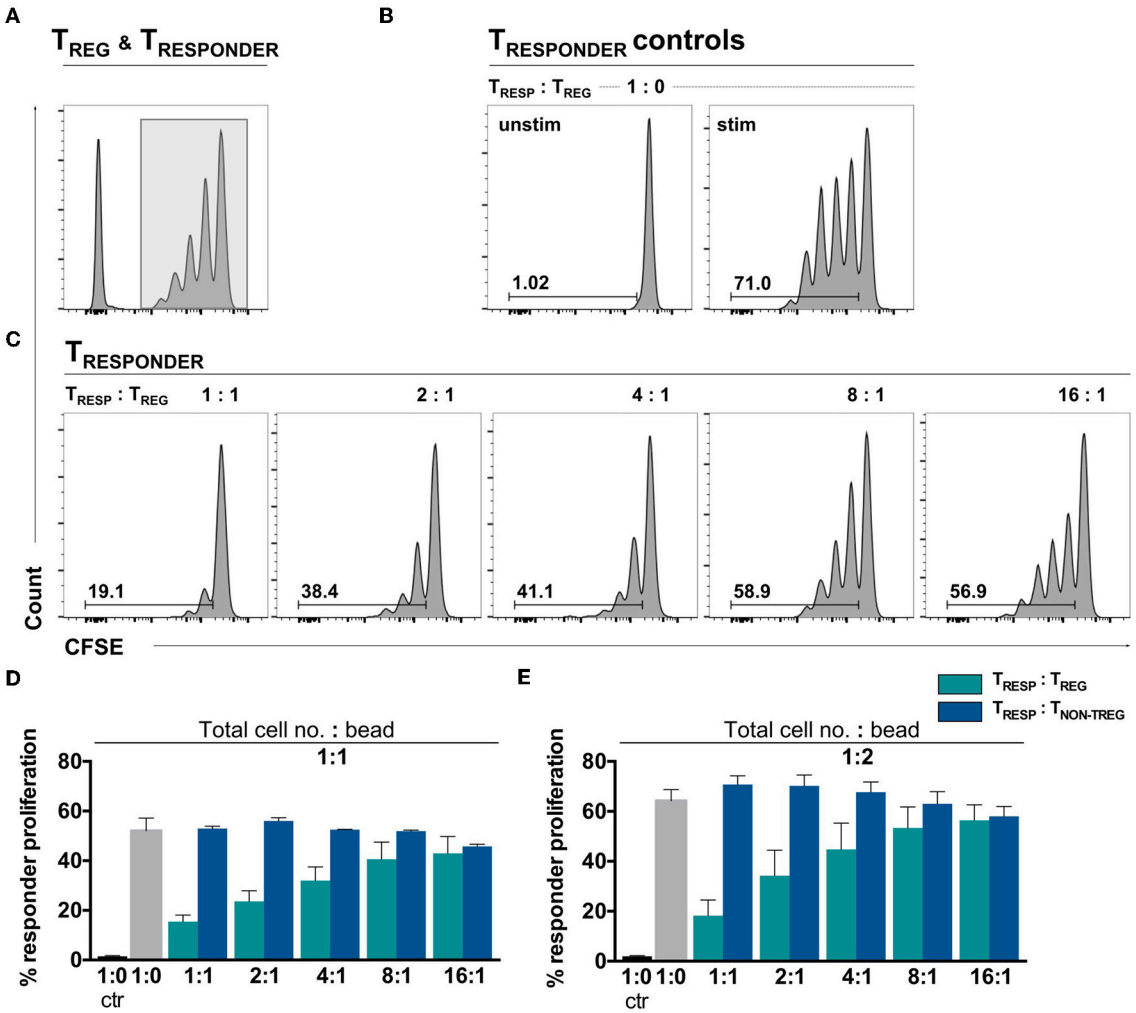

FIGURE 2 | *Ex vivo* isolated T_{REG} demonstrate a dose-dependent T_{RESP} proliferation suppression in a bead-uncompetitive setting. FACSorted $CFSE^{+}CD4^{+}CD25^{-}$ T_{RESP} were co-cultured with autologous FACSorted T_{REG} and stimulated with anti-CD3/CD28-coated microbeads for 96 h. T_{RESP} proliferation was analyzed by CFSE dilution. Representative plots depicting **(A)** CFSE-labeling strategy to accurately analyze T_{RESP} proliferation; **(B)** proliferation of unstimulated and stimulated $CFSE^{+}T_{RESP}$ cultured without T_{REG} and **(C)** $CFSE^{+}T_{RESP}$ proliferation after co-culture with different T_{REG} ratios. **(D)** Percentage of T_{RESP} proliferation after co-culture with decreasing T_{RESP}:T_{REG} ratios (green bars) and T_{RESP}:$T_{NON^{-}TREG}$ ratios (blue bars) stimulated with a total cell number:bead ratio of 1:1. $n = 7$ T_{RESP}:T_{REG} co-cultures, $n = 3$ T_{RESP}:$T_{NON-TREG}$ co-cultures. **(E)** Percentage of T_{RESP} proliferation after co-culture with decreasing T_{RESP}:T_{REG} ratios (green bars) and T_{RESP}:$T_{NON^{-}TREG}$ ratios (blue bars) stimulated with a total cell number:bead ratio of 1:2. $n = 3$. Median data of independent experiments are shown and error bars represent SEM.

cultures without T_{REG} (**Figure 1D**, quantified in E, F, blue bars), indicating that indeed T_{RESP} and T_{REG} compete for CD3/28-binding microbeads. Serving as a negative control, we co-cultured T_{RESP} with $CD4^{+}CD25^{-}$ non-T_{REG}/effector T_{CELLS} in place of T_{REG}. When the bead number was adjusted to T_{RESP} only we observed similar reductions of CD154 and CD69 expression (**Figures 1G,H**, red bars) as when T_{RESP} were co-cultured with T_{REG} (**Figures 1E,F**, red bars). Correspondingly, when adjusting the bead number to the total cell number (**Figures 1E,H**, blue bars), the expression of CD154 and CD69 is similar to the conditions with T_{RESP} only (**Figures 1E–H**, gray bars). To mimic the competition for the activating microbead stimuli, we stimulated T_{RESP} with different amounts of αCD3/28-coated microbeads in the absence of T_{REG}. We set the actual bead/T_{CELL} ratio according to the published T_{RESP}/T_{REG} co-culture approach, in which the activation bead/T_{RESP} ratio is adjusted to T_{RESP} only, i.e., calculated the actual bead/T_{CELL} ratio in each setting. CD154 and CD69 expression decreased in a dose-dependent manner with highest expression levels at a bead/T_{RESP} ratio of 0.4 (69.83 and 89.47%, respectively) and lowest at a ratio of 0.1 (37.80 and 53.33%, respectively). The T_{RESP} activation pattern with the different bead ratios ranging from 0.1 to 0.194 indicate a strong bead/T_{RESP} ratio dependency (**Figures 1I,J**).

T_{REG} Demonstrate a Dose-Dependent T_{RESP} Proliferation Suppression in a Bead-Uncompetitive Setting

To confirm T_{REG} functionality in an environment where the number of αCD3/28-activation microbeads is adjusted to the total cell number, the gold-standard T_{RESP} proliferation suppression assay was performed. The proliferation assay was conducted with T_{CELLS} of the same donors in parallel to the experiments shown in **Figure 1**. Following activation, T_{RESP} proliferation alone yielded 52.03% and dose-dependently decreased in the presence of T_{REG} to 15.51% at a T_{RESP}/T_{REG} ratio of 1:1 (**Figures 2A–C**, quantified in **Figure 2D**, green bars). Thus, we conclude that the T_{REG} employed in this study are able to suppress T_{RESP} proliferation in a standardized bead-competitive setting. To ascertain the reduction of proliferation to be T_{REG}-mediated, we have added non-T_{REG}/effector T_{CELLS} instead of T_{REG} to T_{RESP} and observed no decrease in T_{RESP} proliferation, indicating the suppression of T_{RESP} proliferation to be a T_{REG}-attributable effect (**Figure 2D**, blue bars). Even when T_{CELLS} are stimulated with twice the number of activating αCD3/CD28 microbeads, the T_{REG}-specific impact in suppressing T_{RESP} proliferation can be seen (**Figure 2E**).

DISCUSSION

In conclusion, when adjusting the αCD3/28-bead numbers to only T_{RESP} in co-cultures of T_{RESP} and T_{REG}, activation marker expression was comparable to approaches where T_{RESP} were cultured alone at same bead/total cell ratio present in the T_{RESP}/T_{REG} co-culture. When normalizing αCD3/28-bead competition by adjusting the bead number to total cell numbers, T_{REG}-mediated suppression of activation marker upregulation is nullified. Even more strikingly, when titrating non-T_{REG}/effector T_{CELLS} to T_{RESP} and adjusting the αCD3/28-bead numbers to T_{RESP} only, we observe the same decrease in activation marker expression as in T_{RESP}:T_{REG} co-cultures. We thereby demonstrate that the suppression of activation marker expression on T_{RESP} observed in co-cultures with T_{REG} are due to competitive T_{CELL} receptor and CD28 engagement limited by αCD3/28 microbead availability rather than by suppressive activity of T_{REG} (**Supplementary Figure 1**). There is a pressing demand for a fast assay to evaluate T_{REG} functionality, especially in the light of upcoming clinical trials needing a robust diagnostic test to assess the suppressive function as a release criterion for their T_{REG} cell products. Nonetheless, the T_{RESP} proliferation suppression analysis should still be considered as the gold-standard T_{REG} functional assay as it is performed by adjusting the activation bead to T_{CELL} ratios in experimental setups with decreasing T_{REG} cell numbers (to assess T_{REG} dose-dependent suppression). Since we firmly believe that activation bead to T_{CELL} receptor competition should be kept constant throughout all conditions within a T_{REG} functional assay, we claim that the rapid assessment for human T_{REG} function proposed by Canavan et al. (5) and Ruitenberg et al. (6) does not result in reliable evidence of functional suppression since the putative T_{REG}-mediated suppression of T_{RESP} activation is to be ascribed to competitive T_{CELL} receptor and CD28 engagement. Hence, we suggest that the previously published protocol is unsuitable as a diagnostic test to assess suppressive T_{REG} function.

ETHICS STATEMENT

The Charité Ethics Committee (IRB) approved the study protocol and all blood donors provided written informed consent.

AUTHOR CONTRIBUTIONS

DW designed the research, performed experiments, analyzed and interpreted the data, and wrote the manuscript. LA performed experiments and revised the manuscript. SS performed statistical analyses. PR revised the manuscript. H-DV interpreted the data and revised the manuscript. MS-H led the project, designed the research, analyzed and interpreted the data, and wrote the manuscript.

FUNDING

The study was generously supported in parts by the Deutsche Forschungsgemeinschaft (DFG-SFB-TR36-project A3–H-DV, PR, and MS-H) and the German Federal Ministry of Education and Research (Berlin-Brandenburg Center for Regenerative Therapies grant—all authors). We acknowledge support from the German Research Foundation (DFG) and the Open Access Publication Fund of Charité—Universitätsmedizin Berlin.

ACKNOWLEDGMENTS

We acknowledge the assistance of the BCRT Flow Cytometry Core Lab, Dr. D. Kunkel and J. Hartwig. Mathias Streitz for flow cytometry assistance. Karolina Grzeschik and Anke Jurisch for technical assistance. Dr. Toralf Roch for critical discussions. We likewise acknowledge support from the German Research Foundation (DFG) and the Open Access Publication Fund of Charité — Universitätsmedizin Berlin.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00150/full#supplementary-material

REFERENCES

1. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* (2008) 133:775–87. doi: 10.1016/j.cell.2008.05.009

2. Desreumaux P, Foussat A, Allez M, Beaugerie L, Hébuterne X, Bouhnik Y, et al. Safety and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn's disease. *Gastroenterology* (2012) 143:1207–17.e1–2. doi: 10.1053/j.gastro.2012.07.116

3. Brusko TM, Hulme MA, Myhr CB, Haller MJ, Atkinson MA. Assessing the *in vitro* suppressive capacity of regulatory T cells. *Immunol Invest.* (2007) 36:607–28. doi: 10.1080/08820130701790368
4. Venken K, Thewissen M, Hellings N, Somers V, Hensen K, Rummens J-L, et al. A CFSE based assay for measuring CD4+CD25+ regulatory T cell mediated suppression of auto-antigen specific and polyclonal T cell responses. *J Immunol Methods* (2007) 322:1–11. doi: 10.1016/j.jim.2007.01.025
5. Canavan JB, Afzali B, Scottà C, Fazekasova H, Edozie FC, Macdonald TT, et al. A rapid diagnostic test for human regulatory T-cell function to enable regulatory T-cell therapy. *Blood* (2012) 119:e57–66. doi: 10.1182/blood-2011-09-380048
6. Ruitenberg JJ, Boyce C, Hingorani R, Putnam A, Ghanekar SA. Rapid assessment of *in vitro* expanded human regulatory T cell function. *J Immunol Methods* (2011) 372:95–106. doi: 10.1016/j.jim.2011.07.001
7. Landwehr-Kenzel S, Issa F, Luu S-H, Schmück M, Lei H, Zobel A, et al. Novel GMP-compatible protocol employing an allogeneic B cell bank for clonal expansion of allospecific natural regulatory T cells. *Am J Transplant.* (2014) 14:594–606. doi: 10.1111/ajt.12629
8. Pedersen AE, Holmstrøm K, Jørgensen F, Jensen SS, Gad M. Development of assay platforms for *in vitro* screening of Treg modulating potential of pharmacological compounds. *Immunopharmacol Immunotoxicol.* (2014) 37:63–71. doi: 10.3109/08923973.2014.977449
9. R C Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing (2017).
10. Ruhnau J, Schulze J, von Sarnowski B, Heinrich M, Langner S, Pötschke C, et al. Reduced numbers and impaired function of regulatory T cells in peripheral blood of ischemic stroke patients. *Mediat Inflamm.* (2016) 2016:2974605. doi: 10.1155/2016/2974605
11. Noguchi K, Gel YR, Brunner E, Konietschke F. nparLD: an R software package for the nonparametric analysis of longitudinal data in factorial experiments. *J Stat Softw.* (2012) 50, 1–23. doi: 10.18637/jss.v050.i12

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.