Aus dem Institut für Medizinische Immunologie

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Regulatory T cells in adoptive immunotherapy: from subset characterization to functional testing

Regulatorische T-Zellen in der adoptiven Zelltherapie: Von der Charakterisierung von Subpopulationen bis zur funktionellen Testung

zur Erlangung des akademischen Grades

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Zusammenfassung

Die aus dem Thymus stammenden regulatorischen T-Zellen (tT_{REG}) spielen eine wichtige Rolle bei der Unterdrückung unerwünschter Immunantworten *in vivo*. Immuntherapien mit humanen tT_{REG} sind daher vielversprechende und langfristige Strategien zur Verhinderung von Autoimmunität und Transplantatabstoßung. Sie stellen derzeit ein progressives Forschungsgebiet dar. Erste klinische Studien mit *in vitro* expandierten polyklonalen, autologen tT_{REG}, appliziert nach einer Nieren-Lebendspende, konnten ohne Patientengefährdung durchgeführt werden. Diese Studien zeigen erste Hinweise auf die Wirksamkeit der tT_{REG}-basierten Therapien. Die Anwendung dieses Therapieansatzes kann jedoch noch verbessert werden. Zum einen würde ein besseres Verständnis über die verschiedenen Differenzierungsstadien der humanen tT_{REG} das Wissen über die tT_{REG}-Biologie weiter vertiefen. Des Weiteren würde die Voraussage über die Lokalisation und das Verhalten dieser Zellen nach dem adoptiven tT_{REG}-Transfer verbessert werden. Für die routinemäßige klinische Anwendung wäre die Entwicklung eines schnellen und robusten Testsystems zur Bewertung der suppressiven Funktion der tT_{REG} als Freigabekriterium in der adoptiven Immuntherapie vonnöten. Mithilfe der Anwendung von Markern zur Bestimmung von Effektor-T-Zelldifferenzierungsstadien haben wir tT_{REG}-Subpopulationen definiert. Durch umfangreiche phänotypische, funktionelle und epigenetische Untersuchungen konnten wir zeigen, dass das tT_{REG}-Kompartiment in ähnliche Subpopulationen wie sein Effektor-T-Zell-Pendant unterteilt werden kann. Hier wiesen die tT_{REG} Subpopulationen unterschiedliche Merkmale hinsichtlich Phänotyp, Stabilität des Zelltyps/Differenzierungsgrades, funktioneller Kapazität und epigenetischem Profil auf. Das deutet darauf hin, dass auch tT_{REG} einem Muster der linearen Differenzierung unterliegen. Bemerkenswert ist auch, dass wir eine bisher unbeschriebene Subpopulation mit Markern und Eigenschaften von Gedächtniszellen innerhalb des naiven tT_{REG} Kompartiments identifizieren konnten. Es wäre folglich wichtig, die Zusammensetzung der Zellprodukte bezüglich der tT_{REG} Subpopulation für die adoptive Immuntherapie aufgrund ihres möglichen Einflusses auf die Wirksamkeit zu berücksichtigen.

Hinsichtlich der Entwicklung eines aussagekräftigen, robusten und klinisch praktikablen tT_{REG}-Testsystems haben wir einen veröffentlichten tT_{REG}-Funktionsassay getestet, der für eine schnelle Freigabe von tT_{REG}-Produkten vorgeschlagen wurde. Im Verlauf unserer Untersuchungen stellten wir allerdings fest, dass dieser Test für die Beurteilung der Suppression von früh exprimierten Effektor-T-Zell-Aktivierungsmakern und ihrer proinflammatorischen Zytokinproduktion zur Bestimmung der tT_{REG}-Funktionalität ungeeignet ist.

Unsere Daten deuten darauf hin, dass tT_{REG} keine mit den bisher angewendeten Techniken messbaren oder suppressiven Effekte auf die frühe Effektor-T-Zellaktivierung zeigen. Daher besteht weiterhin die Notwendigkeit, existierende Protokolle zu verbessern oder neue, aussagekräftige, robuste und praktikable Ansätze zu entwickeln, um die tT_{REG}-Funktion mit Hilfe eines GMP-konformen tT_{REG}-Produktfreigabetests zu bestimmen.

Abstract

Thymus-derived regulatory T cells (tT_{REG}) play an important role in suppressing unwanted immune responses *in vivo*. Therefore, immunotherapies applying human tT_{REG} are promising long-term strategies for preventing autoimmunity and allograft rejection and are currently a progressive area of investigation. First clinical trials applying *in vitro* expanded autologous and polyclonal tT_{REG} following living-donor kidney transplantation have proven safety and demonstrate first hints of efficacy. The value of these approaches can, however, be improved upon: – Firstly, a better understanding of the human tT_{REG} mode of differentiation would further expand the knowledge about tT_{REG} biology as well as help to predict their fate following application in adoptive tT_{REG} transfer. – Secondly, the development of a yet missing robust and short-term test system for evaluating tT_{REG}-mediated suppressive function as a release criterion for their application in adoptive immunotherapy would facilitate routine clinical application.

Applying effector T cell differentiation-determining markers to define tT_{REG} subsets and by extensive phenotypic, functional and epigenomic description, we could demonstrate that the tT_{REG} compartment can be divided into similar subpopulations as their effector T cell counterparts. Hereof, the tT_{REG} subsets present with distinct characteristics in terms of phenotype, lineage stability, functional capacities and epigenomic profile suggesting that also tT_{REG} underlie a pattern of linear differentiation. Of note, we also identified a previously undescribed subset within the naïve tT_{REG} compartment expressing certain memory markers and characteristics. Because of the putative impact on cell product efficacy, the tT_{REG} subset composition should be taken into account for adoptive immunotherapy.

For the development of a robust and clinically feasible tT_{REG} test system, we challenged a published tT_{REG} functional assay suggested for rapid cell product release. Thereby, we demonstrated that assessing suppression of early effector T cell activation markers and their pro-inflammatory cytokine production to be inappropriate measures to determine tT_{REG} functionality.

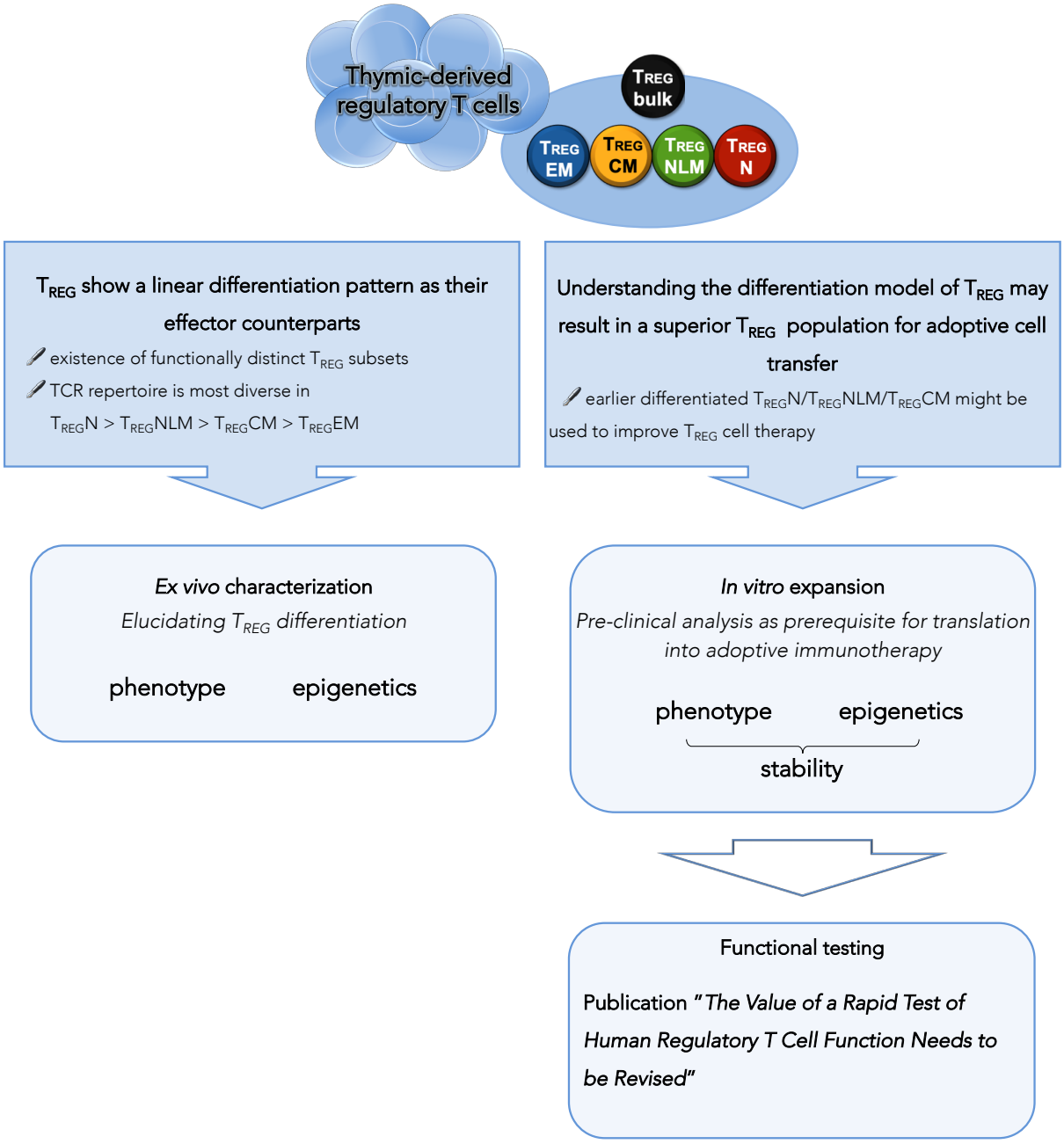
Our data suggest that tT_{REG} do not show measurable suppressive effects on early effector T cell activation, hence there is continuing pressure to improve current protocols or develop novel, robust and feasible approaches to determine tT_{REG} function suitable for a GMP-compliant tT_{REG} product release assay.

Index of Abbreviations

α	alpha
APC	antigen presenting cell
AVTT	adoptive anti-viral T cell therapy
CCR7	C-C chemokine receptor type 7
CD	cluster of differentiation
CFDA-SE	carboxyfluorescein diacetate succinimidyl ester
CFSE	carboxyfluorescein succinimidyl ester
CM	central memory
CO_2	carbon dioxide
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cell
DNA	deoxyribonucleic acid
EM	effector memory
EMRA	effector memory expressing CD45RA (terminally-differentiated effector)
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FoxP3	forkhead box P3
GITR	glucocorticoid-induced TNFR-related protein
GMP	good manufacturing practice
GvHD	graft versus host disease
HMD	highly methylated domains
HSCT	hematopoietic stem cell transplantation
ICOS	inducible T-cell co-stimulator
IFNγ	interferon gamma
IgG1	immunglobulin G 1
IL	interleukin

Ki-67	antigen Ki-67
MHC	major histocompatibility complex
mL	milliliter
mRNA	messenger ribonucleic acid
N	naïve
NLM	naïve-like memory
OX40	tumor necrosis factor receptor superfamily, member 4 / CD134
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PMA	phorbol 12-myristate 13-acetate
PMD	partially methylated domains
rh	recombinant human
RNA	ribonucleic acid
RRBS	reduced representation bisulfite sequencing
rtPCR	real-time polymerase chain reaction
SCM	memory stem T cell
SDS-Page	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEM	standard error of the mean
SOT	solid organ transplantation
T_{CONV}	conventional T cell
TCR	T cell receptor
TGFβ	transforming growth factor beta
TSDR	T_{REG} specific demethylation region
tT_{REG}	thymic-derived regulatory T cell

Schematic Overview of this PhD Thesis



TCR: T cell receptor | N: naïve | NLM: naïve-like memory | CM: central memory | EM: effector memory

Introduction

Thymus-derived $CD4^+CD25^+FoxP3^+$ regulatory T cells (tT_{REG}) can modulate effector immune responses and thus play an essential role in the induction and maintenance of immunological tolerance to self-antigens. Also, other types of suppressive T_{REG} have been described, such as peripherally induced T_{REG}, Type 1 regulatory T cells (Tr1) and Type 3 Helper cells (TH3). Fatal autoimmune diseases and other pathogenic disorders in both humans and murine models occur due to the loss in tT_{REG} cell number and/or function. Among it is the genetic disease immunodysregulation called polyendocrinopathy enteropathy X-linked (IPEX) as well as numerous autoimmune diseases, including type 1 diabetes, relapsing-remitting multiple sclerosis, psoriasis, myasthenia gravis and rheumatoid arthritis[1].

A diverse arsenal of T_{REG}-mediated suppressive mechanisms has been identified in the past years. Inhibitory cytokines, most importantly IL-10 and TGFβ, act as short-range soluble factors and function in suppressing conventional T cell (T_{CONV}) proliferation and pro-inflammatory cytokine release[2]. Further, T_{REG} have demonstrated the capacity to perform cytolysis of T and B cells *e.g.* in a granzyme B-dependent and perforin-(in)dependent manner[3–5]. Another mode of T_{REG}-mediated suppression is by metabolic disruption, which is, among other mechanisms, performed by ATP or IL-2 deprivation due to T_{REG}-expressed ectonuclease activity and a constitutively high expression of the IL-2 receptor α-chain CD25, respectively. This mechanism enables T_{REG} to withdraw IL-2 from the inflamed environment of T_{CONV}, thereby leading to the prevention of T_{CONV} proliferation and activating T_{CONV} apoptosis[6]. Another inhibitory mechanism of T_{CONV} activation is T_{REG}-mediated targeting of antigen presenting cells (APCs), e.g. by reducing the contact period of APCs and $CD4^+$ T cells[7,8] or by competitively binding co-stimulatory CD28 preventing binding to their shared APC ligands CD80 and CD86[9]. Only recently it was demonstrated that T_{REG} possess the capacity to remove MHC class II:cognate antigen complexes from dendritic cell (DCs) surfaces, thereby reducing the DCs' capacity to function as APCs[10].

Due to the immunoregulatory functions of T_{REG}, the transfer of human T_{REG} has become an appealing therapeutic alternative to improve the long-term outcome in transplantation and thereby reducing the side-effects of conventional immunosuppressive drugs. This approach would be of great benefit for patients since a major challenge in hematopoietic stem cell transplantation (HSCT) and solid organ transplantation (SOT) is the induction of tolerance, enabling the long-term allograft survival without the necessity for lifelong immunosuppression. In several mouse models, in addition to their protective role in autoimmunity, tT_{REG} have been shown to play a key role in the induction and maintenance of tolerance to alloantigens, thereby controlling allograft rejection and graft versus host disease (GvHD)[1].

Similarly, in humans, the adoptive transfer of donor-derived tT_{REG} has been shown to prevent GvHD after HSCT in the absence of any post-transplantation immunosuppression[11]. Furthermore, in patients

suffering from type I diabetes, tT_{REG} were well tolerated and showed dose-related hints of efficacy[12–14]. A principal clinical phase I/IIa study (ONE study) on the therapeutic application of tT_{REG} in SOT has been initiated within a global network with the ultimate goal of inducing allograft tolerance in renal SOT patients[15]. First clinical data of the adoptive transfer of polyclonally *in vitro* expanded tT_{REG} demonstrated safety and first hints of efficacy. However, monitoring the fate of the transferred tT_{REG} revealed a limited survival (Landwehr-Kentzel *et al*., in revision).

To this end, adoptive immunotherapeutic tT_{REG} applications entail polyclonally *in vitro* expanded tT_{REG} in their bulk entity. From proof-of-concept studies of adoptive anti-viral T cell therapy (AVTT), we learned that controlling the patient's viral load was unsuccessful over an extended period of time due to the limited persistence of adoptively transferred T cells[16–18]. For adoptive immunotherapeutic applications, *ex vivo* isolated T cells have to be stimulated for excessive *in vitro* expansion and by this acquire a proliferation-induced late differentiation state[19]. The late differentiation state of the adoptively administered T cells may have led to the limited *in vivo* survival of the transferred cells and thus it is strongly believed that defining a distinct composition of enriched T cell memory subsets with increased longevity potential will ultimately lead to an increased quality of adoptively transferred T cell products. For the conventional effector T cell compartment it is well established that central memory T cells (T_{CM}) and memory stem T cells (T_{SCM}) possess a great proliferative potential, self-renewal capacity and have been demonstrated to show superior survival, persistence and engraftment than further differentiated memory T cells, such as effector memory (T_{EM}) and terminally-differentiated effector (T_{EMRA}) T cells[20–24].

In contrast to conventional T cells, convincing data on the existence of different tT_{REG} subsets based on distinct differentiation states do not exist. From several murine studies T_{REG} memory was proposed due to long-term persistence of antigen-specific tT_{REG} exhibiting potent immunosuppressive properties despite the elimination of their cognate antigens[25–27]. However, human data on functional tT_{REG} memory is still missing and even though a rising number of memory effector T cell markers have been identified, similar indicators of functional T_{REG} memory are less clearly defined. One of the limitations is the fact that only a few tT_{REG} intrinsic molecules associated with their role in immunosuppression are expressed *de novo* upon activation since tT_{REG} activation commonly increases the expression of protein molecules, which they already express in the steady state (*e.g.* CTLA-4, CD25, ICOS, GITR)[28,29]. Yet, the greatest challenge in defining human memory tT_{REG} has been the lack of evidence that tT_{REG} can persist for prolonged periods of time in the absence of their respective cognate antigen.

Therefore, **one aim** of my PhD project was the characterization and understanding of particular tT_{REG} subsets at distinct differentiation states regarding their stability, function and phenotype to identify prospective approaches for advancing adoptive tT_{REG} cell products.

In vitro polyclonally expanded bulk tT_{REG} have already presented as auspicious candidates for immunotherapeutic application. For the ultimate deployment of tT_{REG} cell products, the functional capacity of tT_{REG} has to be assured before adoptive transfer, yet a test system meeting all clinical, laboratory handling and regulatory authority requirements is still missing. Within my PhD project, the **second aim** was to assess the *in vitro* functional capacities of the tT_{REG} subsets and to challenge tT_{REG} functional assays relating to translation into a robust clinically feasible and Good Manufacturing Practice (GMP)-compliant processes. For this, two protocols were compared back-to-back: one following a long-published 'gold standard' protocol assessing the suppression of autologous responder T cell proliferation and a second published protocol evaluating suppression of autologous responder T cell activation offering essential advantages with regards to clinical translation such as time kinetics. Ultimately, the aim was to define a GMP-compliant functional tT_{REG} assay as a release criterion for tT_{REG} cell products.

Methodology

Subjects

Peripheral blood mononuclear cells (PBMCs) were obtained from either healthy volunteers (20 male : 33 female donors, age range from 20 to 82 years) or buffy coats from the German Red Cross (DRK) society. The relevant institutional review boards approved the study and all subjects gave their written informed consent according to the 'Declaration of Helsinki'.

PBMC Isolation

PBMCs were isolated from blood by means of density gradient centrifugation. In brief, heparin-supplemented blood was diluted 1:2 with sterile PBS (Gibco) and carefully added onto a layer of Biocoll (Biochrom) solution. After centrifugation at 800x *g* for 20 minutes at room temperature (RT) without deceleration, the interface of PBMCs between separated blood plasma and Biocoll (Biochrom) was collected and washed twice with a large amount of PBS at 400x g for 10 minutes at 4°C with full deceleration. Cell numbers were determined using a Neubauer chamber after diluting the cells 1:2 with trypan blue (Sigma-Aldrich). Cells were cultured in incubators at 37°C and 5% CO_2 in X-Vivo15 medium (Lonza) supplemented with 10% FCS (Biochrom), 100 U/mL penicillin and 100 µg/mL streptomycin (both Biochrom).

$CD4^+$ T cell enrichment by magnetic-activated cell sorting (MACS)

$CD4^+$ T cell enrichment was performed prior to FACSorting of various T cell populations by incubating PBMCs with antiCD4-coupled magnetic microbeads (Miltenyi Biotec), which were transferred onto a column within a strong magnetic field. There, $CD4^+$ T cells bound to microbeads were retained within the column and unlabeled $CD4^-$ cells were collected as negative fraction. Finally, the column was taken out of the magnetic field and $CD4^+$ T cells were eluted. Human CD4 MicroBeads were used according to the manufacturers' protocol.

Isolation of untouched $CD3^+$ T cell enrichment by modified density-based negative selection protocol

Enrichment of $CD3^+$ T cells was performed for obtaining responder T cells autologous to the expanded tT_{REG} subsets in order to perform functional proliferation suppression assays. For this, fresh blood was taken from the respective tT_{REG} donor and incubated with "RosetteSep™ Human T Cell Enrichment Cocktail" (StemCells) at 20µL/mL blood and further treated according to the manufacturers' protocol. The "RosetteSep™ Human T Cell Enrichment Cocktail" consists of mouse IgG1 antibodies specific for human lineage antigens (CD16, CD19, CD36 and CD56). These antibodies are crosslinked to mouse

IgG1 antibodies targeting human glycophorine on erythrocytes by means of rat anti-mouse IgG1 secondary antibodies, thus forming bispecific tetrameric antibody complexes. These complexes crosslink all unwanted nucleated cells to multiple erythrocytes by forming erythrocyte rosettes around the targeted mononucleated cell, thereby increasing the density of the unwanted cells, such that they pellet along with the free erythrocytes when centrifuged over a density gradient medium. The desired $CD3^+$ T cells remain free of antibody and can be collected as enriched population at the interface between the plasma and the density medium.

Isolation of bulk tT_{REG}, tT_{REG} subsets and responder T cells

Freshly isolated PBMCs were enriched for $CD4^+$ T cells *via* positive selection by MACS. $CD4^+$ T cells were rested in PBS/0.5% FCS at 4°C O/N. Subsequently, the cells were labelled with monoclonal antibodies and FACSorted. In brief, cells were gated according to forward/sideward scatter and $CD4^+$ T cells selected to further define bulk tT_{REG} by $CD25^{high}$ and $CD127^{low}$ expression. Further, tT_{REG} subsets were isolated according to their respective CD45RA, CCR7, CD45RO and CD95 expression. In the case of functional assays, responder T cells were additionally isolated defined by their $CD4^+CD25^{low}$ expression.

Expansion of tT_{REG}

FACSorted tT_{REG} were suspended in X-Vivo15 medium supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin (complete medium) at a maximum of 10^5 tT_{REG} in 200µL medium per 96 well U-bottom cell culture plate (Falcon). 500U/mL rhIL-2 (Proleukin S, Novartis Pharma) and 100µM Rapamycin (Pfizer) was complemented to the medium. On day 1 of expansion, T_{REG} expansion beads (Miltenyi Biotec), particles loaded with activating antiCD3/CD28 antibodies, were added at a bead-to-cell ratio of 4:1. On day 7, the cells were re-stimulated at a bead-to-cell ratio of 1:1 and the medium was replaced when splitting the cells or when the medium had changed color from red to yellow, thereby indicating that the pH had changed due to extensive metabolic processes.

FACS staining, data acquisition and analysis

For extracellular staining, cells were incubated with appropriate antibodies at 4°C for 30 minutes and subsequently washed with PBS at 400 *g* for 10 minutes if not stated otherwise.

Prior to cytokine staining, cells were stimulated with 10ng/mL PMA and 1µg/mL Ionomycin (both Sigma-Aldrich) for 4 hours, after which 4µg/mL Brefeldin A (Sigma-Aldrich) was added for further 2 hours. For intranuclear and intracellular staining, cells were fixed and permeabilized using the "Foxp3/Transcription Factor Staining Buffer Set" (eBioscience) according to the manufacturers' instruction. Intracellular antibody staining was performed at 4°C for 30 minutes. Additionally, in all

intracellular staining protocols, CD3, CD4 and CD95 molecules were stained intracellularly to enhance the intensity by also staining internalized receptors.

Live/Dead discrimination was performed using LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Molecular probes, life technologies) together with extracellular antibodies prior to fixation and permeabilization at 4°C for 30 minutes.

CD154 staining for the functional tT_{REG} activation suppression assay was performed by adding CD154 antibody into the culture medium at the start of stimulation to ensure antibody binding also to transiently expressed CD154 molecules.

Flow cytometry data acquisition was performed using a BD LSR Fortessa with software FACS Diva (BD). Data analysis was performed by FlowJo (TreeStar) software.

FACSorting

FACSorting was performed by the Flow Cytometry Core Facility of the "Berlin-Brandenburg Center for Regenerative Medicine" and supported by Dr. Désirée Kunkel and Dr. Jens Hartwig. Antibody staining for FACSorting was performed as described above.

CFDA-SE staining for detection of cell proliferation

Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) enters cells by diffusion through the cell membrane and is subsequently cleaved by intracellular enzymes to form an amine-reactive product, carboxyfluorescein succinimidyl ester (CFSE), forming covalent bonds with intracellular molecules. When CFSE-labelled cells divide, they pass on half the number of CFSE-labelled molecules to their progeny. Therefore, each cell division can be assessed by measuring the corresponding decrease in cell fluorescence intensity *via* flow cytometry hence being a useful tool to assess T cell proliferation. For tT_{REG} proliferation suppression assays, responder T cells were stained with 10µM CFDA-SE/PBS for 3 minutes, followed by the addition of 10mL cold FCS. The cells were washed twice with cold complete medium. For tT_{REG} activation marker suppression assays, responder T cells were stained with 2µM CFDA-SE/PBS for 1 minute, followed by the addition of 10mL cold FCS. The cells were washed twice with cold complete medium.

Marker	Fluoro-chrome	Clone	Company	Marker	Fluoro-chrome	Clone	Company
CCR7	PE	G043H7	Biolegend	CD45RA	PE-Dazzle 594	HI100	Biolegend
CCR7	APC-Cy7	G043H7	Biolegend	CD45RO	BV785	UCHL1	Biolegend
CD127	APC-AF700	R34.34	Beckman Coulter	CD69	APC-Cy7	FN50	Biolegend
CD134	PE	Ber-ACT35	Biolegend	CD8	BV510	RPA-T8	Biolegend
CD154	BV711	24-31	Biolegend	CD95	PE-Cy7	DX2	Biolegend
CD25	APC	2A3	BD	FoxP3	AF488	259D	BD
CD3	BV650	OKT3	Biolegend	IFNγ	BV605	4S.B3	Biolegend
CD31	PE	WM59	Biolegend	IL-2	BV421	MQ1-17H12	Biolegend
CD4	PerCp-Cy5.5	SK3	Biolegend	Ki-67	AF700	Ki-67	Biolegend

Table 1 List of used antibodies

Quantitative real-time PCR

Total RNA from FACSorted T cells was isolated using the QIAamp RNA Blood Mini Kit (Qiagen) and transcribed into cDNA using the QIAamp QuantiTect® Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The mRNA expression of genes was analyzed by quantitative rtPCR performed as described previously[30] using an Applied Biosystems (ABI) 7500 cycler using TaqMan Universal PCR Mastermix (Applied Biosystems) with the following thermal protocol: 2 min at 50 °C, 10 min at 95 °C, 42 x (15 s at 95 °C, 60 s at 60 °C). Analysis was performed using the 7500 Real Time Analysis software (Applied Biosystems).

T cell receptor sequencing

Genomic DNA from cells of interest was obtained using the QIAamp DNA Blood Mini Kit (Qiagen) and sent to Adaptive Biotechnologies, Seattle, USA for TCRβ sequencing. Analysis of TCR clonality was performed using the Adaptive Biotechnologies 'ImmunoSEQ' platform.

Cytokine Multiplex Assay

Cytokine analyses were performed using the 'Meso Scale U-Plex Kit' (Meso Scale Discoveries) following the manufacturer's instructions. Supernatants were diluted 1:4 with the appropriate buffer contained within the kit.

Proliferation suppression assay

Described in detail within the publication.

DNA methylation analysis by Reduced Representation Bisulfite Sequencing (RRBS)

Frozen cell pellets from *ex vivo* FACSorted tT_{REG} subsets were sent to the University of Saarland for DNA methylation analysis. In short, genomic DNA was isolated and subjected to enzymatic digestion obtaining a library of short DNA fragments. After specific size selection by SDS-Page, DNA was treated for bisulfite conversion followed by PCR amplification and final sequencing. Bioinformatic analyses were performed by the team of Dr. Gilles Gasparoni at the Institute for Genetics/Epigenetics at the University of Saarland.

Methylation analysis of T_{REG} specific demethylation region

Genomic DNA from cells of interest was obtained using the QIAamp DNA Blood Mini Kit (Qiagen) and subjected to bisulfite conversion using the EpiTect kit (Qiagen) according to the manufacturer's instructions. A minimum of 60ng bisulfite-treated genomic DNA was used in a PCR to quantify the Foxp3 TSDR. rtPCR was performed in a final reaction volume of 20 µL containing 10 µL FastStart universal probe master (Roche Diagnostics), 50 ng/µL lamda DNA (New England Biolabs), 5 pmol/µL methylation or nonmethylation-specific probe, 30 pmol/µL methylation or nonmethylation-specific primers and 60 ng bisulfite-treated DNA or a respective amount of plasmid standard. The samples were analyzed in triplicates on an ABI 7500 cycler.

Statistical analysis

GraphPad Prism V8 was used for graph generation and Kolmogorov-Smirnov testing was performed to assess normal Gaussian distribution. To determine significance, experiments with $n \geq 6$ were subjected to a paired one-way ANOVA test comparing the mean of each column with the mean of every other column with additional Tukey testing correcting for multiple comparisons using statistical hypothesis testing. Experiments with n=3 were analyzed by a paired two-tailed *t*-test to determine significance. *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; ****: $P \leq 0.0001$

Results

1. Phenotypic characterization of thymic-derived T_{REG} subsets

1.1. tT_{REG} phenotyping reveals distinct subsets when applying classical / naïve memory differentiation marker profiles

T lymphocyte differentiation of naïve into functional effector cells is essential for optimal protection against different classes of pathogens as well as for the development of immunological memory, which is classically defined on the basis of our comprehension about memory effector T cells. Whereas over the last decades an increased number of indicators for the reliable identification of effector T cell subsets based upon their differentiation state have been identified, comparable markers for defining functional tT_{REG} subsets are less clearly defined to date. This is mostly due to the fact that there are only a scarce number of tT_{REG}-specific molecules being expressed *de novo* upon tT_{REG} activation. Two of the few cell surface proteins being differentially expressed on effector as well as regulatory T cells are CD45RA[31,32] and CCR7[33]. On this basis, tT_{REG} subsets were largely classified as their effector T cell counterparts.

For this *ex vivo* investigation of the phenotype and pro-inflammatory cytokine profile of effector T cells and tT_{REG}, an extensive flow cytometry panel has been established, defining subsets within both T cell lineages. In brief, freshly isolated PBMCs of 53 healthy donors were polyclonally stimulated with PMA/Ionomycin, as well as left untreated and labelled with a selection of monoclonal antibodies for flow cytometric analysis (Fig. 1). For all flow cytometry-based analyses, the gating strategy commenced with lymphocyte discrimination, doublet exclusion, followed by the selection of living $CD3^+CD4^+$ T cells (Fig. 1 A). Thereafter, two major gating strategies were employed:

1) From bulk $CD4^+$ T cells, tT_{REG} were gated based on their high expression of CD25 and FoxP3. Further, tT_{REG} subsets (T_{REG}CM, T_{REG}EM, T_{REG}TEMRA) were defined according to CD45RA and CCR7 expression. After stringent elimination of any memory T cells by excluding $CD45RO^+CD62L^-$ T cells, T_{REG}NLM and T_{REG}N were defined based on their differential expression of CD95 and CCR7 (Fig. 1 B).

2) Bulk $CD4^+$ T cell subsets (TCM, TEM, TEMRA) were defined according to their CD45RA and CCR7 expression. Once having excluded $CD45RO^+CD62L^-$ memory T cells, the subsets TSCM and TN were defined by means of their differential expression of CD95 and CCR7 (Fig. 1 C).

For the analysis of pro-inflammatory cytokine profiles, PBMCs were polyclonally stimulated with PMA/Ionomycin and intracellularly stained for IFNγ and IL-2. Setting the threshold by means of an

unstimulated control, the expression of IFNγ and IL-2 was assessed on both, bulk $CD4^+$ T cell and tT_{REG} subsets (Fig. 1 D).

As strikingly demonstrated in figure 2 A and B, the central memory compartment within the bulk $CD4^+$ T cells compares to only halve the frequency of $T_{REG}CM$. Thereby, the majority of tT_{REG} exhibit a central memory phenotype, suggesting their circulation between peripheral blood and lymphoid organs. Further, TN of bulk $CD4^+$ T cells are twice as frequent compared to the naïve cells within the T_{REG} population. Noting that naïve and central memory composition differs strongly between bulk $CD4^+$ T cells and tT_{REG}, it is all the more intriguing that the frequencies of TSCM within the bulk $CD4^+$ T cell compartment strongly compare to that of $T_{REG}NLM$, a tT_{REG} subpopulation within the naïve compartment expressing the memory T cell marker CD95. On this basis, the newly mentioned tT_{REG} subset was termed as 'naïve-like memory' tT_{REG} population ($T_{REG}NLM$).

Non-production of pro-inflammatory cytokines, including IFNγ and IL-2, is a hallmark of regulatory T cells. Therefore, the PBMCs were polyclonally stimulated with PMA/Ionomycin for 6 hours in order to validate the authenticity of the tT_{REG} subsets by demonstrating the absence of pro-inflammatory cytokine production. As a positive stimulation control, the cytokine production profile of the bulk $CD4^+$ T cell subsets was also assessed.

As anticipated, the data shown in figure 2 C and D demonstrate that none of the tT_{REG} subsets produce mentionable amount of both IFNγ and IL-2, whilst bulk $CD4^+$ T cells generate considerable amounts of both cytokines, thereby serving as a positive control for polyclonal stimulation. Expectedly, the highest IFNγ and IL-2 producers present to be effector memory T cells, followed by comparable frequencies from TCM and TSCM and lowest cytokine production by naïve $CD4^+$ T cells (Fig. 2 C,D).

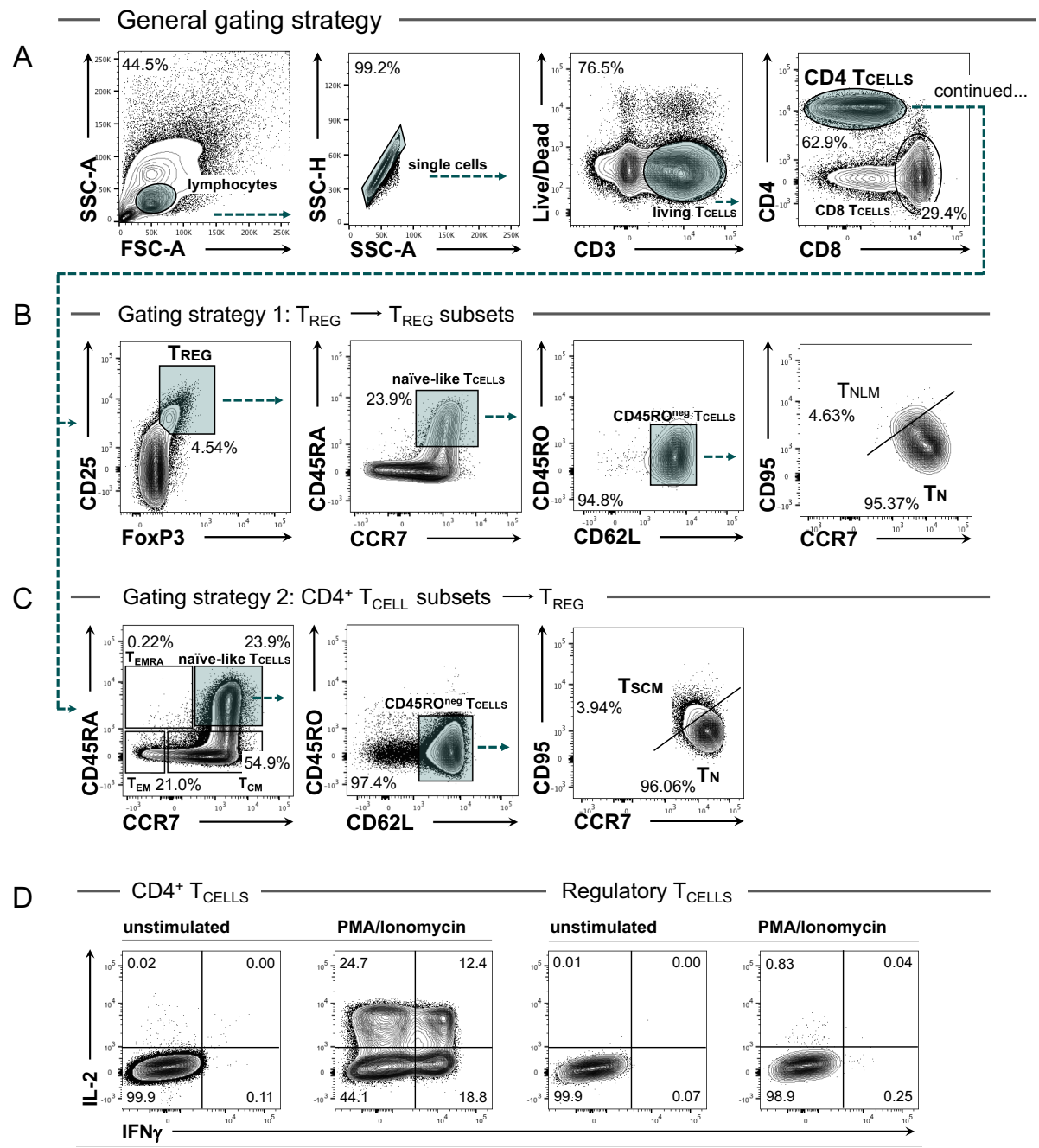


Figure 1. Gating strategy for defining effector and thymic-derived regulatory T cell subsets.

(A) Strategy for analyzing effector T cell and tTREG subsets from PBMCs, starting from lymphocytes followed by doublet discrimination, selection of living CD3+CD4+ T cells. (B) Strategy for studying tTREG subsets based on their expression of CD25 and FoxP3. tTREG subsets were set according to their expression of CD45RA, CCR7, CD45RO and CD95. (C) Strategy for analyzing bulk CD4+ T cells according to their expression of CD45RA, CCR7, CD45RO and CD95. tTREG within the CD4+ TSCM compartment were further characterized upon their expression of CD25 and FoxP3. (D) PBMCs were stimulated with PMA/Ionomycin for 6 h, permeabilized and additionally stained for IFNγ and IL-2. Illustrated are density plots of flow cytometry data of one representative donor.

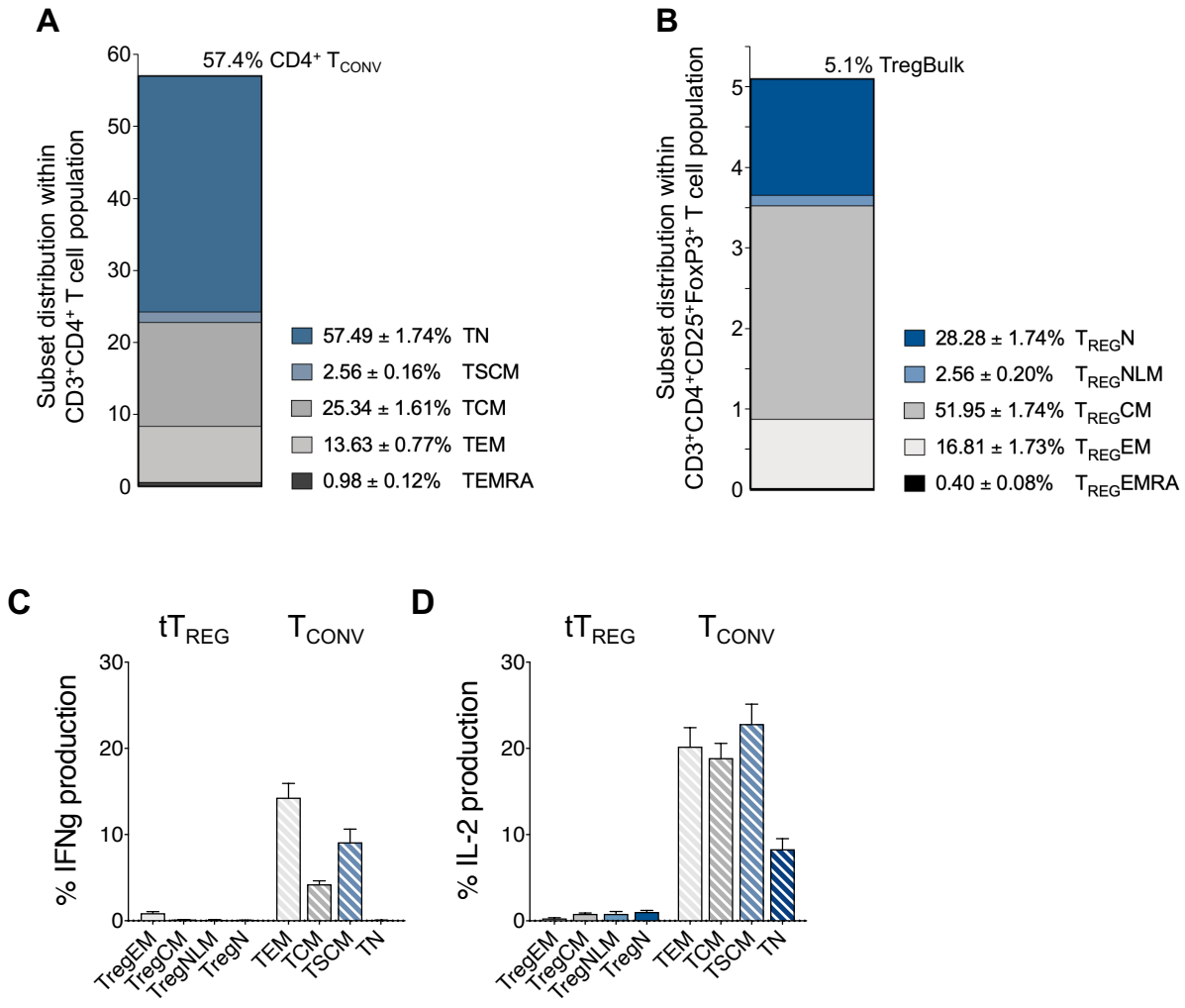


Figure 2. Composition of subsets defining T cell differentiation stages distinctly differs between bulk CD4⁺ T cells and tT$_{REG}$.

(A) Summary of ex vivo investigation of bulk CD4⁺ conventional T cells and (B) tT$_{REG}$ subset distribution of freshly isolated PBMCs based on flow cytometry data (Fig. 1 A-C). Cells were polyclonally stimulated with PMA/Ionomycin for 6 h and cytokine production was assessed following intracellular cytokine staining (Fig. 1 D). The production of the mentioned pro-inflammatory cytokines by tT$_{REG}$ subsets and their CD4⁺ conventional T cell counterparts is summarized in (C) and (D). 20 male : 33 female donors, age range from 20 to 82 years. n=53 ± SEM.

1.2 tT$_{REG}$ depict distinct extracellular and intracellular marker expression with regard to their recent thymic emigration, active proliferation state, prior activation and ectonuclease activity potential

Investigating potential differentiation-related protein expression patterns by tT$_{REG}$ subsets with regard to specific markers playing a role in T cell development, homeostasis, activation and function, tT$_{REG}$ and conventional CD4⁺ T cells were analyzed. At first, the cell adhesion molecule PECAM-1, also known as CD31, which is expressed on naïve CD4⁺ T cells after their development and egress from the thymus into the periphery was examined[34]. Upon TCR engagement during the initial priming of naïve CD4⁺ T cells by their cognate antigen, CD31 is rapidly downregulated[35]. We see a similar pattern within the

tT_{REG} and T_{CONV} population: naïve T cells express significantly higher frequencies of CD31 compared to TSCM/NLM and TEM. Contrarily to the $CD4^+$ T_{CONV}, T_{REG}NLM and T_{REG}CM express similar CD31 frequencies. In both compartments, TEM express lowest CD31 frequencies (Fig. 3 B).

Since proliferation and clonal expansion of antigen-specific T cells are important functions for conferring immunity and immunological memory, the nuclear protein Ki-67 was investigated within $CD4^+$ T_{CONV} and T_{REG} subsets. Ki-67 plays a role in the regulation of cell division and is expressed during active phases of cell division yet is absent in quiescent T cells[36]. Our data demonstrate that within both T cell compartments EM express highest frequencies of Ki-67, being significantly higher than expression in CM, SCM/NLM and N. Also, very similar in both compartments is the lowest Ki-67 expression by the naïve T cells, followed by the SCM/NLM subsets (Fig. 3 C).

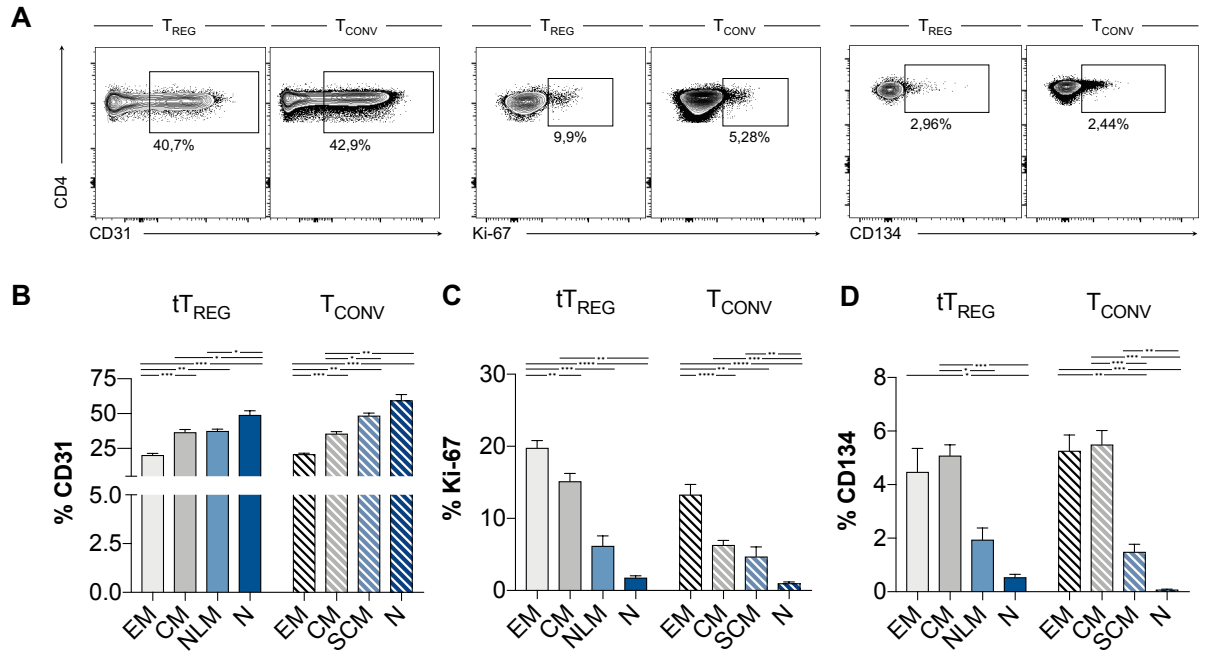


Figure 3. tT_{REG} depict distinct extracellular and intracellular marker expression with regard to recent thymic emigration, active proliferation state, prior activation.

*Ex vivo isolated and unstimulated PBMCs were analyzed by flow cytometry based on extracellular, intracellular and intranuclear proteins labelled with fluorochrome-conjugated monoclonal antibodies. **(A)** Representative FACS plots are shown for the flow cytometric detection of CD31, Ki-67, CD134 in tT_{REG} and T_{CONV} compartments. Summary of frequencies within tT_{REG} and conventional $CD4^+$ T cell subsets of CD31 **(B)**, Ki-67 **(C)** and CD134 **(D)**. n=6. Results are presented as mean ± SEM. Normal distribution of data points was tested with Kolmogorov-Smirnov test and significance was determined by paired one-way ANOVA test comparing the mean of each column with the mean of every other column with additional Tukey testing correcting for multiple comparisons using statistical hypothesis testing. *: P≤0.05; **: P≤0.01; ***: P≤0.001; ****: P≤0.0001*

Next, we determined the expression of CD134, also called OX40, on the surface of conventional $CD4^+$ T cells and tT_{REG} subsets. CD134 represents a major co-stimulatory receptor and was demonstrated, opposed to CD28, to not be constitutively expressed on naïve $CD4^+$ T cells, yet is induced 1-2 days after TCR engagement[37]. Matching other groups' findings, we could demonstrate that naïve T cells of both

compartments, having not yet encountered their cognate antigen and thus not yet experienced TCR engagement, are negative in CD134 expression. The subsets CM and EM within both compartments express significantly higher frequencies of CD134 than N and SCM/NLM. Strikingly, SCM/NLM express low but significantly higher frequencies of OX40 than N (Fig. 3 D).

1.3 tT_{REG} subsets demonstrate strong differences in TCR repertoire diversity

TCR repertoire analysis is a common method for analyzing clonal expansion of T cells after cognate antigen encounter giving us the opportunity to study different T cell lineages in their process of differentiation. This was of great interest as to help in elucidating the identity of different tT_{REG} subsets and to shed some more light onto the path of tT_{REG} differentiation. We therefore analyzed the clonality of the entire repertoire of the different tT_{REG} subsets. Clonality expresses the statistics for how much the TCR repertoire is made up of expanded clones, *i.e.* the degree to which one or a few clones (cells sharing identical TCR sequences) dominate the repertoire. The analysis defines the measure of clonality with a value of 0 to 1, where 0 represents a flat distribution with each clone appearing only once and 1 being an entirely monoclonal sample, *i.e.* only one clone was found and all reads come from that one clone[38]. Our TCR sequencing data reveal that the further differentiated tT_{REG} subsets T_{REG}CM and T_{REG}EM display the least diverse repertoire, fitting the fact that cells from these subsets have undergone vast clonal expansion after antigen encounter, leading to the emerge of single TCR clones. On the other end, the slightly greater clonality of T_{REG}NLM compared to T_{REG}N interprets as that the T_{REG}NLM population has undergone clonal expansion (Fig 4 A).

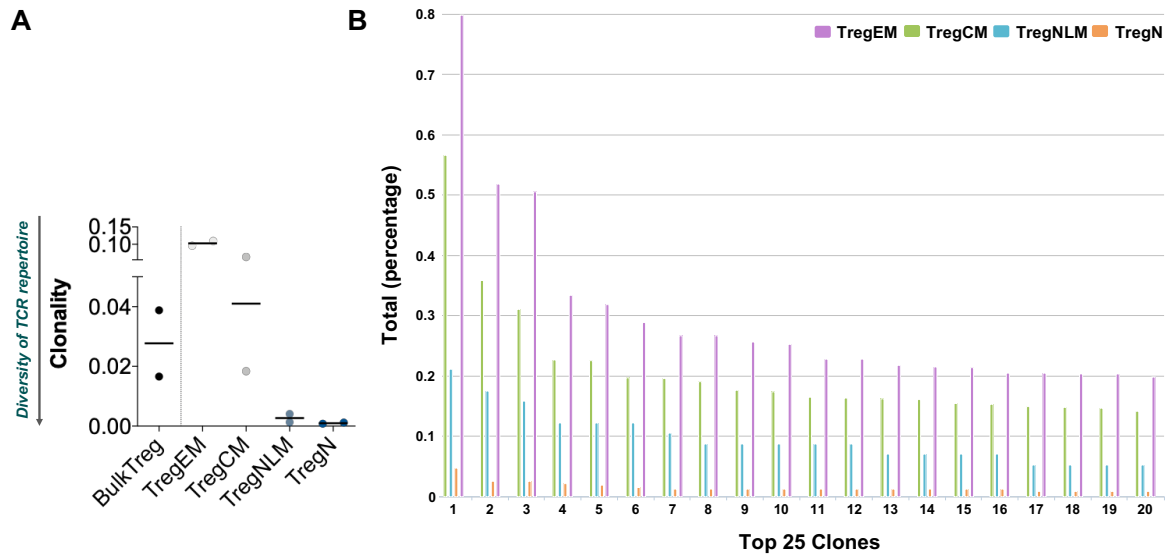


Figure 4. TCRβ repertoire analysis reveals disparate TCR repertoire among tT_{REG} subsets.

***(A)** TCRβ repertoire analysis of ex vivo FACSorted tT_{REG} subsets. n=2, mean data shown. **(B)** Intuitive view depicting the relative proportions of the top 25 clones of each tT_{REG} subset of one donor.*

1.4 tT_{REG} subsets demonstrate variations within their epigenetic profile

In 2008, Polansky-Biskup *et al.* described the *T_{REG}-specific demethylation region* (TSDR), an evolutionary conserved CpG-rich element within the Foxp3 locus, to be selectively demethylated in permanently differentiated FoxP3$^+$ tT_{REG}[39]. For the investigation of potential differences in tT_{REG}-lineage stability among tT_{REG} subsets, the degree of TSDR demethylation of *ex vivo* tT_{REG} sub-populations was analyzed. Our observed mean TSDR demethylation of tT_{REG}Bulk was in agreement with data of Polanksy-Biskup *et al.*[39]. Within the tT_{REG} compartment, tT_{REG}CM demonstrated the lowest TSDR demethylation whereas early differentiated subsets showed the greatest degree of stability implying that the degree of FoxP3 stability differs according to the tT_{REG}'s stage of differentiation (Fig. 5 A).

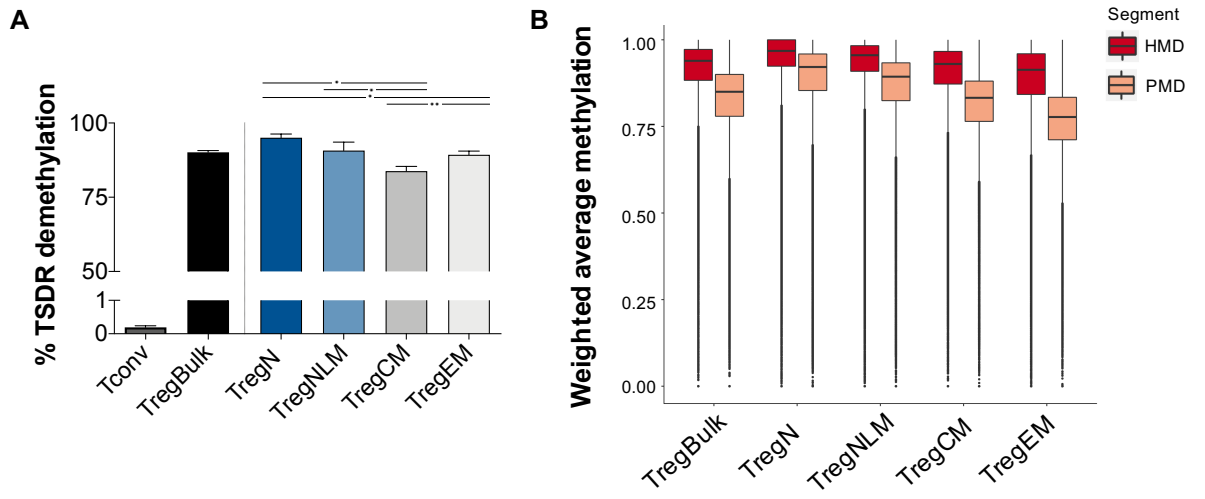


Figure 5. tT_{REG} subsets demonstrate variations within their epigenetic profile.

*Ex vivo FACSorted tT_{REG} cell subsets were analyzed by **(A)** qPCR to define the percentage of TSDR demethylation (n=3, results are presented as mean ± SEM) and **(B)** RRBS to determine the weighted average DNA methylation across defined DNA segments. n=6. Normal distribution of data points was tested with Kolmogorov-Smirnov test and significance was determined by paired two-tailed t-test. *: P≤0.05; **: P≤0.01; ***: P≤0.001; ****: P≤0.0001*

As part of the German Epigenome Program (DEEP), in 2016, Polansky-Biskup *et al.*, demonstrated a proliferation-associated genome-wide loss of DNA methylation during CD4$^+$ effector memory T cell differentiation[40]. In cooperation with Dr. Polansky-Biskup and Dr. Gasparoni from the Institute of Genetics/Epigenetics of the University of Saarbrücken we analyzed the DNA methylation pattern of tT_{REG} subsets by Reduced Representation Bisulfite Sequencing (RRBS). Within the partially methylated domains (PMD), which were shown to have the greatest loss of methylation of all analyzed segments[40], also the tT_{REG} compartment demonstrates a progressive loss of DNA methylation from early to phenotypically defined late differentiated subsets (Fig. 5 B).

Since tT_{REG} are expanded *in vitro* for the application in adoptive immunotherapy in order to obtain sufficient cell numbers, we next expanded FACSorted tT_{REG} subsets stimulated with α(anti)CD3/CD28 expansion microbeads and in the presence of high-dose IL-2 and Rapamycin for 21 days. The obtained

data (not shown) demonstrate that 1. T_{REG}CM-derived cells, followed by T_{REG}NLM-derived and T_{REG}N-derived exhibit the greatest proliferative capacity, 2. early differentiated tT_{REG} subsets show the most stable T_{REG} ($CD25^{+}FoxP3^{+}$) phenotype over the duration of expansion, 3. T_{REG}EM-derived, as the only tT_{REG} subset, produce substantial amounts of pro-inflammatory cytokines IFNγ and IL-2, and 4. early differentiated tT_{REG} subsets retain the greatest degree of TSDR demethylation, i.e. tT_{REG} lineage stability upon *in vitro* expansion (*manuscript in progress*).

2. Functional characterization of thymic-derived T_{REG} subsets

Since immunosuppressive functionality is a defining characteristic of tT_{REG} rather than their definition by means of phenotypic markers, none of which are exclusive to the tT_{REG} lineage, suppressive capacities of *ex vivo* isolated and *in vitro* expanded tT_{REG} were studied next. For this, we started off with the gold-standard proliferation suppression functional test where the proliferation of autologous responder T cells (T_{RESP}) incubated with different ratios of tT_{REG} and polyclonally stimulated with αCD3/CD28 microbeads was assessed by means of a CFSE dilution-based *in vitro* assay.

2.1 Gold-standard proliferation suppression assay reveals greatest capacity to suppress autologous responder T cell proliferation by early differentiated tT_{REG}

Since low peripheral tT_{REG} cell numbers obtained from a small volume of blood available from patients requires *in vitro* GMP-compliant expansion of tT_{REG}, we aimed at analyzing any differential suppressive capacities between the different tT_{REG} subsets after they have been expanded for 21 days. This *in vitro* expansion time period is in accordance with our GMP facility protocol applied for clinical adoptive tT_{REG} cell products. Collectively, it is to note that after 3 weeks of expansion, early differentiated tT_{REG}-derived subsets most potently suppressed autologous T_{RESP} proliferation while phenotypically defined late differentiated EM-derived tT_{REG} showed only little suppressive capacity (Fig. 6), which goes along with their substantial conventional cytokine secretion and loss in tT_{REG}-specific phenotype.

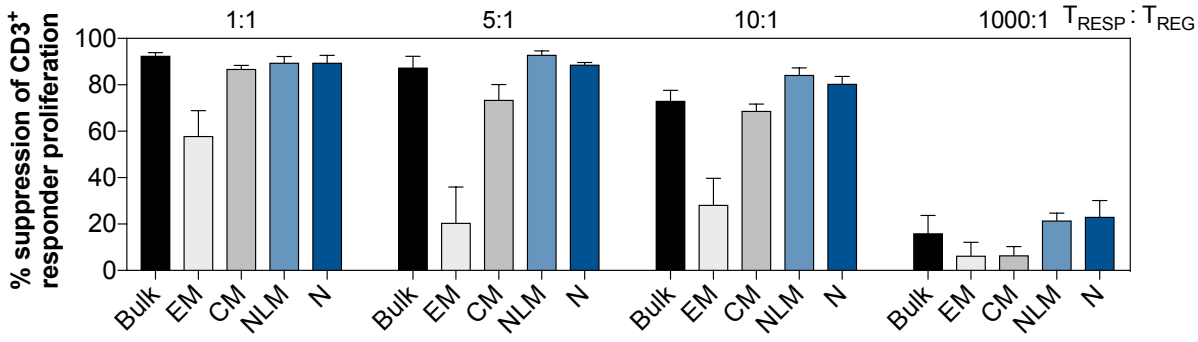


Figure 6. Early differentiated tT_{REG} demonstrating greatest capacity to suppress autologous responder T cell proliferation.

Freshly isolated autologous $CD3^+$ T_{RESP} were co-cultured with 3-week in vitro expanded tT_{REG} subsets at different T_{RESP}:T_{REG} ratios and stimulated with αCD3/CD28-coated microbeads at a total cell number:bead ratio of 1:1 for 96 hours. n=6. Results are presented as mean ± SEM.

The gold-standard responder cell proliferation suppression assay, as demonstrated above, is a robust assay to assess tT_{REG} functionality, yet is subject to certain limitations including a delicate intracellular CFSE-labelling step and, most importantly, a 4-day incubation period until performance readout. The last-mentioned aspect is of particular disadvantage, as functionality should be assessed as close to cell product administration as possible.

2.2 Suppression of early activation marker expression by tT_{REG} cannot be determined by means of a short-term functional assay

As until today expanded tT_{REG} cannot be frozen and thawed without loss of function, we made use of a published protocol to evaluate tT_{REG} potency within a much shorter period of time based on the assessment of activation marker CD69 and CD154 expression by autologous responder T cells. Shortly after the first experiments were performed according to protocol, we revealed a potential drawback of this procedure, which was extensively followed up on and published in the peer-reviewed journal '*Frontiers in Immunology*' with the title "*The value of a rapid test of human regulatory T cell function needs to be revised*". This publication lays the foundation of this PhD thesis and can be found on page 32-38. Briefly summarizing, we were able to demonstrate that the tT_{REG}-mediated suppression of activation marker expression proposed by the published protocol is purely attributable to competition for αCD3/CD28 activating microbead-binding by responder T cells and tT_{REG} as opposed to tT_{REG} functionality. For this publication, my contributions were the design and implementation of the experiments, interpretation of the results and writing of the manuscript.

2.3 Initiation of pro-inflammatory cytokine production by effector T cells is not suppressed by tT_{REG}

After appreciating that assessing suppression of early activation marker expression as a readout for tT_{REG} function in addition to the continued pressing need for a reproducible short-term potency test for tT_{REG}, we investigated whether tT_{REG} possess an immune repressive influence on cytokine production by autologous effector T cells in an uncompetitive activating microbead environment. This tT_{REG} test system protocol was designed to meet several criteria necessary for successful GMP-compliant translation, such as no need for intracellular cell labelling, easy handling by the experimenter, short incubation period of 7 hours and a standardized multiplex readout system.

After 7 hours tT_{REG}/T_{RESP} co-culture, pro-inflammatory cytokines IFNγ, TNFα and IL-2 were analyzed in the supernatant. However, the results reveal no suppressive effect of tT_{REG} on conventional T cell effector cytokine production in this experimental setup (Fig. 7 A). For the sake of interest, we also incubated the co-cultures for 24 hours (Fig. 7 B) and likewise observed no tT_{REG}-mediated suppression of conventional T cell effector cytokine production (Fig. 7 C,D).

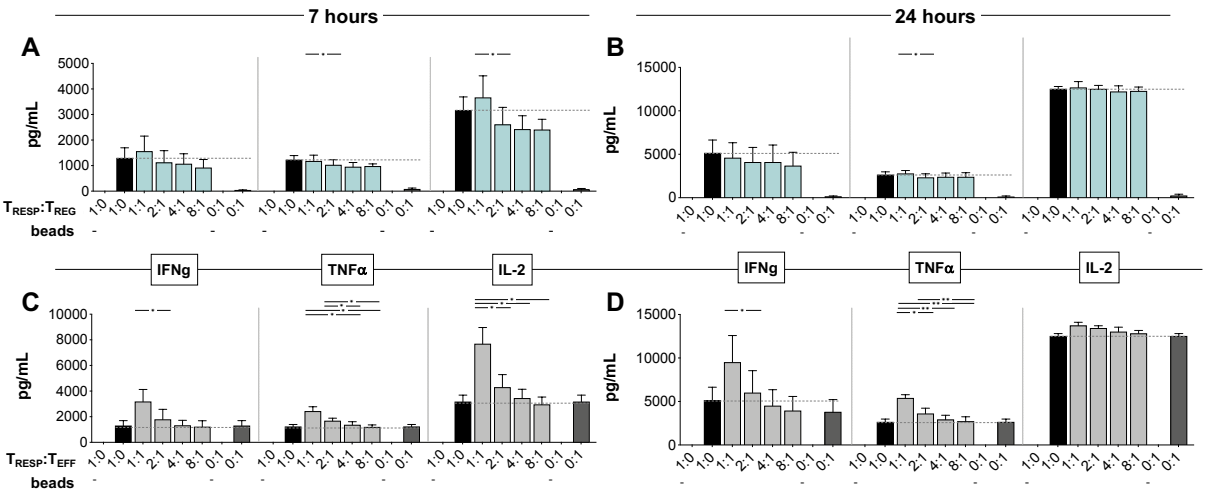


Figure 7. Effector T cell cytokine production is not suppressed by tT_{REG} after 7 nor after 24 hours.

*Ex vivo FACSorted $CD4^+CD25^-$ T_{RESP} were co-cultured with autologous bulk tT_{REG} (green bars) and stimulated with αCD3/CD28-coated microbeads at a total cell number:bead ratio of 5:1 for **(A)** 7 hours and **(B)** 24 hours. Confirming potential tT_{REG} specificity, co-cultures were performed with $CD4^+CD25^-$ effector T cells (T_{EFF}) (grey bars) instead of tT_{REG} and incubated for **(C)** 7 hours and **(D)** 24 hours. Cytokines were assessed by Mesoscale Multiplex analysis. n=3. Results are presented as mean ± SEM. Normal distribution of data points was tested with Kolmogorov-Smirnov test and significance was determined by paired two-tailed t-test. *: P≤0.05; **: P≤0.01; ***: P≤0.001; ****: P≤0.0001*

Discussion

According to the objectives outlined within the **first aim** of this PhD thesis, this work was able to describe distinct subsets making up the human tT_{REG} compartment. By applying recognized differentiation lineage markers defining conventional T cell subsets, e*x vivo* characterization of tT_{REG} disclose similar subset distributions, including a T cell memory marker expressing tT_{REG} population within the naïve tT_{REG} compartment (T_{REG}NLM). Further investigations support the as such defined tT_{REG} subsets by their differential expression of proteins associated with their recent thymic emigration, current state of proliferation as well as prior activation. Supportingly, T cell receptor sequencing demonstrates strong differences in TCR repertoire diversity between tT_{REG} populations, where the least differentiated subsets T_{REG}N and T_{REG}NLM present the greatest TCR diversity. Most strikingly and in accordance with data published for $CD4^+$ T_{CONV} within the German Epigenome Program (DEEP)[40], tT_{REG} subsets, as previously shown for $CD4^+$ T_{CONV} subsets, demonstrate a genome-wide loss in DNA methylation, which, combined with the data of *ex vivo* characterization, suggest being associated with previous proliferation and differentiation.

In vitro expansion of tT_{REG} subsets points out considerable differences between tT_{REG} populations with the essence of effector tT_{REG} predominantly losing tT_{REG}-specific characteristics, such as their phenotype, inability for pro-inflammatory cytokine production, lineage stability, suppressive function and proliferative potential over the time of *in vitro* expansion. Since on the other hand, early differentiated tT_{REG} subsets demonstrate a stable tT_{REG}-characterizing phenotype, these data could be of value for clinical translation as to possibly deplete the effector memory or enrich for the central memory tT_{REG} subset before *vitro* expansion. In addition to an increase in patient safety by preventing tT_{REG}EM converting to conventional T cells during *in vitro* expansion, this could also lead to a cell therapeutic product being superior in potency compared to bulk-only tT_{REG} cells. Supporting this implementation, tT_{REG}CM demonstrate a greater capacity to suppress autologous T_{RESP} proliferation at a high T_{RESP}:T_{REG} ratio compared to bulk tT_{REG}. These findings suggest that fewer early differentiated tT_{REG} cell numbers might be sufficient for successful application in adoptive immunotherapy. With regard to clinical implementation, requiring fewer tT_{REG} cell numbers for a successful therapy may imply that less patient material would be necessary for *in vitro* expansion or even that possibly a shorter expansion period for reducing proliferation induced differentiation / senescence could be sufficient. In addition, the cell product would be ready for application at an earlier time point reducing the production costs and making the cell product available for the patient at an earlier time point.

Regarding the **second aim** of the project - challenging tT_{REG} functional assays relating to their translation into a robust GMP-compliant and clinically feasible protocol – it can be concluded that the previously published protocol on the assessment of tT_{REG}-mediated suppression of early activation makers on T_{CONV} cannot be used to evaluate tT_{REG} functionality as release criterion for adoptive tT_{REG} immunotherapy. Even after protocol adjustments to attain bead-uncompetitive co-culture conditions, data demonstrate that tT_{REG} do not suppress early activation marker expression on autologous responder T cells. Likewise, tT_{REG}-mediated suppression of pro-inflammatory cytokine production by autologous responder T cells cannot be observed within the first 7 hours of activation. Hence, tT_{REG} do not exert short-term suppressive effects on conventional effector T cells. However, these data can be subjected to critical review since the immense supra-physiological magnitude of T cell activation, as shown by roughly 90% of T_{RESP} expressing CD69 as demonstrated in the publication, could be too strong for tT_{REG} to mediate suppressive effects. Nonetheless it is difficult to determine the physiologically relevant strength of T cell activation. Therefore, titrating T_{RESP}-activating microbeads in the presence of tT_{REG}, with the readouts of suppressing early activation marker expression and/or pro-inflammatory cytokine production, could be interesting for future investigations. Secondly, tT_{REG}-specific mechanisms of immune response dampening include the indirect inhibition of effector T cell responses by interfering with APC-effector T cell priming. On this account, adding APCs, for example whole PBMCs, to the co-culture and using physiologically presented antigens for stimulation could potentially create optimized conditions for assessing tT_{REG}-mediated suppression of pro-inflammatory cytokine production and/or early activation marker expression by effector T cells.

To date, there are still no reliable short-term functional assays to assess tT_{REG} function. The gold-standard protocol to evaluate tT_{REG}-mediated suppression of T_{RESP} proliferation should still be considered the most robust assay, yet due to its 4-day incubation period, does not present as a feasible candidate for clinical application. In conclusion, future efforts should be concentrated to optimize existing or develop novel clinically-feasible protocols to reliably asses tT_{REG} functionality. For this, the suppression of conventional effector T cell function by means of other tT_{REG} mechanisms of immunosuppression, *e.g.* the CTLA-4 ↔ CD80/CD86 interaction, could be explored.

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„Ich, Désirée Jacqueline Wendering, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: "***Regulatory T cells in adoptive immunotherapy: from subset characterization to functional testing / Regulatorische T-Zellen in der adoptiven Zelltherapie: Von der Charakterisierung von Subpopulationen bis zur funktionellen Testung***" selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Datum	Désirée J. Wendering

Detailed Statement of Contribution

For the successful publication of the article 'The Value of a Rapid Test of Human Regulatory T Cell Function Needs to be Revised', I first of all challenged a previously published protocol for the assessment of human regulatory T cell function by reproducing their results followed by critical questioning of the protocol's central aspect, namely T cell activation. Consequently, I generated the hypothesis, study conceptualization, experiment planning and implementation, data analysis, data presentation and interpretation and writing of the manuscript.

For obtaining human T cells, I recruited healthy donors, collected venous blood and isolated peripheral mononuclear cells. For fluorescence activated cell sorting, I designed the panel setup and prepared the cells for the sorting process, which was finally performed by the Core Facility for Cell Sorting of the Berlin-Brandenburg Center for Regenerative Therapies. For the T cell co-cultures, I was responsible for conducting the experiments and optimization of co-culture conditions, such as determining the ideal intracellular CFSE-cell-tracing protocol for human T cells. For flow cytometric analysis, I established and compensated panels together with Leila Amini. Finally, I stained and analyzed the cells at the flow cytometer. I analyzed all acquired data, which lead to figure 1 and 2, as well as created all data plots, figures and schematic diagrams as seen in figure 1 and 2, in addition to the supplementary figure. I composed and wrote the entire original manuscript, which was proof-read and adjusted by all co-authors, yet mainly by Dr. Schmück-Henneresse.

Dr. Schlickeiser was responsible for the statistical analysis in figure 1 E and F. For this, he performed 3 different and in-depth statistical evaluations requested during the reviewing process.

Prof. Dr. Volk and Prof. Dr. Reinke kindly acquired the financial resources and supported my publishing process with valuable guidance and critical discussions.

Dr. Schmück-Henneresse was part of designing of the project. Further, for flow cytometric data, I discussed gating strategies with Dr. Schmück-Henneresse and elaborately discussed the acquired data.

Following the reviewing process, I replied to all the reviewer's comments as well as adjusted the manuscript accordingly. Dr. Schmück-Henneresse and I formulated the rebuttal letter. Dr. Schmück-Henneresse was responsible for manuscript submission and communication with the editor and reviewers.

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Datum Désirée J. Wendering

Extract of Journal Summary List

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI
Selected Categories: **"Immunology"** Selected Category Scheme: WoS
Gesamtanzahl: 155 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS IMMUNOLOGY	39,215	41.982	0.085360
2	Annual Review of Immunology	17,086	22.714	0.028800
3	NATURE IMMUNOLOGY	41,410	21.809	0.102290
4	IMMUNITY	46,541	19.734	0.136360
5	TRENDS IN IMMUNOLOGY	11,204	14.188	0.026850
6	JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY	49,229	13.258	0.083800
7	Lancet HIV	1,476	11.355	0.007950
8	JOURNAL OF EXPERIMENTAL MEDICINE	62,537	10.790	0.078310
9	IMMUNOLOGICAL REVIEWS	14,555	9.217	0.028540
10	Cancer Immunology Research	4,361	9.188	0.021180
11	CLINICAL INFECTIOUS DISEASES	61,618	9.117	0.120010
12	AUTOIMMUNITY REVIEWS	8,956	8.745	0.020990
13	Journal for ImmunoTherapy of Cancer	1,675	8.374	0.007130
14	CURRENT OPINION IN IMMUNOLOGY	9,275	7.932	0.020120
15	JOURNAL OF AUTOIMMUNITY	6,410	7.607	0.015490
16	Cellular & Molecular Immunology	3,633	7.551	0.008300
17	EMERGING INFECTIOUS DISEASES	29,657	7.422	0.057980
18	Mucosal Immunology	6,105	7.360	0.021860
19	SEMINARS IN IMMUNOLOGY	4,552	7.206	0.010950
20	EXERCISE IMMUNOLOGY REVIEW	740	7.105	0.001110
21	Journal of Allergy and Clinical Immunology-In Practice	2,802	6.966	0.009670
22	CLINICAL REVIEWS IN ALLERGY & IMMUNOLOGY	2,741	6.442	0.005880
23	Seminars in Immunopathology	2,967	6.437	0.009290
24	BRAIN BEHAVIOR AND IMMUNITY	12,583	6.306	0.026850
25	ALLERGY	16,476	6.048	0.025790
26	Emerging Microbes & Infections	1,318	6.032	0.005910
27	Advances in Immunology	2,423	5.935	0.004250
28	Current Topics in Microbiology and Immunology	5,633	5.829	0.011740
29	World Allergy Organization Journal	1,352	5.676	0.003800
30	Frontiers in Immunology	16,999	5.511	0.067470

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Selected JCR Year: 2017; Selected Categories: "IMMUNOLOGY"

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

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ORIGINAL RESEARCH
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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

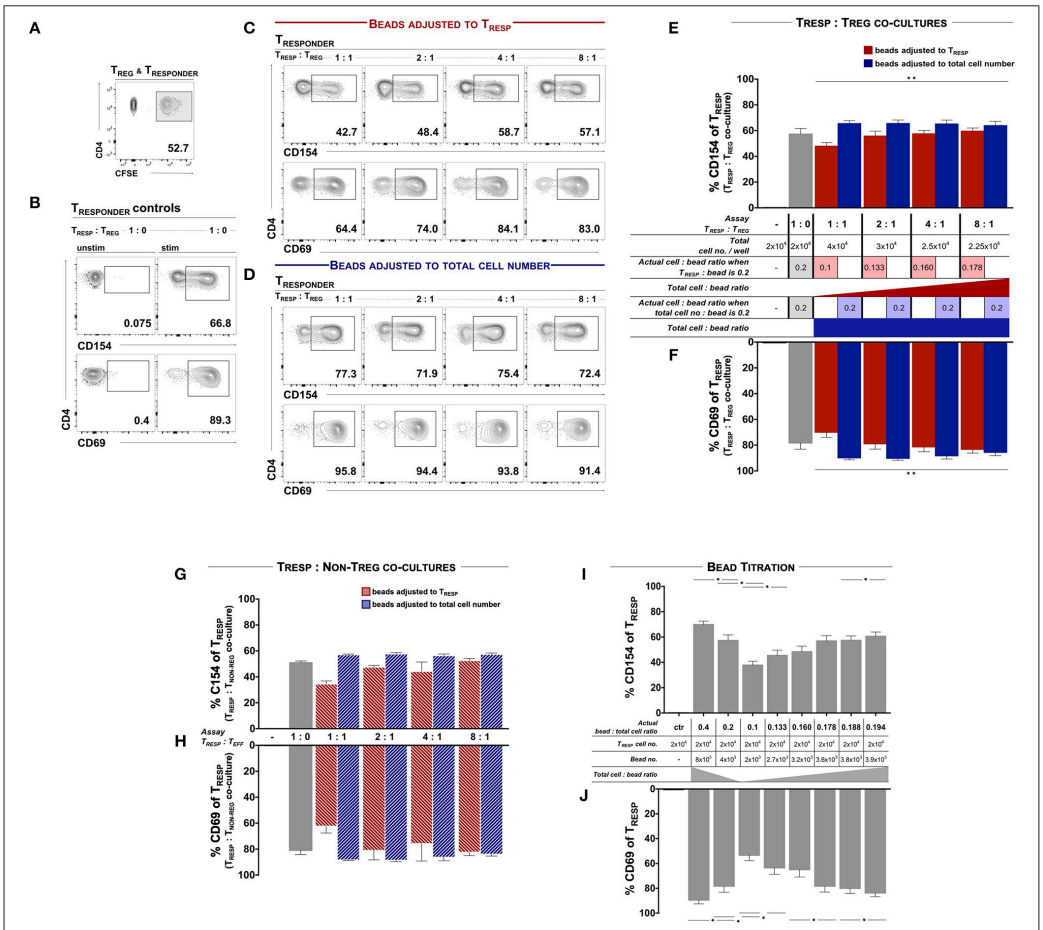

FIGURE 1 | T_{CELL} early activation marker expression is dependent of TCR engagement and cannot be used for T_{REG} functional evaluation. FACSorted $CD4^+CD25^-$ T_{RESP} with and without autologous T_{REG} co-culture were stimulated with anti-CD3/CD28-coated microbeads and analyzed for early activation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

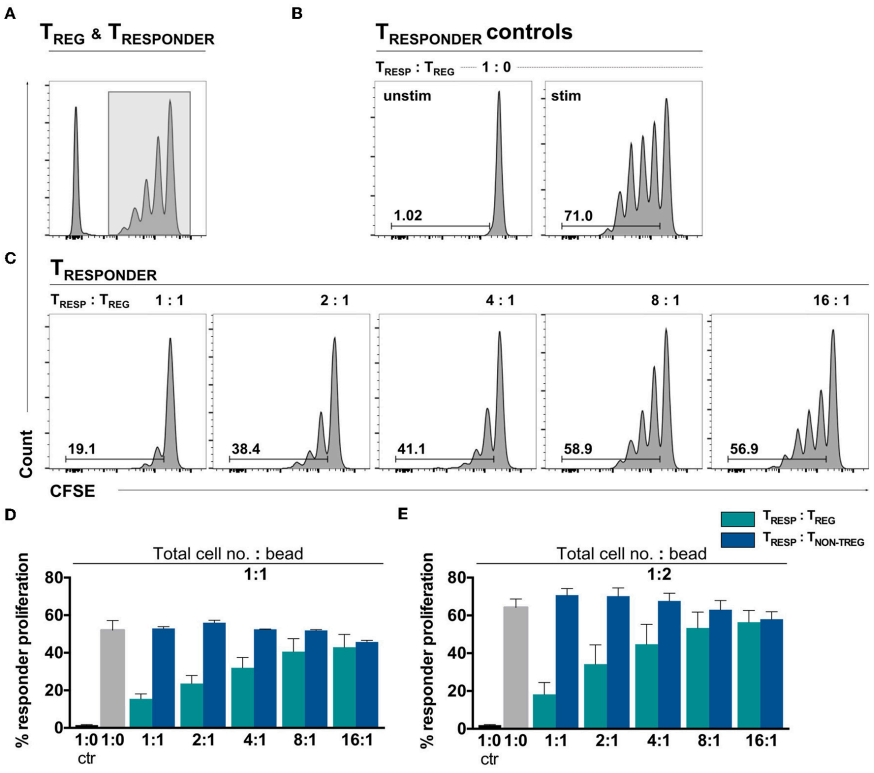

FIGURE 2 | *Ex vivo* isolated T_{REG} demonstrate a dose-dependent T_{RESP} proliferation suppression in a bead-uncompetitive setting. FACSorted $CFSE^{+}CD4^{+}CD25^{-}$ T_{RESP} were co-cultured with autologous FACSorted T_{REG} and stimulated with anti-CD3/CD28-coated microbeads for 96 h. T_{RESP}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

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Supplementary Material

The value of a rapid test of human regulatory T cell function needs to be revised

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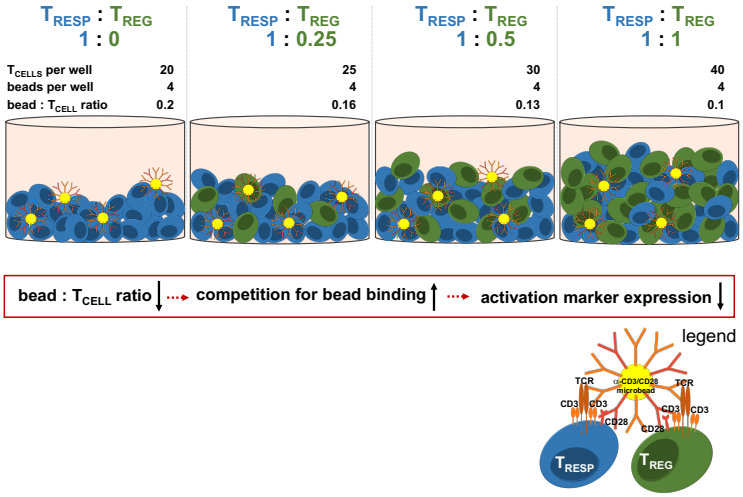

Supplementary Figure 1. Schematic diagram of T_{CELL} / αCD3/28-coated activating microbead competition in dependence of microbead/T_{CELL}

Since T_{RESP} cell numbers are kept constant and T_{REG} are titrated to T_{RESP} in a functional T_{RESP}/T_{REG} co-culture assay, also activating microbead numbers are constant when adjusted to T_{RESP} only – in this case 4 beads per 20 T_{RESP} and therefore a microbead/T_{CELL} ratio of 0.2. When the same amount of T_{REG} is added to T_{RESP} in a 1:1 co-culture setting, the actual microbead /T_{CELL} ratio is halved, i.e. decreased to 0.1. By decreasing the T_{REG} number to demonstrate a T_{REG}-specific effect, the total T_{CELL} numbers per well decrease and consequently lead to an increase in microbead /T_{CELL} ratio. Both, T_{RESP} and T_{REG} express the signaling molecule CD3 and T_{CELL} co-stimulatory receptor CD28 on their cell surface. As therefore both T_{CELL} populations compete for binding αCD3/CD28-coated activating microbeads, highest microbead availability per T_{CELL} would predict the greatest T_{CELL} activation and hence the lowest T_{CELL} activation when bead availability is lowest.

Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publication List

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