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

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$CD4^+CD25^+FoxP3^+$ 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**

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## 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

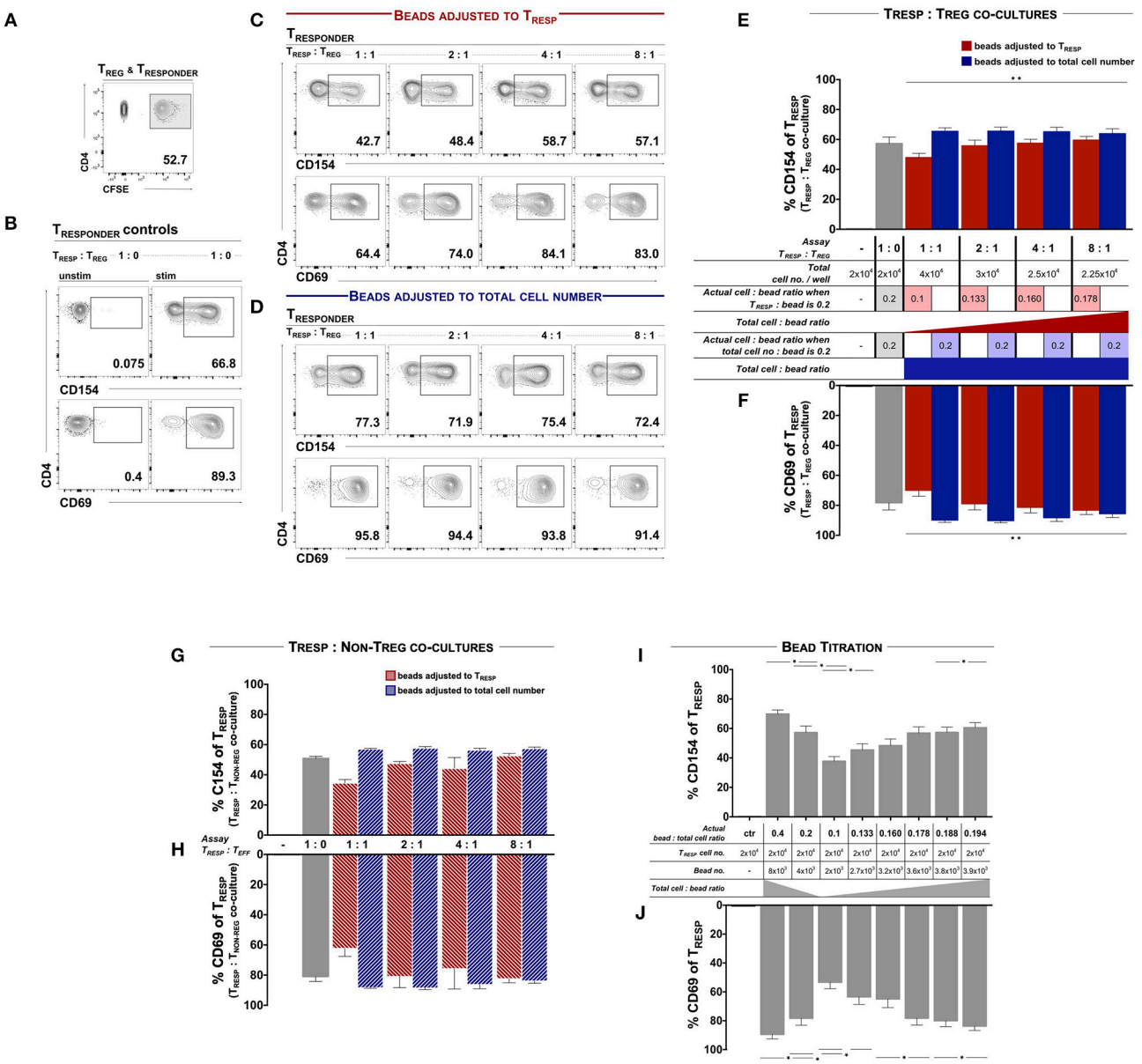


**FIGURE 1 |** $T_{CELL}$ early activation marker expression is dependent of TCR engagement and cannot be used for $T_{REG}$ functional evaluation. FACSorted $CD4^+CD25^-$ $T_{RESP}$ with and without autologous $T_{REG}$ co-culture were stimulated with anti-CD3/CD28-coated microbeads and analyzed for 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 $T_{RESP}$

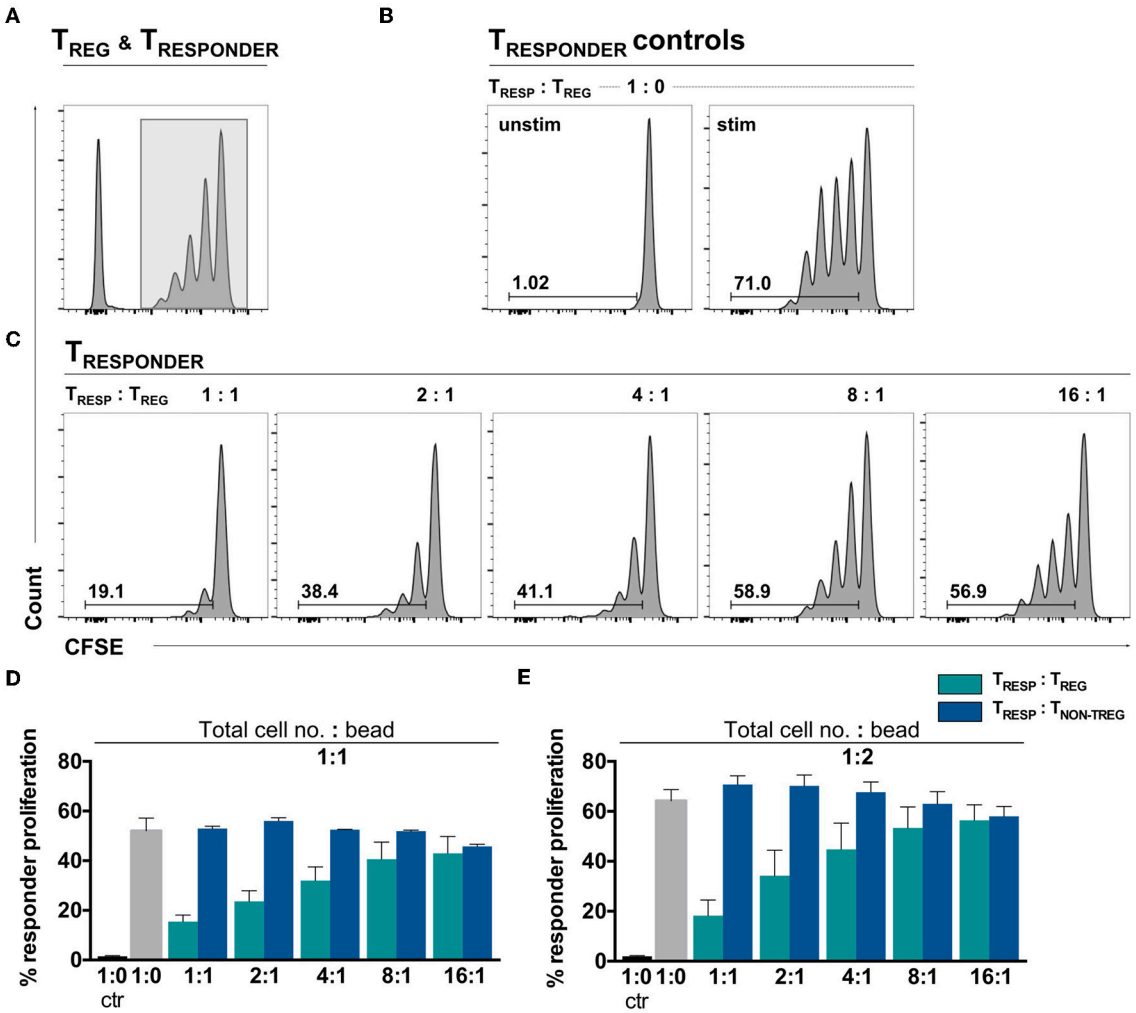


**FIGURE 2 |** *Ex vivo* isolated $T_{REG}$ demonstrate a dose-dependent $T_{RESP}$ proliferation suppression in a bead-uncompetitive setting. FACSorted $CFSE^{+}CD4^{+}CD25^{-}$ $T_{RESP}$ were co-cultured with autologous FACSorted $T_{REG}$ and stimulated with anti-CD3/CD28-coated microbeads for 96 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.

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## 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

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**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.