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SUMMARY

Regulatory T cells (Treg) offer new immunotherapeutic options to control undesired immune reactions, but the heterogeinetiy of Treg raises the question which Treg population should be used for clinical translation “from bench to bedside”, especially to solid organ transplantation (SOT) patients. Based on this final goal, this project involves three main parts: i) investigating Treg frequency and subsets distribution with age in healthy donors and transplant (Tx) patients; ii) comparing the suppressive capacity of Treg subsets and expanding them *in vitro* without losing functionality; iii) clarifyjing the differiation relationship of Treg subsets and their relation to conventional T cells (Tconv) by T cell receptor (TCR) repertoire analysis.

From both healthy donors and Tx patients, an age-dependent shift from naïve Treg (TregN) to memory Treg (TregM) was observed, dominated by the central-memory (CM) phenotype over less abundant effector-memory (EM) population; compared to age matched healthy donors, Treg in Tx patients contained less naive but more EM cells, moreover, they were pre-activated by expression of CD137 due to the exposure to allo antigens, thus more susceptible to activation-induced cell death. Regarding control of early Tconv activation, TregCM showed enhanced suppressive capacity compared to TregN; furthermore, only TregCM could induce apoptosis of responder cells especially in NK cells and B cells populations while TregN could not, which may result from cell-cell interactions as TregM have mugh higher cytotoxic T-lymphocyte antigen 4 (CTLA-4) expression than TregN. Following *in vitro* expansion of the Treg subsets with rapamycin, however, TregN converted mainly into TregCM phenotype with enhanced suppression activity comparable to the level of freshly isolated and expanded TregCM. The poor proliferation capacity of TregEM might indicate EM as the terminal differential stage. Expansion of Treg in the gas-permeable and GMP-compliant G-Rex device could further enhance the expansion without losing phenotype and functionality. These data suggest that expansion with total Treg is optimal for adoptive Treg therapy as the majority of them are the highly potent TregCM.

Lastly, TCR repertoire study by next generation sequencing (NGS) indicated that TregM derived from TregN rather than Tconv in an antigen-driven process. The highest similarity of the TCR repertoires was observed between TregCM and TregEM. These data reveal new insights for the first time into the distinct TCR repertoires of Treg subsets and Tconv in human by NGS technology.

ZUSAMMENFASSUNG

Regulatorische T-Zellen (Treg) eröffnen neue immuntherapeutische Wege zur Kontrolle unerwünschter Immunreaktionen, jedoch wirft die Heterogenität dieser Zellen die Frage auf, welche Treg-Population für die klinische Anwendung im Rahmen der „bench to bedside“ - Anwendung, besonders im Bereich der Organtransplantation, am geeignetsten erscheint. Darauf basierend werden in dieser Arbeit drei Fragestellungen bearbeitet: i) Bestimmung der Häufigkeit von Tregs und deren Subpopulationen in verschiedenen Altersgruppen bei Empfängern einer Organtransplantation (Tx) und einer gesunden Kontrollgruppe; ii) Vergleich der Suppressorkapazität verschiedener Treg-Populationen und in vitro-Expansion der Zellen unter Erhaltung ihrer Funktionalität; iii) Klärung der Differenzierungsmerkmale von Tregs und deren Verknüpfung mit konventionellen T-Zellen (Tconv) mittels Analyse des T-Zell-Rezeptor- (TCR) Repertoires.

Sowohl bei gesunden Probanden als auch bei Tx-Empfänger konnte eine altersabhängige Verschiebung von naiven (TregN) hin zu dominant zentralen Gedächtnis-Zellen (TregCM) beobachtet werden, Treg von Tx-Empfängern hatten mehr Effektor-Memory-Zellen (EM) und sie waren mehr aktiviert. In Bezug auf die Kontrolle der frühen Tconv zeigen TregCM eine erhöhte Suppressorkapazität im Vergleich zu TregN. Außerdem sind im Gegensatz zu TregN nur TregCM dazu in der Lage, Apoptose bei Responderzellen zu induzieren. Der Grund hierfür könnte in der stärkeren Expression von CTLA-4 auf TregM liegen. Die Expansionskultur führte zur phänotypischen Veränderung der TregN, deren Umwandlung in TregCM mit einer verbesserten Suppressoraktivität verbunden ist, welche mit der von frisch isolierten und expandierten TregCM vergleichbar ist. Die schwache Proliferationskapazität der TregEM könnte die Effektor-Memory-Zellen als begrenzende Phase der Zelldifferenzierung ausweisen. Die Daten legen nahe, dass das Expandieren mit gesamt Treg für die Adoptive-Treg-Therapie optimal sind, da sie der größte Anteil von ihnen die hochpotenten TregCM sind.

TCR-Studien mittels Next Generation Sequencing zeigen weiter, dass TregM aus TregN entstehen, anstatt aus Tconv, in einem Antigen-gesteuerten Prozess. Die größte Ähnlichkeit des TCR-Repertoires wurde hierbei zwischen TregCM- und TregEM-Zellen nachgewiesen. Diese Daten belegen erstmalig neue Erkenntnisse hinsichtlich der Unterschiede der TCR-Repertoires von TregM und Tconv beim Menschen.

ABBREVIATIONS

ADCC	antibody dependent cellular cytotoxicity
APC	antigen presenting cells
ATP	adenosine triphosphate
BCR	B cell receptor
BFA	Brefeldin A
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CDC	complement dependent cytotoxicity
cDNA	complimentary DNA
CDR3	the third complementarity-determining region
CFSE	arboxy fluorescein succinimidyl ester
CTL	cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DC	dendritic cells
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
Foxp3	forkhead box 3
FSC	forward scatter
GVHD	graft versus host disease
hAB serum	human serum AB
HLA	human leucocyte antigen
HSCT	hematopoetic stem cell transplantation
IDO	Indolamin-2,3-Dioxygenase
IFN	interferon
IL	interleukin
iTreg	induced regulatory T cells
IU	international unit
L/D	LIVE/DEAD
LFA-1	Lymphocyte function-associated antigen 1
mAb	monoclonal antibody
MACS	magnetic activated cell sorting
MFI	mean fluorescence intensity
MH index	Morisita-Horn similarity index
MHC	major histocompatibility complex
NET	neutrophils extracellular traps
NFAT	Nuclear factor of activated T-cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

NGS	next generation sequencing
NK cells	natural killer cells
nTreg	natural regulator T cells
OL	overlap
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
PRRs	pattern recognition receptors
SEM	standard error mean
SOT	solid organ transplant
SSC	side scatter
ST	stimulated
Tconv	conventional T cells
TconvM	memory conventional T cells
TconvN	naïve conventional T cells
TCR	T-cell receptor
TGF-ß	tumor growth factor beta
Th1	type 1 T helper cells
TLR	Toll-like receptor
TNF	tumor necrosis factor
Tr1	type 1 regulatory T cells
TregCM	central memory Treg cells
TregEM	effector memory Treg cells
TregM	memory regulatory T cells
TregN	naïve regulatory T cells
TSDR	Treg specific demethylation region
Tx	transplantation
UN	un-stimulated
V ß	T-cell receptor variable ß chain

1. INTRODUCTION

1.1 General aspects of Treg development and suppressive functionality

1.1.1 Innate and adaptive immune system

Human immune system is an extremely complex and powerful network, which functions like a strong army to protect us from infections. It can not only augment immune responses upon "foreign agents" like viruses; but also dampens reactions to "self-tissues"; resulting in a formidably active but homeostatic "protecting" environment. This powerful network consists of two distinct but intimately correlated systems: innate immune system and adaptive immune system.

Briefly, when we got infections, innate immune system firstly recognizes the conserved components in many microorganisms via pattern recognition receptors (PRRs), e.g. Toll-like receptors (TLR) and works immediately in a general manner by mainly phagocytosis and complement dependent cytotoxicity (CDC) (1-3). Leucocytes especially neutrophils, macrophages, dendritic cells, NK cells and mast T cells are involved in these processes. As the majority of phagocytes, neutrophils are one of the first "soldiers" arriving at an infection site under chemotaxis (4). On one hand, they kill the invading microbes directly by engulfment, secretion of anti-microbials and generation of neutrophils extracellular traps (NET) (5); on the other hand, neutrophils also secrete cytokines to recruit and activate other immune cells. In addition, macrophages present antigens to activate adaptive immune system like the big "presenter", dendritic cells, but also produce many chemicals like enzymes to "digest" invading microbes directly (6).

However, innate immune system is far from enough for protecting organism from infections because lots of pathogens could escape it. Luckily, we got second the protection layer, adaptive immune system, which can be activated by innate immune responses thus "adapt" stronger responses and reinforce it when same antigens are met again by "memory" in a specific manner (7). Lymphocytes, especially B cells and T cells, are involved in these responses. On one hand, cytotoxic T cells can kill infected cells directly by releasing cytotoxins when they are activated by TCR binding to specific-antigen and MHC-I complex and CD8 molecule; on the other hand, helper T cells can recognize antigens with MHC-II

complex and get activated with releasing several cytokines to enhance the activity of macrophages and cytotoxic T cells for direct killing. Another big player in adaptive immune system is B cells(8). They recognize pathogens by their specific receptors on the cell surface and uptake and process it into many peptides, which are subsequently presented by these B cells with their MHC-II molecule to helper T cells, making these helper cells release cytokines and again stimulate B cells to become plasma cells producing large amount of antibody. These antibodies will again promote complement system by antibody-dependent cell mediated cytotoxicity (ADCC) or direct phagocytosis. In addition, some of activated T or B cells will become memory cells and response immediately next time when the same antigens were met (8).

The tight collaboration of innate and adaptive immune system provides a powerful network to protect us. More importantly, they can also discriminate "self" from "non-self" to minimize possible self-reactive damage in several ways. In brief, self-reactive clones in lymphocytes are neutralized mainly by clonal deletion, abortion and anergy when potential auto-reactive cells encounter antigens or by its regulatory mechanism when those cells escape thymus censorship (9, 10). In this regulatory mechanism, "suppressor T cells", later called regulatory T cells (Treg), are proved to be one of the most important regulators in keeping immune homeostasis (11).

1.1.2 General finding and development of Treg

Treg are a specialized subpopulation of T cells that play a central role in maintaining homeostasis within immune system. The concept of suppressor T cells was raised in early 1970s by Gershon and Kondo (12). They found that one population of T cells could not only enhance immune activity, but also suppress it, keeping immune responses more balanced. Massive researches regarding this T cell population, now called regulatory T cells, were conducted subsequently all over the world.

Like many other seminal findings in science, the exploration of Treg with significant importance in clinical adoptive cell therapy nowadays, also has ups and downs (13). From late 1960s to early 1980s, Treg attracted much interest as the concept of the "suppressor T cells" was raised. During this period, it was found by many researchers that thymectomy of normal mice and rats produced damage in "self" organs like ovaries and led to the development of thyroiditis accompanied by autoantibodies production and so on (14-16). Type-1 diabetes was

also found to be induced with same methods in both mice and rats later (17, 18). More interestingly, inoculation of normal T cells from healthy mice or rats could prevent these autoimmune diseases in syngeneic animals (19, 20). Taken together, Sakaguchi et al.. proposed that the coexistence of potential self-reactive CD4 T cells mediating autoimmune diseases and other CD4 T cell population mainly suppressing them in periphery of healthy mice and rats (21).

From mid 1980s to early 1990s, research of suppressor T cells was hindered by the big issue regarding I-J region of mouse MHC gene and collapsed in this period. The I-J region was believed to encode important molecules expressed by suppressor T cells and pivotal to the suppression functionality (22). However, this region was not found in mouse MHC gene by molecular biology techniques (23), suggesting the previous concept of "suppressor T cells" might be false . Together with paucity of specific cell markers, research of suppressor T cells went downhill (13).

In the second half of the nineties, a milestone of suppressor T cells research was achieved mainly by the discovery of CD25, the α chain of interleukin 2 (IL2) receptor, which was found as a useful surface marker for the suppressive T cells, later called regulatory T cells (Treg) (24-26). Thus, Treg research was rising again with intensive further studies regarding the pivotal functional role of IL2 and CD25 for Treg survival (27-29). Furthermore, the discovery of CD25 also made isolation of Treg feasible, thus the investigation of Treg function *in vivo* with adoptive Treg cell transfer became achievable.

Since 2000s, massive investigations regarding Treg development and function have been conducted. Another mile stone was achieved by the discovery of transcriptional factor forkhead box P3 (FoxP3), also called Scurfin, as an important regulator for Treg development and function in 2003 (13). In the same year, several groups reported simultaneously that FoxP3 mRNA could be expressed by $CD4^+CD25^+$ peripheral T cells and $CD4^+CD8^-CD25^+$ thymocytes, but not by $CD4^+CD25^-$ conventional T cells; moreover, ectopic expression of Foxp3 conferred suppressor function on peripheral $CD4^+CD25^-$ T cells (30-32). Later on, more and more data have proved that FoxP3 is a master controller of Treg development in thymus and function, thus the most reliable molecular Treg marker so far (33). Indentifying FoxP3 as the specific Treg marker further broadened the way nowadays for Treg research and benefited the clinical translation significantly as well.

1.1.3 Classification of thymus derived natural Treg and peripheral induced Treg

Naturally occurring Treg (nTreg) derives in thymus. When a T cell precursor is produced in bone marrow and transferred into thymus, it will receive very strict “training” or “education” through several complicated selection processes. Only minority of the survivors can become final Treg cell and enter into periphery. To recognize millions of antigens presented by major histocompatibility complex (MHC) on the antigen-presenting cells, it is firstly essential for T cells to have enormous T cell receptor (TCR) repertoire which are mainly produced by stochastic V(D)J recombination of gene segments in the α and β chain of their TCR in the thymus as well as the random insertion and deletion of nucleotides at the segment junction sites. The clonal diversity of the TCR repertoire is further enhanced by different pairing of α and β chain. Subsequently, the whole repertoire is shaped by positive and negative selections. Positive selection selects single positive CD4 or CD8 T cells while negative selection further deletes the clones with high affinity to self peptide and MHC complexes (34). These two processes result in conventional T cells (Tconv) repertoire with low affinity to self-antigens but high-affinity to foreign antigens (35). Some self-reactive cells with intermediately high affinity can escape from negative selection and divert into nTreg cell lineage, which is mainly responsible for maintaining homeostasis within immune system. The lineage-specific transcription factor that determines the fate of Treg lineage is FoxP3 (36), its expression on developing thymocytes seems to depend chiefly on the intensity of TCR stimulation (37, 38). The demethylation of FoxP3 locus, Treg specific demethylation region (TSDR), depends on the duration of TCR stimulation, thus TSDR of nTreg are totally demethylated (39).

Additionally, $FoxP3^+$ Treg can also be induced from naïve Tconv with presence of cytokines and low amount of antigens in periphery (40-42), that is so-called induced Treg (iTreg). Unlike nTreg, TSDR of iTreg are not completely demethylated (43), although they also have FoxP3 expression. As nTreg mainly prevent autoimmunity to keep immune tolerance, iTreg are believed to mainly control chronic allergic inflammation and be one of the important barriers for tumor cells (43). Besides Th3 cells, CD4 typ1 regulatory T (Tr1) cells are another type of adaptive Treg produced out of thymus. They don’t express FoxP3, but secrete IL10 and express granzyme B to kill myeloid antigen presenting cells (44, 45). The basic differentiation of different T cell lineages is shown in Figure 1. This project focuses on nTreg research.

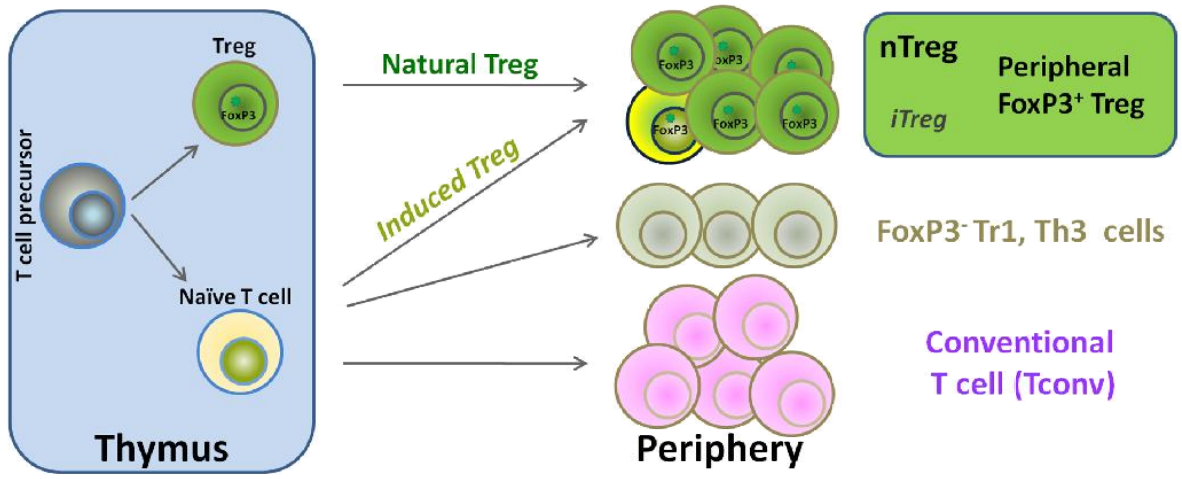

Figure 1: Differentiation processes of different Treg and Tconv lineages. Each color represents one type of cells: Gray - T cell precursor; green - natural Treg; light yellow - naïve T cell; bright yellow – induced Treg; pink - Tconv.

1.1.4 Treg Suppression mechanism

Treg do the suppression job by several mechanisms. They can inhibit activation and proliferation of Tconv directly or indirectly via decreasing the antigen presentation ability of APCs (Figure 2). Firstly, Treg can secrete several immunosuppressive cytokines including TGFβ, IL10 and IL35, whose deficiency in mice could result in either autoimmunity or reduced suppressive activity (46, 47). Secondly, Treg can express granzyme and kill responder cells in perforin-dependent manner in human and mice (48, 49). Treg can also express Galectin-1[1], which is another important molecule during interaction of Treg-DC and Treg-Tconv (50). Thirdly, Treg have higher expression of CD25, the α-chain of IL2 receptor, thus they compete IL2 with Tconv under certain conditions, resulting in suppression of Tconv activation and proliferation (51, 52).

From the anti-inflammatory aspect, Treg express ectoenzymes CD39 and CD73, which could hydrolyze extracellular ATP and AMP to produce the immunosuppressive molecule adenosine (53-55). Adenosine may inhibit DC and Tconv by increasing cyclic AMP (cAMP) through adenosine-A2A adenosine receptor signals (56, 57). cAMP is another key component of Treg mediated suppression as antagonism of cAMP could partly abrogate IL2 transcription and proliferation (58). Higher amount of cAMP in DC cocultured with Treg contributes to

[1] Galectin-1: a member of a highly conserved family of β-galactoside binding proteins.

suppression of DC in a mice model (59). Furthermore, cAMP in suppressed Tconv induces expression of the inducible cAMP early repressor (ICER), which acts as a repressor at IL2 and IL4 gene loci (60). Additionally, Treg suppress particular TCR signaling pathways in Tconv initially by suppression of calcium signaling, resulting in immediate inhibition of NFAT, NF-κB activation and then IL2 transcription (61).

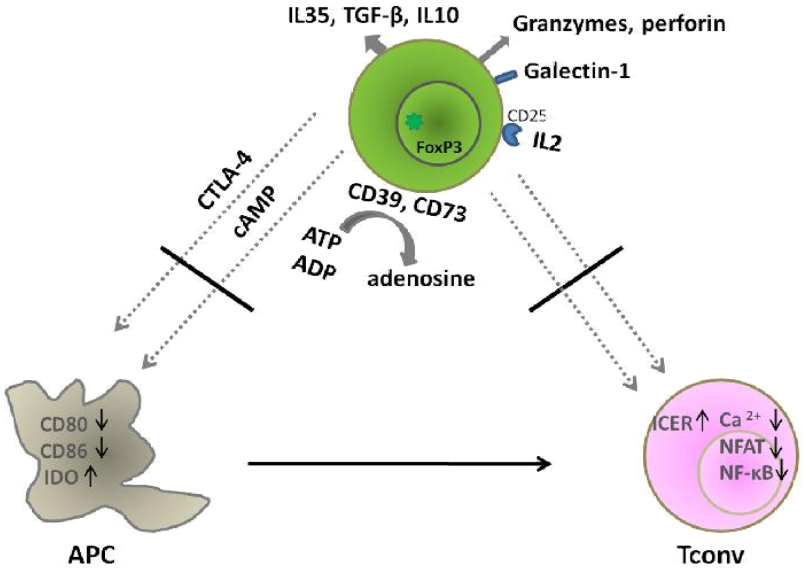

Figure 2: Different mechanisms of Treg-mediated Tconv suppression, adapted from Schmidt et al. (62). Treg have different suppression mechanisms under different situations. They can secrete inhibitory cytokines (TGFβ, IL10 and IL35) and kill Tconv by expressing granzymes in a perforin dependent manner. Treg also suppress Tconv proliferation by IL2 consumption due to higher amount of CD25 expression. Treg can rapidly suppress TCR-induced Ca^{2+}, NFAT and NF-κB signal pathway. Treg can produce immunosuppressive molecule adenosine and transfer cAMP to Tconv or APCs. Furthermore, they can decrease antigen presentation ability of APCs via higher expression of CTLA-4.

Regarding antigen presenting cells (APC), Treg also suppress Tconv in an indirect manner by inhibiting the antigen presentation ability of APC. Both murine and human Treg express high amount of inhibitory molecule CTLA4 [2] (63, 64), which compete for binding to CD80/CD86 with costimulatory molecule CD28 partly depending on adhesion molecule LFA-1[3], thus decrease the cell-cell contact between Tconv and APCs *in vitro* and *in vivo* (65-68). On the other hand, Treg can also increase the expression of the enzyme indoleamine 2, 3-dioxygenase (IDO) in DC via CTLA-4 induced signaling, resulting in starvation of Tconv and

[2] CTLA4: Cytotoxic T-Lymphocyte Antigen 4, also known as CD152.

[3] LFA-1: Lymphocyte function-associated antigen 1.

arrest of cell cycle, as IDO can catalyze degradation of the essential amino acid, tryptophan and also induce iTreg generation (69, 70).

1.1.5 Cross talk between Treg and non-T cells

In addition to the suppression of Tconv, Treg also interact with many other cell types including DC, B cells, NK cells, macrophages, osteoblasts, mast cells and NK T cells (71). Most importantly, Treg can decrease antigen presentation ability of DC as described previously. Additionally, it is reported that Treg derived from the tumor environment are able to lyse NK cells and CTL [4] in the granzyme B and perforin dependent manner (72). Recently, Gasteiger et al. found that restraint of NK cell cytotoxicity by Treg is related with limiting of IL2 availability (73). CD8 Treg have also been reported that they can down regulate the immune responses of macrophages in mice and human (74, 75). Regarding bone cells, Treg have been shown that they could suppress osteoclasts differentiation by secretion of cytokines like TGF-β *in vitro* in 2007 (76, 77). Several years later, protection of local and systematic bone destruction by Treg was observed as well *in vivo* by the same authors (78, 79), indicating further crosstalk between skeletal system and immune system. Regarding B cells, although they might be affected by immune suppressive cytokines secreted by Treg, it's not clear so far whether Treg are able to induce apoptosis or cell death of B cells in human.

1.2 Heterogeneous Treg for clinical translation

1.2.1 Treg for adoptive cell therapy

Since the discovery of CD25 as a new Treg surface marker in 1995 (24), worldwide substantial efforts have been made in understanding the mechanism of Treg suppression and exploring related clinical application for cellular therapy. Data from animal models have proved that adoptive transfer of Treg can prevent several autoimmune diseases and block GvHD [5] and allograft rejection after transplantation (80, 81). Therefore, regarding the essential role of Treg in keeping immune tolerance, there are several different translational therapy strategies from different aspects, as shown in Figure 3(82).

[4] CTL: Cytotoxic T Lymphocyte, also known as $CD8^+$ T cell or killer T cell

[5] GvHD: Graft versus Host Diease.

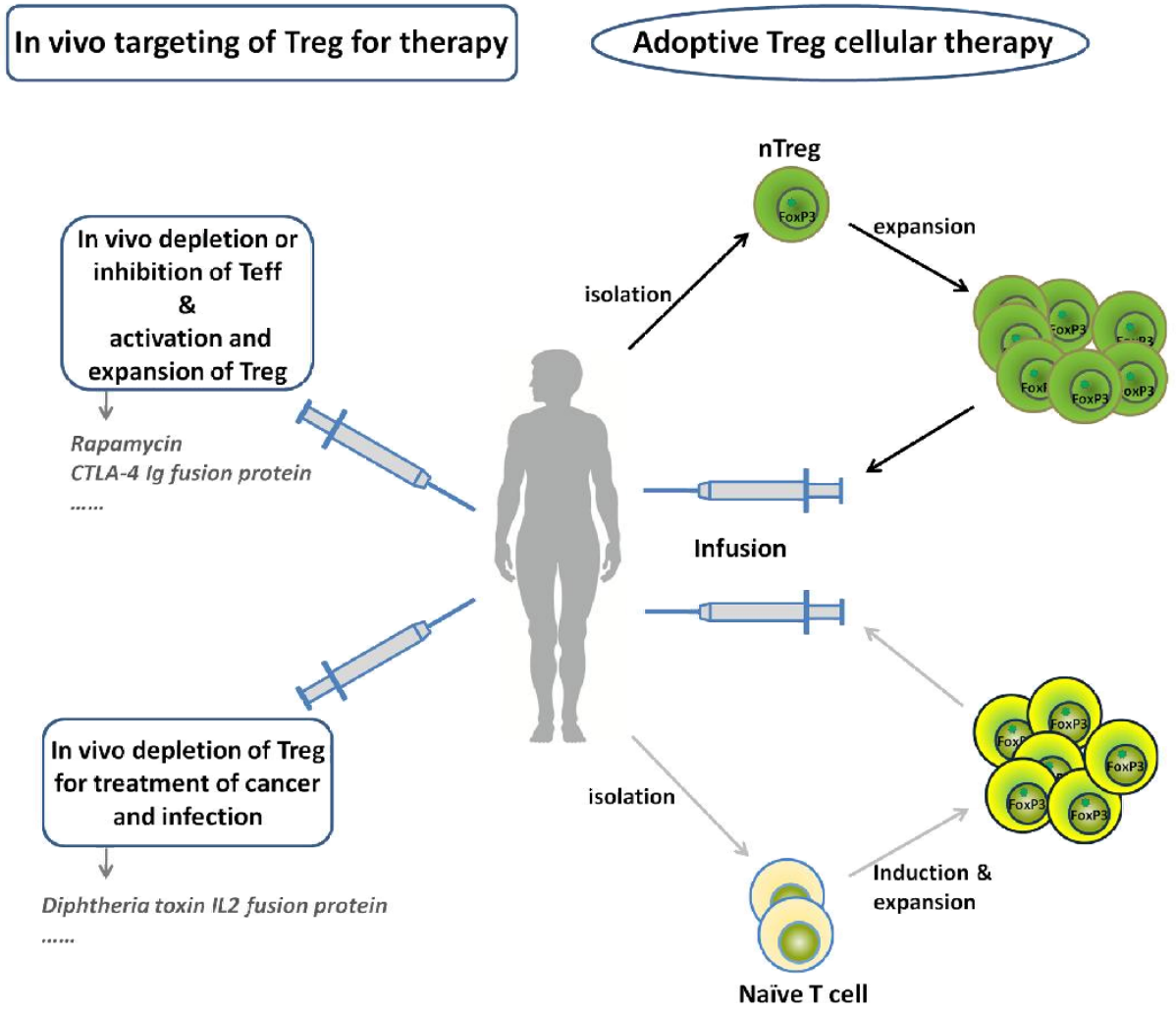

Figure 3: Treg immunotherapy, adapted from Wang et al. (82).

In vivo targeting of Treg for therapy has two aspects: i) *in vivo* depletion of Treg could be used for treatment of cancer and chronic infection by injection of diphtheria toxin-IL-2 fusion protein, which can bind to CD25, internalize cells (mostly Treg) and kill these cells, resulting in augmenting anti-tumor and anti-viral function of Tconv (bottom left in Figure 3Figure 3) (83); ii) *in vivo* activation and expansion of Treg with depletion or inhibition of Tconv by using of rapamycin or CTLA-4-Ig fusion protein could induce tolerance to allograft and reduce several autoimmune diseases (top left in Figure 3) (84). However, regarding adoptive cellular therapy, nTreg can be isolated from peripheral blood, expanded *in vitro* to large numbers with keeping the phenotype and suppression activity, then infused back to the patients (top right in Figure 3); alternatively, due to the induction of Treg from naïve Tconv, naïve Tconv could also be isolated and induced into iTreg *in vitro*, then expanded to large cell numbers with keeping immunosuppressive capacity and infused back to patients, leading to

the final tolerance to allograft or reduce autoimmune responses (bottom right in Figure 3) (82).

So far, several proof of concept clinical studies on adoptive transfer of different Treg population have been conducted after allergenic bone marrow transplantation, which have proved that adoptive Treg therapy is safe and effective to treat several diseases (85-88). However, regarding more clinical relevance in autoimmune diseases like type-1 diabetes and solid organ transplantation (SOT), more new clinical trials are on the ways. Currently, we and other five European institutions are collaborating on the "ONE study" to translate adoptive Treg therapy into kidney transplantation. Thus, as Leslie et al. have proposed, Treg therapy may "get their chance to shine" and achieve a new exciting milestone for human immune tolerance induction in the future (89).

1.2.2 nTreg heterogeneity in periphery

Despite the promising prospect of Treg for adoptive cell therapy, there are still lots of open questions regarding Treg heterogeneity and stability for real clinical translation. Similar with Tconv, Treg also consist of naïve and memory sub-populations according to the expression of CD45RA (90, 91). Furthermore, according to the report from Sakaguchi group, nTreg could be divided into 3 subsets: $CD45RA^{+}Foxp3^{lo}$ resting Treg (rTreg) and $CD45RA^{-}Foxp3^{hi}$ effector Treg (eTreg) and $CD45RA^{-}Foxp3^{lo}$ cells, since rTreg and eTreg are immunosuppressive *in vitro* but $CD45RA^{-}Foxp3^{lo}$ cells are not, they are regarded as non-Treg, also due to their secretion of IL17, IL2 and IFN-γ. Interestingly, rTreg can convent into eTreg after T cell receptor (TCR) stimulation, but eTreg are more prone to apoptosis (92). In mice, FoxP3 expression in Treg are proved to be unstable *in vivo*, they may even get lost due to epigenetic modifications of the gene, thus these "unstable" Treg could differentiate into memory effector cells as a huge "danger" for potential adoptive Treg transfer (93), which makes Treg cell therapy even more complicated.

Regarding stability of Treg, Edinger group suggested naïve Treg as the most stable subset for adoptive Treg therapy as they could keep FoxP3 expression after strong and repeated TCR stimulation during *in vitro* expansion (94). However, majority of Treg express the "memory" phenotype, particularly in patients. This raises the question: which Treg subset should be used for adoptive cellular therapy with Treg in SOT patients? Thus, to translate Treg "from bench

to bedside", it is pivotal to investigate the heterogeneous composition of Treg population and their functionality including stability more into details.

1.2.3 *In vitro* isolation and expansion of nTreg

Natural Treg are hypo-proliferative *in vitro* while Tconv are not, thus expansion of pure nTreg population to large numbers with keeping FoxP3 expression and suppression function is the biggest obstacle for clinical translation. Regarding isolation of pure nTreg, the discovery of CD25 made the isolation feasible and the involvement of CD127 enhanced the purity significantly. This is due to CD127 expression is inversely correlates with FoxP3 expression and Treg suppressive capacity. Therefore, sorting of $CD4^{+}CD25^{hi}CD127^{lo}$ cells becomes another isolation strategy for Treg (95). Except from the commonly used isolation methods like activated cell separation (MACS) and Fluorescence Activated Cell Sorting (FACS), IBA GmbH (Goettingen, Germany) has manufactured streptamer® reagents, including anti-CD4-Fab-Streptamer, anti-CD25-Fab-Streptamer and anti-CD45RA-Fab-Streptamer, for isolation of Treg by several labeling and positive selection processes. After each selection, tagged cells are liberated from the magnetically tagged Fab-Streptamers by incubation with D-biotin, which is a competing Streptactin ligand and can cause dissociation of the Fab-Streptamer label from the cell surface. This isolation method provides a new option for Treg isolation (81).

Expansion of Treg *in vitro* without losing their phenotype and immune suppressive capacity is also essential for successful adoptive Treg therapy due to the low number of circulating Treg in periphery. From our data, only 0.5-1% total peripheral blood mononuclear cells (PBMC) are Treg by expressing the phenotype "$CD4^{+}CD25^{hi}FoxP3^{+}CD127^{lo}$" (Results 4.1.1). Several groups have proved that culturing nTreg *in vitro* with anti-CD3 and anti-CD28 monoclonal antibody-coated beads and high dose of IL-2 in presence of rapamycin can successfully expand them keeping high purity and suppression function (96, 97). However, since FoxP3 expression may get lost after strong and repeated stimulation during expansion (94), new strategy of Treg expansion with moderate stimulation conditions or in more effective cell culture devices are required for the future Treg expansion.

1.3 T cell receptor repertoire of Treg and Tconv

1.3.1 T cell receptor (TCR) repertoire analysis methods

As mentioned in 1.1.3, due to several selection processes, the T cell receptor (TCR) repertoire is highly diverse, 2.5×10^7 for human naïve T cells (98). To analyze such huge and diverse TCR repertoire, the conventional methods are mainly focused on measuring the length of the third complementarity-determining region (CDR3), the most variable region of TCRβ-chain (99). This analysis method includes several molecular and biological techniques. Complimentary DNA (cDNA) is generated from isolated RNA through reverse transcription process. CDR3-encoding mRNA are then amplified by PCR using specific V and C primers with combination of either fluorescent C or J primer or others like radiographic isotope. The product are later separated by electrophoresis and visualized by, for instance, fluorescent sequencing equipment. Bands of different fluorescence intensity pattern are finally observed, indicating total population of CDR3-encoding mRNA sharing same V-gene or V-J gene but with different length (100). Thus, analysis of CDR3 length distribution provides a basic perception of repertoire variations between different sub-populations and over time. It has been widely used in understanding the diversity of TCR repertoire in different cell types and at different infection periods (101-103).

Although we could get basic interpretation of TCR repertoire composition of a cell population by analysis of CDR3 length distribution, the specific clone information at a sequence level is still missing. Therefore, a new and more powerful technology named next generation sequencing (NGS) is generated and applied to analysis TCR repertoire at a sequence level in this century, which provides us the opportunity to simultaneously analyze single-gene disorders (104, 105).

1.3.2 Treg subsets and Tconv TCR repertoire in mice and human

In addition to the essential role in clinical diagnosis and monitoring during infections, TCR repertoire analysis is also important in studying development of distinct cell lineages and differential status of one specific cell type. Regarding T cells development, in accord with positive and negative selection processes in thymus, several groups have showed TCR repertoire of murine nTreg and Tconv are mostly distinct (106-108). Hindley et al. have analyzed TCR repertoire of tumor-infiltrating Tconv and nTreg from mice and found no

overlap between them, indicating nTreg and Tconv TCR repertoire are influenced differently even in the same tumor microenvironment (107); Relland et al. have also found that TCR repertoire of nTreg and Tconv for same foreign antigen are distinct using CDR3 length distribution analysis by TCR α-chain spectratyping in mice (108). Interestingly, our group have found formerly that antigen specific iTreg and Tconv do share same clones for their TCR repertoire, indicating same origins of iTreg as Tconv, but distinct as nTreg (109). However, TCR data from human nTreg and Tconv are very limited (110), and no data has been available so far using next-generation sequencing. Regarding differential relations of several Treg subsets including naïve and memory cells, TCR repertoire data are also missing.

2. AIMS OF THIS WORK

Due to the promising immune tolerance induction by Treg in several animal models (111-115) and the safety confirmation of Treg therapy in human (85), adoptive cellular therapy with Treg to solid organ transplantation patients (SOT patients) is the final goal of this project. However, to achieve this, more knowledge of Treg cell product composition and function is still needed. Recently, the existence of naïve and memory cells in natural Treg population has been shown and naïve Treg showed superior or equivalent capacity regarding *in vitro* expansion and suppression activity to memory Treg (90, 94). However, majority of Treg express the "memory" phenotype, particularly in SOT patients. This raises the question which Treg subset should be used. Thus, to translate Treg "from bench to bedside", it is pivotal to investigate the heterogeneous composition of Treg population and their functionality with differentiation relationship. Therefore, three main topics should be addressed in this study:

i. Study the heterogeneous composition of Treg in healthy donors and Tx patients.
   - Define total Treg population and subset composition from healthy donors based on age and gender
   - Compare subsets composition and correlations in Treg versus Tconv
   - Compare activation patterns of Treg and Tconv subsets upon *in vitro* TCR-stimulation
   - Compare subsets distribution and activation pattern of Treg in renal transplantation recipients with healthy donors

ii. Compare the functional difference of Treg subsets.
   - Compare suppression activity of Treg subsets regarding activation, proliferation and apoptosis induction of responder cells
   - Study the cross talk between Treg subset and other immune cells
   - Impact of expansion on Treg subsets

iii. Analyze the relationship of Treg subsets with Tconv using TCR repertoire analysis.
   - Study the differentiation relationship of Treg subsets
   - Study the relationship of Treg and Tconv by TCR repertoire

These data that are of importance to decide whether we can use total Treg cell preparation, composed of different subset, or enrichment of particular Treg subset (e.g. naïve cells), are required for optimal adoptive Treg therapy in SOT patients. The TCR repertoire data of this study will shed light on differentiation relationship of human nTreg subsets and Tconv.

3. MATERIAL & METHODS

3.1 Materials

3.1.1 Subjects

Blood samples for Treg phenotypic analysis were collected from healthy donors and renal transplant recipients (Tx patients) after getting written informed consent and approval by the Charité University Medicine Berlin ethics committee (Institutional Review Board). For the study of functionality and TCR repertoires, buffy coat samples of healthy adults from the German Red Cross (DRK) were used.

3.1.2 Equipments and devices

Table 1: List of main equipments and devices

Equipment and Device	Company
Flow Cytometer, FACS LSRII	BD Bioscience
FACS (Cell-Sorter), FACSAria	BD
Quadro MACS™ Separation Unit Octo MACS™ Separation Unit MACSiMAG Separator	Miltenyi Biotec
Cell counter CASY®	Innovatis
CO_2 Incubator	Sanyo
G-Rex10 gas permeable culture device	Wilson Wolf Manufacturing Corporation, USA
FACS-Diva Software	BD BioScience
FlowJo	Tree Star
GraphPad Prism	GraphPad Software

3.1.3 Medias, Buffers and Solutions

Table 2: List of Medias, buffers and solutions

Medium or Buffer	**Description**
RMPI medium:	Rosewell Park Memorial Institute Medium (RPMI) 1640 (Gibco BRL, USA), supplemented with 100 U/mL penicillin and 0.1 mg/ml

	streptomycin and either 10% human AB serum (Lonza, USA) or 10% foetal calf serum (FCS, Sigma Aldrich, Germany)
Proleukin medium	X-Vivo 15 Medium, supplemented with 10% human AB Serum (Lonza, USA) and 500 IU/mL Proleukin with or without 100nm rapamycin
PBS/BSA-buffer:	0.5% Bovine serum albumin (BSA Boehringer-Mannheim, Germany) in PBS-buffer
MACS buffer	2 mM ethylene diaminetetraacetic acid (EDTA) in PBS/BSA buffer
FACS buffer	2 mM ethylene diaminetetraacetic acid (EDTA) in PBS/BSA buffer with 0.1% sodium azide
Fix/Permeabilization buffer for intracellular staining	eBioscience
Annexin V binding buffer	Biolegend

3.1.4 Chemicals and Reagents

Table 3: List of chemicals and reagents

Chemical or reagent	**Company**
Brefeldin A (Bref-A), (5mg/ml in 70% ethanol)	Sigma-Aldrich, Germany
Carboxyfluoresceindiacetat (CFDA), 5mM in Dimethylsulfoxid (DMSO)	Molecular Probes, Netherlands
Monensin	BD Bioscience, Germany
EDTA , (2mM in PBS-Buffer)	Merck, Germany
4,6-Diamidin-2-Phenylindol-Dihydrochlorid (DAPI), (1µg/ml in PBS-Buffer)	Roche, Germany
Paraformaldehyde (PFA)	Sigma Aldrich, Schnelldorf
Phorbol 12-Myristat 13-Acetat (PMA)	Sigma Aldrich, Schnelldorf
Ionomycin	Sigma Aldrich, Schnelldorf
Rapamycin	Alexis Biochemicals

3.1.5 Isolation and stimulation Kits

Table 4: List of isolation and stimulation kits

Name of Kit	Company
$CD4^+CD25^+$ regulatory T cell Isolation Kit	MiltenyiBiotec
$CD4^+CD127^{dim/-}$ regulatory T cell Isolation Kit	MiltenyiBiotec
$CD4^+CD45RA^+$ regulatory T cell Isolation Kit	MiltenyiBiotec
T cell Activation/ Expansion kit	MiltenyiBiotec
Treg Expansion Kit	MiltenyiBiotec
Human CD4 micro beads	MiltenyiBiotec
LIVE/DEAD Fixable Aqua dead cell staining kit	Invitrogen
QIAamp DNA Mini Kit	Qiagen, Germany
QIAamp DNA Micro Kit	Qiagen, Germany
Treg suppression inspector	MiltenyiBiotec
Fast Immune Human Regulatory T Cell Function Kit	BD

3.1.6 Antibodies

Table 5: List of used antibodies

Antibody (Clone)	Conjugate	Company
CD14 (MHCD1430)	Pacific Orange	Invitrogen
CD3 (UCHT1)	PE-Cy7	BD BioScience
CD3 (SK7)	Pacific Blue	BD BioScience
CD4 (RPA-T4)	Alexa 700	BD BioScience
CD8 (APC-Cy7)	SK1	BD BioScience
CD25 (M-A251)	PE	BD BioScience
CD127 (eBioRDR5)	APC-eFlour 780	eBioScience
FoxP3 (259D/C7)	Alexa 488	BD BioScience
Mouse IgG1 k (MOPC-21)	Alexa 488	BD BioScience
Helios (22F6)	Alexa 647	BioLegend
CD45RA (2H4LDH11LDB9)	ECD	Beckman Coulter, Krefeld
CD45RO (APC)	UCHL1	BD BioScience
CD49d (MZ18-24A9)	APC	MiltenyiBiotech
CD62L (DREG-56)	PerCP-Cy5.5	BioLegend
CD31 (WM59)	PE-Cy7	BioLegend
CD152, CTLA-4 (BNI3)	PE-Cy5	BD BioScience
CD154 (CD40L) (24-31)	Pacific Blue	BioLegend
CD137 (4-1BB)	PE-Cy5	BD BioScience

Annexin V	Alexa 647	Biolegend
IFNγ (4S. B3)	APC Cy7	Biolegend
IL-10 (JES3 19F1)	PE	BD BioScience
IL-17 (BL168)	Pacific Blue	Biolegend
IL-2 (MQ1-17H12)	PerCP-Cy5.5	BD BioScience
IL4 (8D4-8)	PE	BioLegend
TNFα (MAb11)	PE-Cy7	BioLegend
FoxP3 (259D/C7)	Alexa 488	BD BioScience
Mouse IgG1 k (MOPC-21)	Alexa 488	BD BioScience
Helios (22F6)	Alexa 647	BioLegend
CD45RA (2H4LDH11LDB9)	ECD	Beckman Coulter, Krefeld
CD45RO (APC)	UCHL1	BD BioScience
CD49d (MZ18-24A9)	APC	MiltenyiBiotech

3.2 Methods

3.2.1 PBMC isolation

Peripheral blood mononuclear cells (PBMC) were separated from erythrocytes, granulocytes and plasma in whole blood using density gradient centrifugation with Biocoll. First, fresh heparin blood was diluted 1:1 by PBS; diluted blood was added carefully to Biocoll solution with a ratio of 2:1. Centrifugation was performed with 340 × g for 30 minutes at room temperature (RT) without brake. Due to different density of different cells, erythrocytes and granulocytes, PBMC were enriched to the interface between plasma (upper layer with thrombocytes) and Biocoll (Granulocytes and erythrocytes were under Biocoll), which were then transferred carefully into a new tube and washed with 50 ml PBS (340 × g, RT, 30 min). Cell pellets were re-suspended in another 50ml PBS and centrifuged again together with supernatant from the last wash. Lastly, cell pellets of one donor from all tubes were pooled and re-suspend in PBS. Subsequent cell counting was performed with CASY® cell counter according to the manual.

PBMC isolation from Buffycoat was performed in a shorter procedure. One Buffycoat (around 80-100ml) was put into 4 50ml-tubes evenly and added PBS to a final 35ml in each tube. This diluted blood was transferred subsequently to cover 15 ml Biocoll in a new tube with care. Centrifugation was done at 800 × g for 20 minutes at room temperature (RT)

without brake. Cells in the interface were transferred and washed twice afterwards as described before.

3.2.2 Flow cytometric staining and analysis

Flow cytometric staining contains surface staining for cell surface molecules and intracellular staining for transcription markers and cytokines. For the surface staining, 250 µl whole blood or 10^6 PBMCs were put in a 5ml FACS-tube and washed twice with 1ml PBS/BSA buffer (4°C, 340 × g, 6 min). Discard supernatant and re-suspend cells in 50 µl staining buffer. Fluorescent dyes conjugated antibodies for surface markers were mixed with staining buffer to a final volume of 50 µl per sample, which was added to each tube, mixed, and incubated with cells on ice for 20 minutes away from light. Wash the cells with 1 ml FACS buffer (4 °C, 340 × g, 6 min), discard supernatant and re-suspend cells in around 100 µl buffer, which were either analysed on flow cytometry (LSR II) immediately or fixed and permeabilized for intracellular staining.

Intracellular staining was done mainly by the Foxp3 staining protocol from eBioscience. Briefly, after surface staining, cells were fixed with 1 ml freshly prepared Fix/Perm buffer (Dilute Fix/Perm concentrate to 1:4 with Fix/Perm dilution buffer), vertex and incubate at room temperature for 30 min away from light. Cells were washed once with FACS buffer first, then twice with permeabilizaiton buffer (Dilute 10× concentrate to 1:10 with water). Prepare 50 µl antibodies mixture to each sample for intracellular staining with diluted permeabilizaiton buffer. Add 50 µl antibodies mixture to each tube and make a final 100 µl volume for staining at room temperature for 30-60 min away from light. Cells were subsequently washed with 1 ml diluted permeabilizaiton buffer and re-suspended in 100 µl buffer. Flow cytometric analysis was performed afterwards on LSR II with FACS DIVA software.

Due to potential influence of Ficoll separation, we compared the staining with the fresh whole blood and the Ficoll-isolated PBMC for Treg phenotype, gating strategy is shown in Figure 4. Firstly, dead cells and monocytes were excluded by Live/Dead aqua and CD14 together in a dump channel. Secondly, we gated roughly on lymphocyte population by forward scatter (FSC) and side scatter (SSC), doublets were excluded further by FSC-A and FSC-H. T cells were gated as $CD3^+$ cells and CD4 T cells as $CD3^+CD4^+$ cells. To obtain pure Treg population, we used CD127 for further gating and defined Treg as

$CD4^+CD25^{hi}FoxP3^+CD127^{lo}$ cells, $CD127^{lo}$ cells on total live PBMC was used as a template for gating. Staining between whole blood and PBMC were comparable; however, for intracellular FoxP3 staining, the positive population was clearer from PBMC staining compared to whole blood (Figure 4)

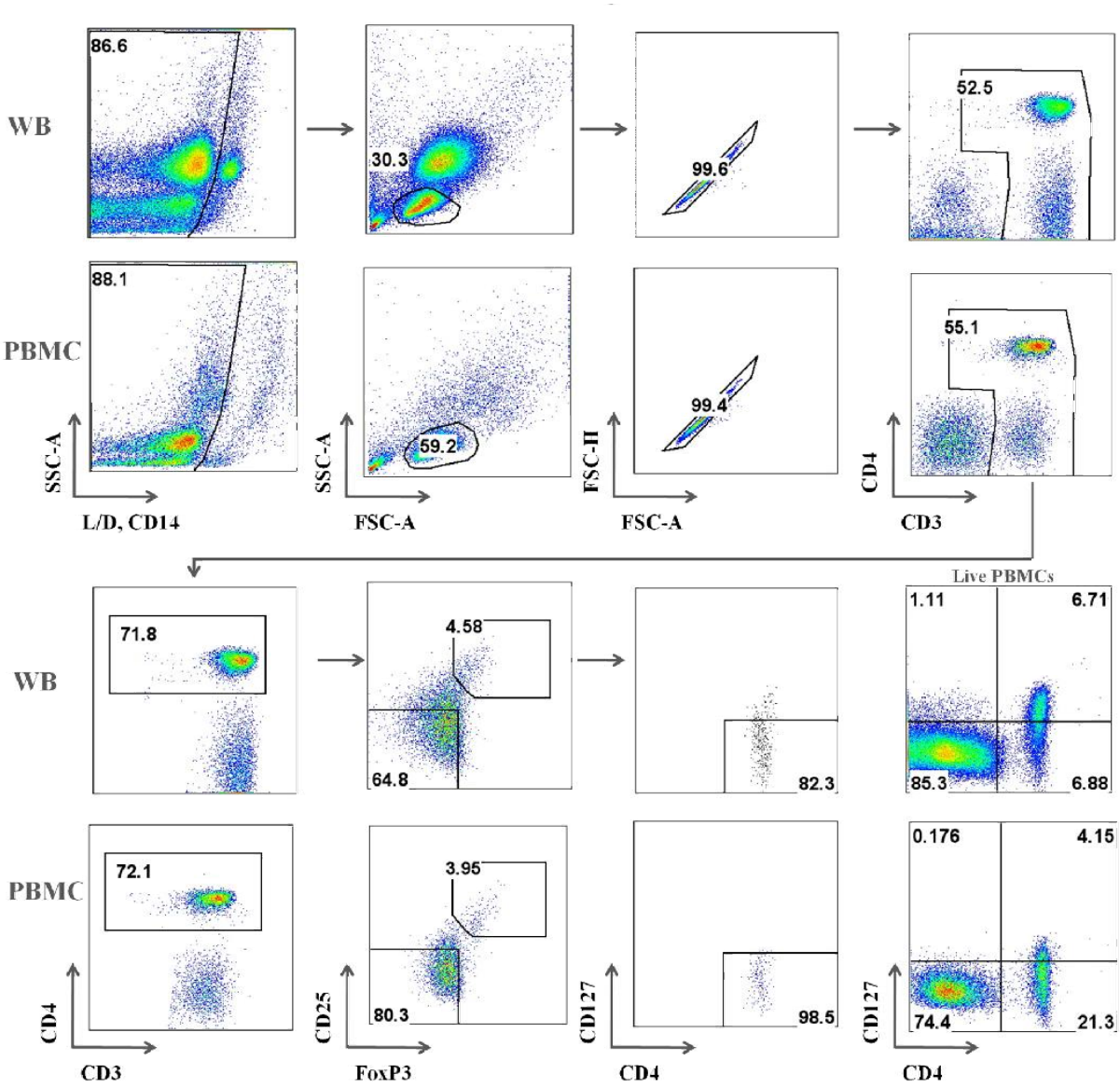

Figure 4: Phenotypic staining of total Treg in fresh whole blood and PBMC. Stepwise phenotypic gating strategy was showed in fresh whole blood and PBMC from one representative donor.

3.2.2.1 FACS panel for Treg phenotypes

Table 6: Antibodies mixture for one sample

Panel 1: Surface staining AB	**[µl]**	**Panel 2: Surface staining AB**	**[µl]**
L/D (PO)	0.5	CD14 (PO)	5
CD3 (PECy7)	4	L/D (PO)	0.5
CD4 (Alexa 700)	2	CD3 (PB)	4
CD25 (PE)	5	CD4 (Alexa 700)	2
CD127 (APC-Alexa 780)	2	CD25 (PE)	5
CD49d (APC)	2	CD127 (APC-Alexa 780)	3
CD45RA (ECD)	2	CD45RA (ECD)	2
/	/	CD31 (PeCy7)	5
/	/	CD62L (PerCP-Cy5.5)	1
Intracellular staining AB	**[µl]**	**Intracellular staining AB**	**[µl]**
Foxp3 (A488)	10	Foxp3 (A488)	10
CTLA-4 (PE-Cy5)	3	Helios (A647)	5

3.2.2.2 FACS panel for Treg activation

Cells were stimulated with CD2/3/28 T cell activation beads with a ratio of 1 cell to 1 bead for 24 hours. After initiating the stimulation for 2 hours, 3µg/ml Brefeldin A was added.

Table 7: Antibodies for Treg activation panel

Surface staining AB	**[µl]**
L/D (PO)	0.5
CD3 (PECy7)	4
CD4 (Alexa 700)	2
CD25 (PE)	5
CD127 (APC-Alexa 780)	2
CD49d (APC)	2
CD45RA (ECD)	2
Intracellular staining AB	**[µl]**
Foxp3 (A488)	10
CD137 (PE-Cy5)	5
CD154 (PB)	1

3.2.2.3 FACS panel for cytokines

Cells were stimulated with 50ng/ml PMA and 600ng/ml Ionomycin for 6 hours, the last 4 hours were accompanied by 2µM Monensin and 7.5µg/ml Brefeldin A.

Table 8: Antibodies for cytokines

Surface staining AB	**[µl]**
L/D (PO)	0.5
CD4 (ECD)	3
Intracellular staining AB	**[µl]**
Foxp3 (A488)	10
Helios (A647)	5
IL2 (PerCP-Cy5.5)	4
IL10 (PE)	10
IL17 (PB)	2
IFNg (Alexa 700)	1
TNFa (Pe-Cy7)	0.5
IL2 (PerCP-Cy5.5)	4

3.2.3 Fluorescence Activated Cell Sorting (FACS)

PBMC were first sorted for CD4 T cells by positive selection with magnetic activated cell separation (MACS). Briefly, cells were washed with 10 ml MACS buffer twice, discard supernatant, then 200 µl human CD4 microbeads were added to 800 µl cells suspension (1: 5), vortex and incubate in the fridge for 15 minutes, then wash them once. Transfer cells from one buffy coat to 2 pre-rinsed LS columns; wash them for 3 times with 3ml MACS buffer. Lastly, the columns were taken out of magnet and put on new Falcon tubes; each column was immediately eluted with 5 ml MACS buffer. Merge eluate from one donor together and centrifuge the cells, i.e., $CD4^+$ fraction.

10^8 MACS sorted $CD4^+$ T cells were stained with 15µl CD4 (Alexa 700), 50µl CD25 (PE), 50µl CD45RA (FITC) and 10µl CD62L (PerCP_Cy5.5) in a final 1ml buffer for 20 minutes in the fridge. Cells were washed and filtered. DAPI was added to cell suspension before sorting with ratio of 3:100. $CD4^+CD25^-$ fraction was sorted as Tconv; $CD4^+CD25^{hi}CD45RA^+$ fraction was sorted as naïve Treg; memory counterpart as $CD4^+CD25^{hi}CD45RA^-$ cells were further separated into $CD62L^+$ (central memory) and $CD62L^-$ (effector memory) fractions.

3.2.4 FoxP3 demethylation assay

The FoxP3 demethylation assay was performed mainly as described before with minor modification (116, 117). Briefly, Genomic DNA of Treg and Tconv subsets were extracted with QIAamp DNA Blood Mini Kit (Qiagen, Germany) and performed for bisulfate conversion subsequently according to the manual (EpiTect, Qiagen). Generally, 60ng bisulfate-treated DNA were used for real-time PCR in a final volume of 20 µl containing FastStart Universal Probe Master, Lamda DNA, methylation or non-methylation-specific probe and primers from Epiontis Company. When more bisulfate-treated DNA (maximal 240ng) was added, the final volume of reaction was also scaled up with the same concentration of other reagents. Final proportion of demethylated FoxP3 locus of TSDR was calculated by dividing the demethylated copy number by the total genomic FoxP3 copy number.

3.2.5 Treg functional assay

3.2.5.1 Suppression assay of activation

The suppression assay was mainly performed as published previously with minor modifications (118). Briefly, 10^5 autologous PBMC were used as responder cells; they were cultured either alone or with 2.5×10^4 or 10^5 Treg, final volume in each well was adjusted to 200µl with RMPI medium. CD2/3/28 beads were added into each well with a ratio of 1 cell to 1 bead. Lastly, add 3µl CD154APC to each well to stain transiently expressed CD154 on cell surface. The whole 96-well plate was centrifuged shortly at room temperature with 500 ×g for 1 minute to spin down the cells. Subsequent activation was done in CO_2 Incubator for 6 hours away from light. Samples without stimulation or with Treg alone were used as controls. 2 repetitions were performed for every condition.

After activation, the cells were washed and stained with 0.5µl Live/Dead Aqua, 8µl CD3 PerCP-Cy5.5/ CD4 FITC/ CD25 PE, 2µl CD69 PE Cy7, 1µl CD8 APC_Cy7 and 1µl CD45RA ECD to a final 50µl volume (20minutes in the fridge, dark). Then the cells were washed and analyzed on LSR II. The gating strategy is showed in Figure 5. Suppression of CD69 or CD154 expression was calculated as (A-B) / A, where A is the mean frequency of positive cells in the sample with responder cells alone and B is the mean frequency of positive cells in the sample cultured with different Treg subset.

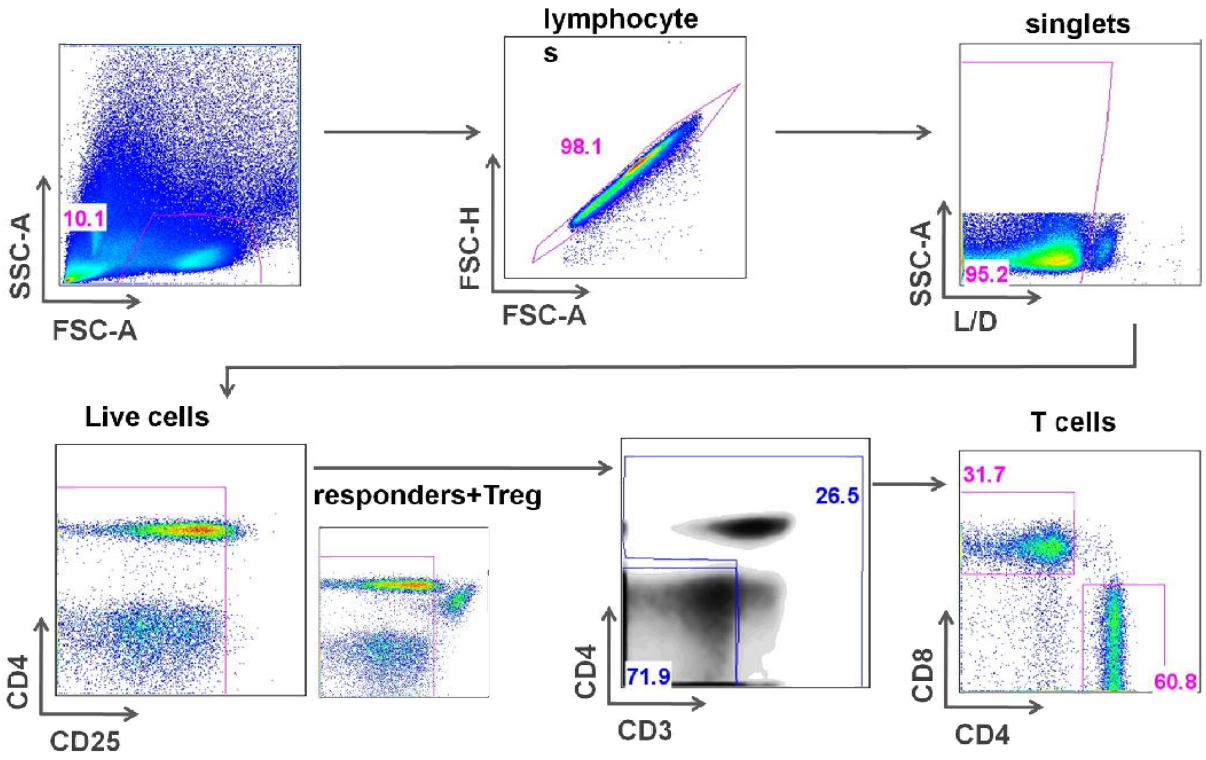

Figure 5: Gating strategy for the suppression assay. Gating strategy is shown from one representative donor with responder cells cultured alone. Treg are excluded from the analysis by gating on $CD25^{lo}$ cells; responder cells co-cultured with TregCM are used to set this gating as shown in the smaller dot plot.

3.2.5.2 Suppression assay of proliferation and apoptosis

Autologous PBMC were used as responder cells and labeled with 2 µM CFDA-SE for 3 minutes, staining was stopped completely by adding 2ml ice-cold FCS for 1 minute. Cells were washed with RMPI medium twice afterwards. The CFSE labeled PBMC were cultured either alone or with Treg at a ratio of 1:1. However, the total number of all cells in each well was 100, 000 and final volume was 200µl with X-vivo medium. 10^5 Treg suppression inspectors were also added to each well for stimulation. Autologous Tconv were cultured with responder cells as well for controls. 2-3 repetitions were performed for every condition. After 3-4 days, the culture is stopped according to the proliferation of the responder cells.

Supernatant of each well was collected and freezed for cytokines detection. Cells were washed with FACS buffer twice, then by Annixin V binding buffer once, discard supernatant and re-suspend cells in Annixin V binding buffer. Stain them with 2µl CD3 PerCP, 1µl CD4 Alexa 700, 1µl CD8 APC_Cy7, 1µl Annexin V APC in a final 50µl for 20 minutes at room

temperature. Add 200µl Annixin V binding buffer to each well to stop the staining, 2µl DAPI was added before analyzing samples on LSR II.

Treg were excluded from the analysis by gating on $CFSE^+$ cells. $DAPI^+Annexin\ V^+$ cells were regarded as dead cells with $DAPI^-Annexin\ V^+$ cells as apoptotic cells. Non-apoptotic cells with $DAPI^-Annexin\ V^-$ populaiton was further analyzed for proliferation by CFDA-SE dilution as shown in Figure 6.

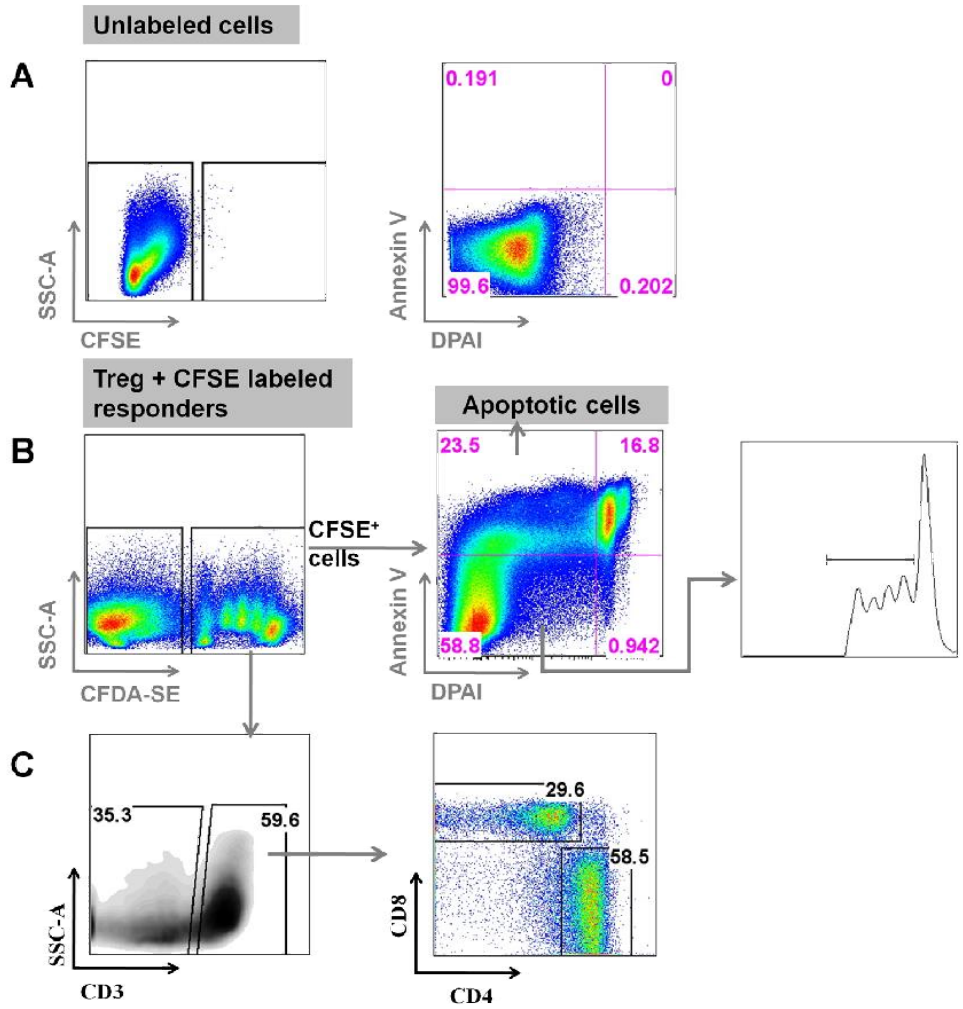

Figure 6: Flow cytometric gating for proliferation and apoptosis assay was showed from a representative donor. (A) Unlabeled cells were used as the control to discriminate Treg from CFDA-SE labeled responder cells. (B) CFDA-SE labeled responder cells were analyzed with DAPI and Annexin V expression. DAPI+Annexin V+ cells were regarded as dead cells and DAPI-Annexin V+ cells were apoptotic cells. Non-apoptotic cells with DAPI-Annexin V- populaiton was further analyzed for proliferation by CFDA-SE dilution. (C) Responder cells were divided into non-T cells by CD3 expression and CD4 and CD8 T cells. All 3 sub-populations undergo same analysis as total PBMC for apoptosis and proliferation.

The percentage of inhibition of proliferation was calculated as (A-B)/A ×100, where A was mean percentage of proliferating cells in responders alone and B was mean percentage of proliferating cells in responders with Treg or Tconv.

Calculation of percentage of apoptosis was done as B'/A' ×100, where A' was mean percentage of apoptotic cells in responders alone and B' was mean percentage of apoptotic cells in responders with Treg or Tconv; i.e. >100 means apoptosis induction.

3.2.6 Treg isolation and expansion

3.2.6.1 Treg isolation

Treg were isolated mainly with the isolation kits from Miltenyi according to the manual by MACS. Taking $CD4^{+}CD127^{dim/-}$ regulatory T cell isolation kit as an example, the procedure is as following: non-CD4 cells and $CD127^{hi}$ cells were deleted by a negative selection with a cocktail antibody of these cells. If starting with 10^{7} cells, cells were mixed with 10µl biotin-antibody-cocktail and incubated for 10 min in fridge, then 30µl MACS buffer and 20µl anti-biotin-microbeads were added and incubated for another 15 minutes in fridge. Cells were washed and re-suspended in 500µl buffer, which were transferred to a pre-rinsed LD column and washed twice with 1ml MACS buffer. Negative fraction was then labeled with 10µl CD25 microbeads in a final 100µl volume. After incubation for 15 minutes in fridge and washing, cells were transferred onto MS column and washed 3 times with 500µl buffer; positive fraction was flushed out into second pre-rinsed MS column with 1 ml buffer out of the magnet. The second column was put back to magnet again and washed 3 times with 500µl buffer. Final eluate from the second MS column was final fraction containing Treg. At last, centrifuge the eluate and re-suspend cells in X-vivo medium.

3.2.6.2 Treg expansion in plate

Treg expansion was mainly done according to the manufacture of human Treg expansion kit (Miltenyi). Briefly, 10^{5} cells were place into one well of 96-well plate with 100 µl X-vivo medium plus 10% human AB serum, 500 IU Proleukin and 100nm Rapamycin. Cells were stimulated with CD3/28 beads with a ratio of 4 beads to 1 cell. Fresh medium were added every 2-3 days and cells were transferred into 24-well plate at day 14. After 3-wk expansion, beads were removed and cells were resting for 4 days.

Regarding removing the beads, re-suspend cells in buffer at a density of up to 2×10^7 cells per 1 mL and vortex thoroughly; place the tube (1.5ml or 5ml) in the magnetic field of the MACSiMAG Separator for 3-5 min. Retaining the tube in the magnet, carefully remove the supernatant containing the MACSiBead-depleted cells and place in a new tube. If necessary, wash the cells and repeat it once.

3.2.6.3 Treg expansion in G-rex10 device

5×10^5 or 10^6 Treg were placed in 20 ml expansion medium in a G-Rex10 device and stimulated with CD3/28 beads with a ratio of 4 beads to 1 cell. 300IU additional proleukin was added every 10 days. After a 3-wk expansion, beads were removed and cells were resting for 4 days.

3.2.7 DNA extraction and next-generation sequencing for TCR repertoire

Total genomic DNA of TregN, CM, EM and Tconv from 6 healthy donors and TregN, M, TconvN, M from additional 4 healthy donors were extracted using QIAamp DNA Blood Mini Kit (QIAGEN). The CDR3 in the TCR-β chain of all these DNA sampls was amplified and sequenced on the ImmunoSEQ platform at Adaptive Biotechnologies using next generation sequencing technology (105, 119).

3.2.8 Computation analysis

The calculation of Shannon entropy (SE) in this study is mainly the same as we described before (30). The SE was normalized, i.e. divided by the natural logarithm (Ln) of the total number of distinct sequences in each sample. Thus, the normalized SE ranges from 0 and 1, where “0” indicates a population dominated by only one clone and “1” indicates the highest diversity, e.g. a cell population with many clones with similar frequencies.

The Moristita-Horn similarity (MH) index is a method to quantify the similarity of two populations such as TCR repertoires (31), it was applied in this research and calculated as described before (32).

3.2.9 Statistical analysis

Statistical tests were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Significance between two groups was determined by paired-t-test if normal distribution is confirmed or Wilcoxon matched pairs test if not. 1-way ANOVA or repeated measures ANOVA analysis with Tukey correction were applied for comparison with more than 3 groups, correlation was determined by Spearman test. Details are noted in each figure legend. Significant difference was defined by the value of probability (p): *P<0.05; **P<0.01; ***P<0.001.

4. RESULTS

4.1 Phenotypic analysis of Treg from healthy donors and Tx patients

To the first aim, 3 FACS panels were established to evaluate the phenotype and activation pattern of Treg. Peripheral blood samples from 60 healthy donors (21 men; 39 women) and 22 renal transplant recipients were collected for this study.

4.1.1 The majority of Treg express central-memory phenotype increasing with age

4.1.1.1 Treg frequency and subsets distribution regarding age and gender

First, we addressed the question whether age or gender has an impact on the distribution of Treg subsets, which might influence the quality of a putative adoptive Treg therapy product. The total Treg ($CD4^+CD25^{hi}FoxP3^+CD127^{lo}$ T cells) frequency in CD4 T cells and the Treg subset composition from 60 healthy donors were analyzed. The gating strategy is shown in Figure 7A. No significant impact of age on the frequency of total Treg in CD4 T cells was found in adults (Figure 7B). There was also no significant difference of the Treg proportion between male and female (Figure 7C). Regarding the heterogeneous composition of Treg in Figure 7A, we performed further analysis on Treg subsets in different age decades. As expected, the proportion of naïve cells ($CD45RA^+CD62L^+$) within the total Treg decreases with increasing age, reaching a plateau at the age period of 40-49 (Figure 7D). Further analysis of TregM subsets reveals that the majority (66.97% on average) of total Treg belongs to the central-memory (CM, $CD45RA^-CD62L^+$) phenotype with a clear impact of age (Figure 7 E), but after the age of 40, the proportion becomes very stable. By contrast, the minority of Treg (12.28% on average) show the effector-memory (EM, $CD45RA^-CD62L^-$) phenotype without significant influence by age in adults (Figure 7F). In addition, Treg of healthy donors contained no terminally differentiated cells (T_{EMRA}) ($CD45RA^+CD62L^-$) at all (Figure 7G). Expression of other important Treg markers are also shown in Figure 7G, e.g., 70.25% of total Treg are $CD49d^-$, 93.60% are $CTLA4^+$ and 78.80% are $Helios^+$.

Compared to Tconv, it is noted that Treg contain significantly less naïve and EM, but more CM pooulation than the respective Tconv (Figure 7H-J). Thus, the majority of Treg exhibit the CM phenotype, circulating mainly between blood and lymphoid organs.

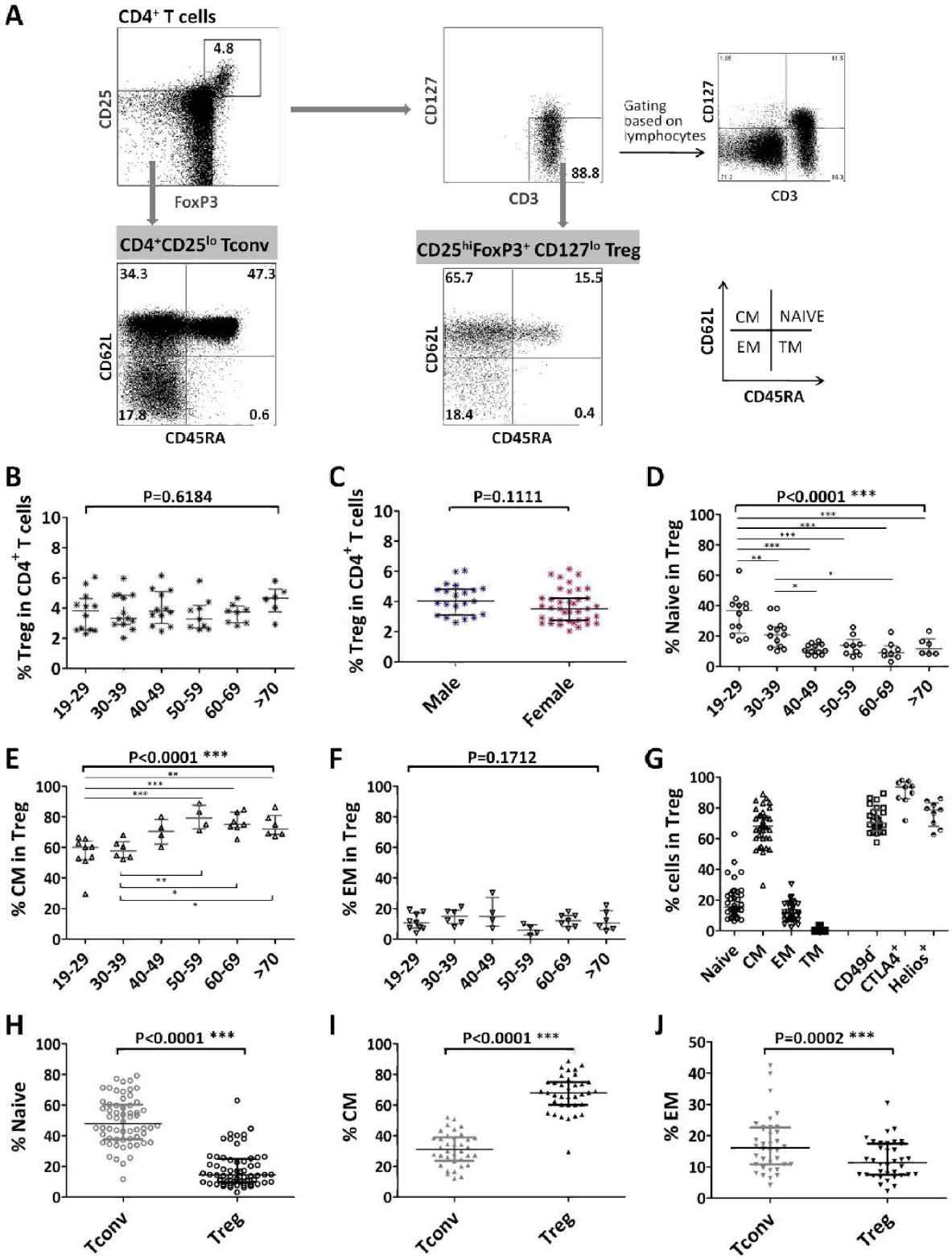
A
CD4+ T cells
4.8
CD25
FoxP3
CD127
88.8
CD3
Gating based on lymphocytes
CD4+CD25lo Tconv
34.3
47.3
17.8
0.6
CD62L
CD45RA
CD25hiFoxP3+ CD127lo Treg
65.7
15.5
18.4
0.4
CM
NAIVE
EM
TM
B
P=0.6184
% Treg in CD4+ T cells
19-29
30-39
40-49
50-59
60-69
>70
C
P=0.1111
Male
Female
D
P<0.0001 ***
% Naive in Treg
E
P<0.0001 ***
% CM in Treg
F
P=0.1712
% EM in Treg
G
% cells in Treg
Naive
CM
EM
TM
CD49d-
CTLA4+
Helios+
H
P<0.0001 ***
% Naive
Tconv
Treg
I
P<0.0001 ***
% CM
J
P=0.0002 ***
% EM

Figure 7: Majority of regulatory T cells express central-memory phenotype increasing with age. (A) Flow cytometric gating scheme for total Treg and subsets: Starting from lymphocytes by FSC and SSC gating, doublets were excluded by FSC-H versus FSC-A gating and dead cells were excluded by live/dead aqua. Cells were further gated on CD3+CD4+, then CD25hiFoxP3+. CD127 was used to define Treg population as CD4+CD25hiFoxP3+CD127lo T cells, gating of this marker was based on total lymphocytes as showed in the small dot plot. Treg and Tconv were further analyzed into subsets. (B, C) There is no significant impact of age and sex on frequency of total Treg in CD4 T cells. (D) Proportion of TregN in total Treg population decreases with age. (E) Majority of Treg express CM phenotype with age. (F) Minority of Treg are EM cells without significant impact from age. (G) Summary of Treg subsets from all donors. (H-J) Treg contain less naive and EM but more CM subsets compared to respective Tconv. Data are from 60 healthy donors from the age of 19 to 87 (21 men; 39 women), distribution of TregCM and TregEM by age is from 36 of total. One-way AVOVA with Tukey correction was used for Figure (B, D, E, F, G); unpaired-t-test was applied for Figure B; Wilcoxon matched pairs test was used for Figure (H-J). (Lei et al. in Preparation)

4.1.1.2 Each Treg subset is weakly positively correlated with Tconv subset regarding the proportion

Compared with the respective Tconv subsets, it was found that the proportion of naïve Treg was positively correlated with that of naive Tconv (Spearman r=0.5627) (Figure 8). CM and EM Treg were also weakly correlated with their respective Tconv counterparts. These results suggest some relation of memory generation between both CD4 populations, Treg and Tconv, as period of antigens exposition or homeostatic expansion over age.

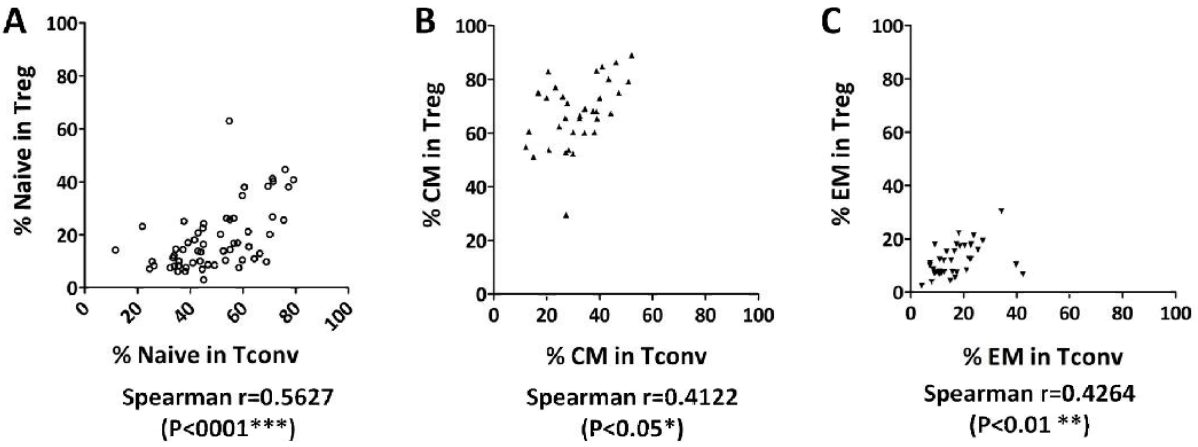

Figure 8: Frequency of NAÏVE, CM and EM cells in Treg were positively correlated with their frequency in donor-matched respective Tconv subsets. (A) Frequency of naïve cells in total Tconv and Treg were showed as values in X-axis and Y-axis separately. (B, C) Frequency of central memory (CM) and effector memory (EM) cells in Tconv and Treg were also compared. Correlation of same cell type in Treg and Tconv was analyzed with Spearman test.

4.1.2 Treg up-regulated only CD137 while Tconv up-regulated both CD137 and CD154 upon TCR stimulation

CD137, also called 4-1BB, is a member of the TNFR super family with co-stimulatory function; 4-1BB stimulation plays an important role in cell survival, proliferation and function in CD4 and CD8 T cells (120). CD154, also named CD40L, is another important activation marker for conventional T cells and its expression can be used to define antigen-reactive T cells (121). T cells activated by CD2/3/28 beads could up-regulate the expression of these two activation markers. Therefore, the expression of CD137 and CD154 were applied to assess the activation status of T cells in the study.

4.1.2.1 Total Treg up-regulated only CD137 expression

Very recently, Schoenbrunn et al. demonstrated a different expression pattern of Treg versus Tconv. Tconv up-regulate CD154 and partly also CD137, while Treg selectively up-regulate CD137 only (122). We have confirmed these data in all our healthy donors. Different activation patterns in Treg and Tconv was showed in Figure 9. Total PBMC were poly-clonally stimulated. Treg and Tconv were further analyzed for expression of CD137 and CD154: i) around 60% of Treg up-regulated CD137 expression with absence of CD154 (Figure 9B, C), which was regarded as pure activated antigen-reactive Treg; ii) almost no $CD154^{+}CD137^{-}$ cells were found in Treg population; iii) <5% of Treg up-regulating both CD137 and CD154 were regarded as “contaminated” cells (Figure 9B, E) because of low expression of Helios and low demethylation status of FoxP3 region. These “contaminated” cells might be induced Treg, as they express the phenotype (109). Activated Tconv, expressed $CD154^{+}$ cells (10% of $CD154^{+}CD137^{-}$ and 40% of $CD154^{+}CD137^{+}$ in Figure 9D, E) and 20% of $CD137^{+}CD154^{-}$ cells (Figure 9C). In summary, Treg, as gated by our strategy (mostly nTreg), do not up-regulate CD154 but CD137 following TCR activation.

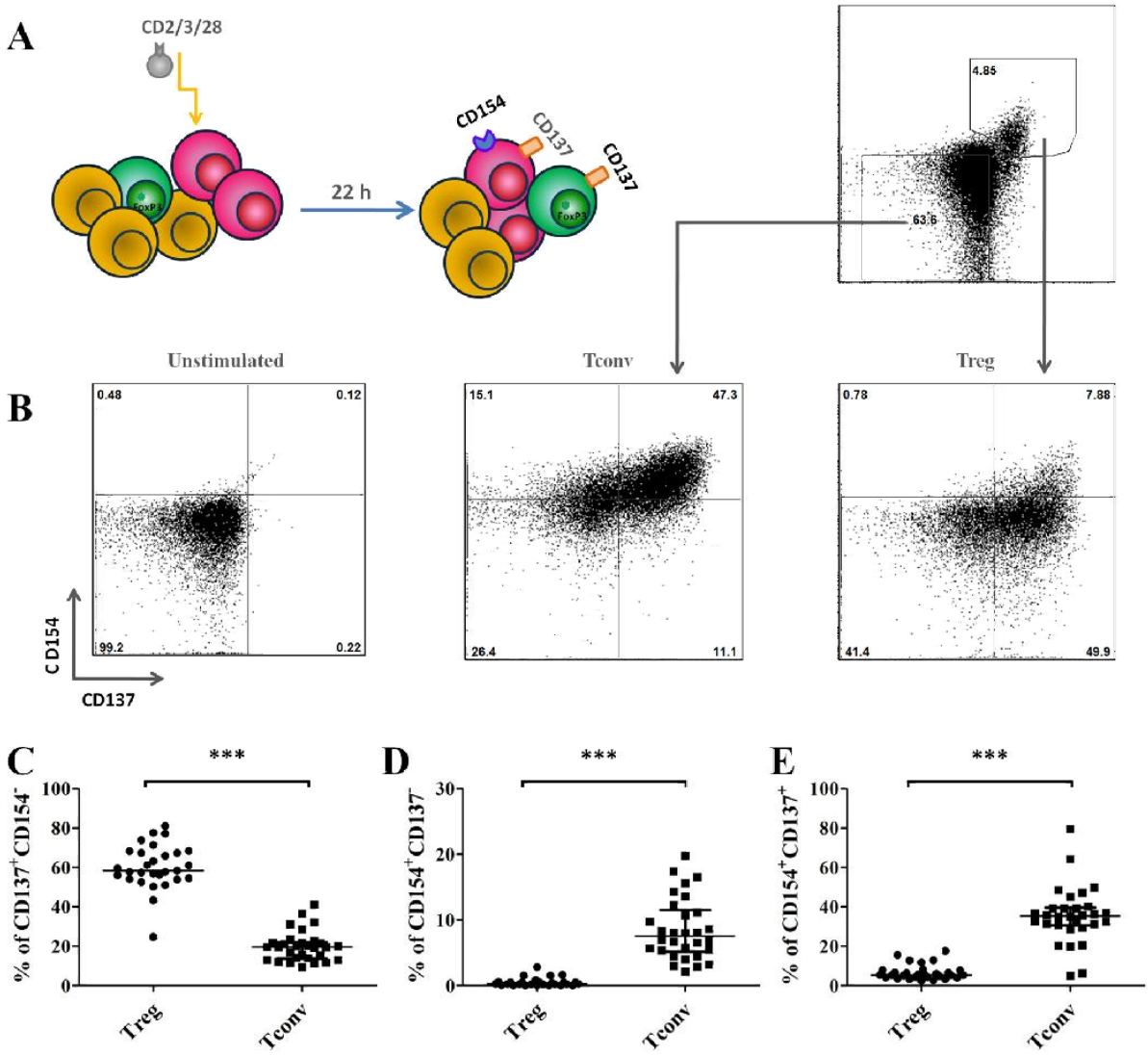

Figure 9: Treg up-regulated only CD137 while Tconv up-regulated both CD137 and CD154 upon TCR stimulation. (A) Schema of activation assay, red cells represent Tconv, green cells with Foxp3 inside represent Treg and orange cells mean all other cell types in PBMC. (B) CD137 and CD154 expression from one representative donor was showed; un-stimulated cells were used as the control. (C) Significant higher proportion of $CD137^+$ cells was found in Treg than Tconv. (D) Tconv expressed CD154 while Treg did not. (E) Large amount of Tconv were also $CD137^+CD154^+$ cells. (n=30, Wilcoxon matched pairs test.)

4.1.2.2 Memory Treg contained higher $CD137^+$ cells, but less $CD137^+CD154^+$ cells than naive Treg upon TCR stimulation

Due to the functional comparison of Treg subsets that would be performed later, here activation patterns in naïve Treg (Treg N), memory Treg (Treg M) and Tconv subsets (Naive/Memory) were compared in Figure 10. Naïve and memory cells were discriminated by further CD45RA expression. We found slightly but significantly higher expression of

$CD137^+CD154^-$ cells, suggesting higher activation level in memory Treg. Interestingly, the proportion of double positive cells ($CD137^+CD154^+$) was even slightly lower in memory than naïve Treg (Figure 10A, B).

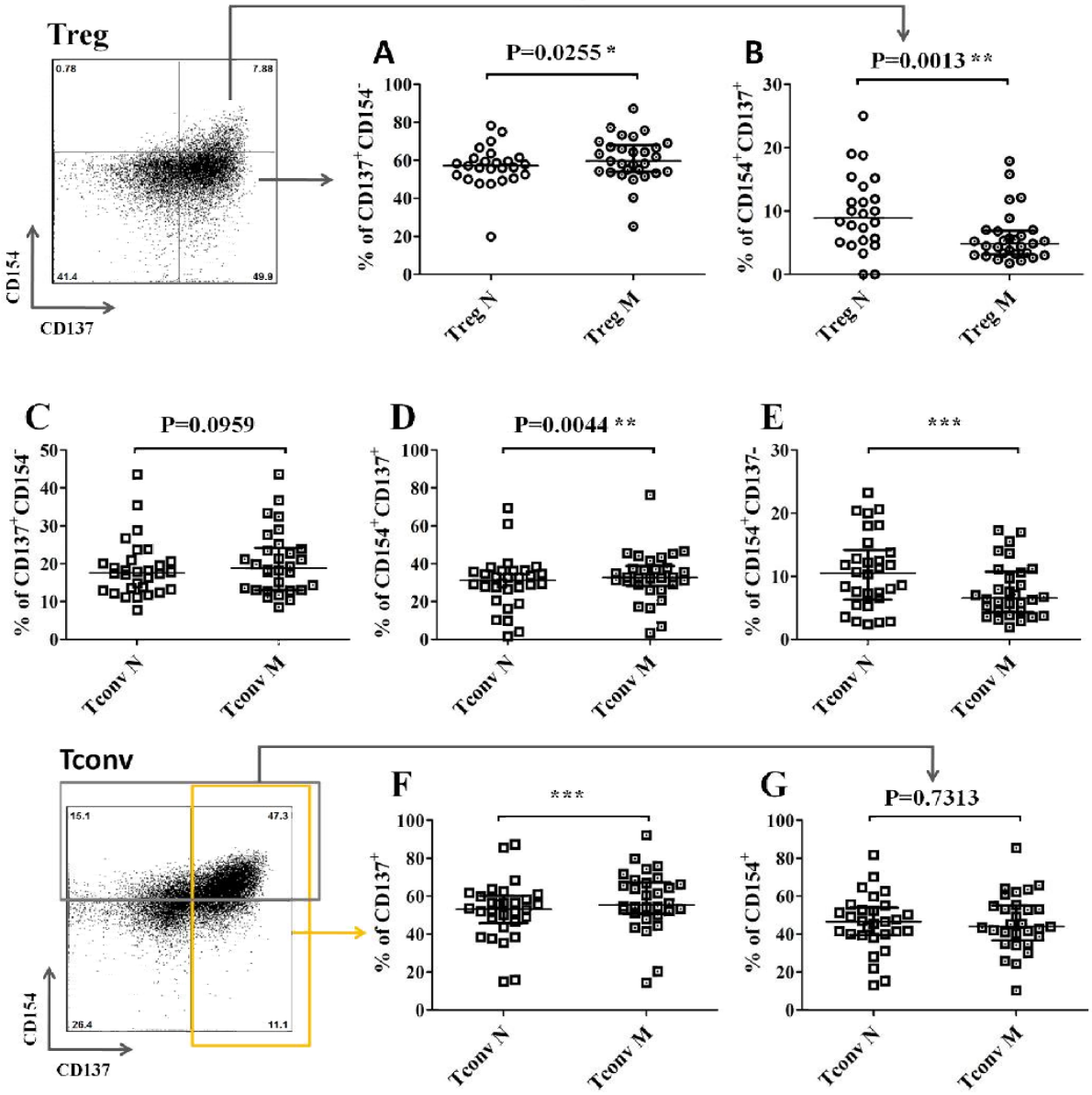

Figure 10: Distinct activation patterns in Treg and Tconv subsets. Treg and Tconv population were further separated into naïve ($CD45RA^+$) and memory ($CD45RA^-$) cells and expression of CD137 and CD154 on the subpopulations were compared. (A, B) Treg M contained slightly higher expression of CD137 while Treg N contained more double positive cells. (C-E) Expression of CD137 and CD154 as single positive and double positive cells on Tconv N/M were compared: Tconv M showed slightly higher fraction of $CD137^+CD154^+$ cells than Tconv N did. (F, G) Tconv M contained more $CD137^+$ cells than Tconv N while no difference was found between them regarding CD154 expression. (n=30, Wilcoxon matched pairs test.) (Lei et al. in Preparation)

Regarding Tconv, majority of activated Tconv were $CD137^+CD154^+$ and significantly higher fraction of double positive cells were found in memory Tconv (mean 37.19%) than naïve Tconv (mean 33.70%) (Figure 10D). As for total $CD137^+$ cells (% $CD137^+CD154^-$ plus % $CD137^+CD154^+$) in both populations, the result was almost the same (Figure 10F). However, lower proportion of $CD154^+CD137^-$ cells were found in memory Tconv than naïve ones (Figure 10E). When only $CD154^+$ cells ($CD154^+CD137^-$ plus $CD154^+CD137^+$) were taken into account, no significant difference was found between Tconv N and Tconv M (Figure 10G).

4.1.2.3 $FoxP3^+Helios^+$ cells may define more pure Treg population regarding CD154 expression

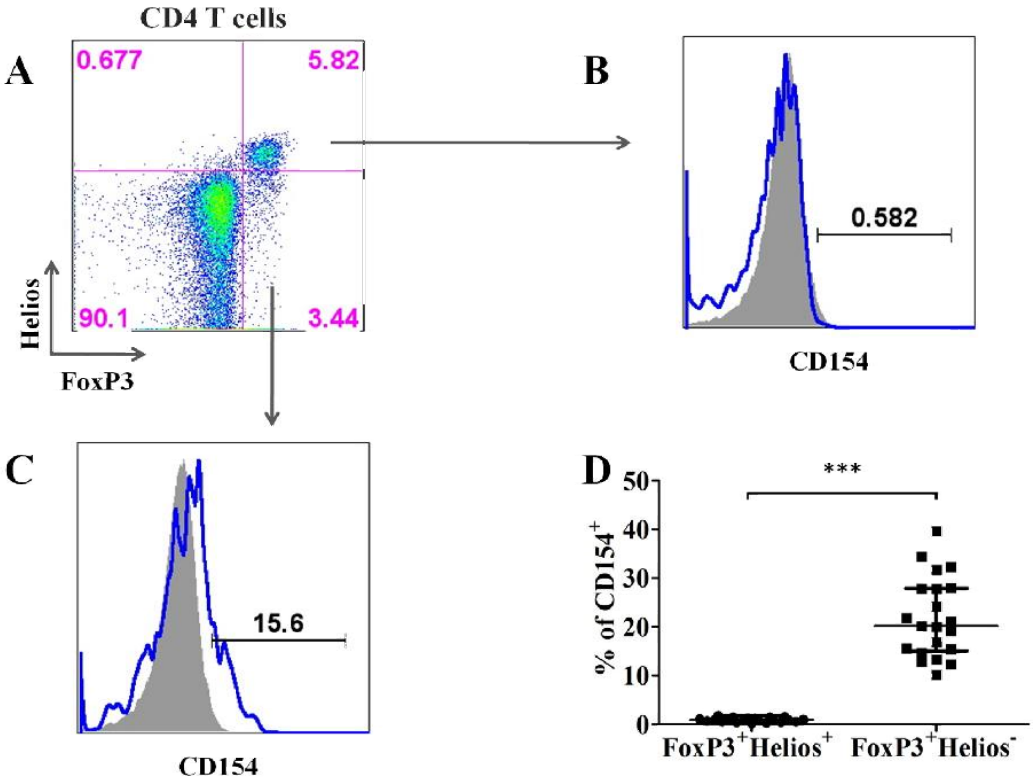

Figure 11: Helios expression is restricted to Treg fraction defined by no CD154 expression. (A-C) Dot plots of CD4 T cells from one representative donor were showed, gray area represents unstimulated sample. (D) $FoxP3^+Helios^-$ cells are able to express CD154 while $FoxP3^+Helios^+$ are not. (n=30, Wilcoxon matched pairs test.)

Helios is an Ikaros transcription factor family member and could up-regulates FoxP3 by binding to the foxp3 promoter (123). Here, when we used CD154 as an exclusion marker for

Treg, almost no CD154^{+} cells were found in FoxP3^{+}Helios^{+} cells while around 15% of cells expressing CD154 were found in FoxP3^{+}Helios^{-} population (Figure 11).

4.1.3 Treg in Tx patients contained more effector-memory cells and were more susceptible to activation-induced cell death

4.1.3.1 Tx patients contained more TregEM than healthy donors with similar age

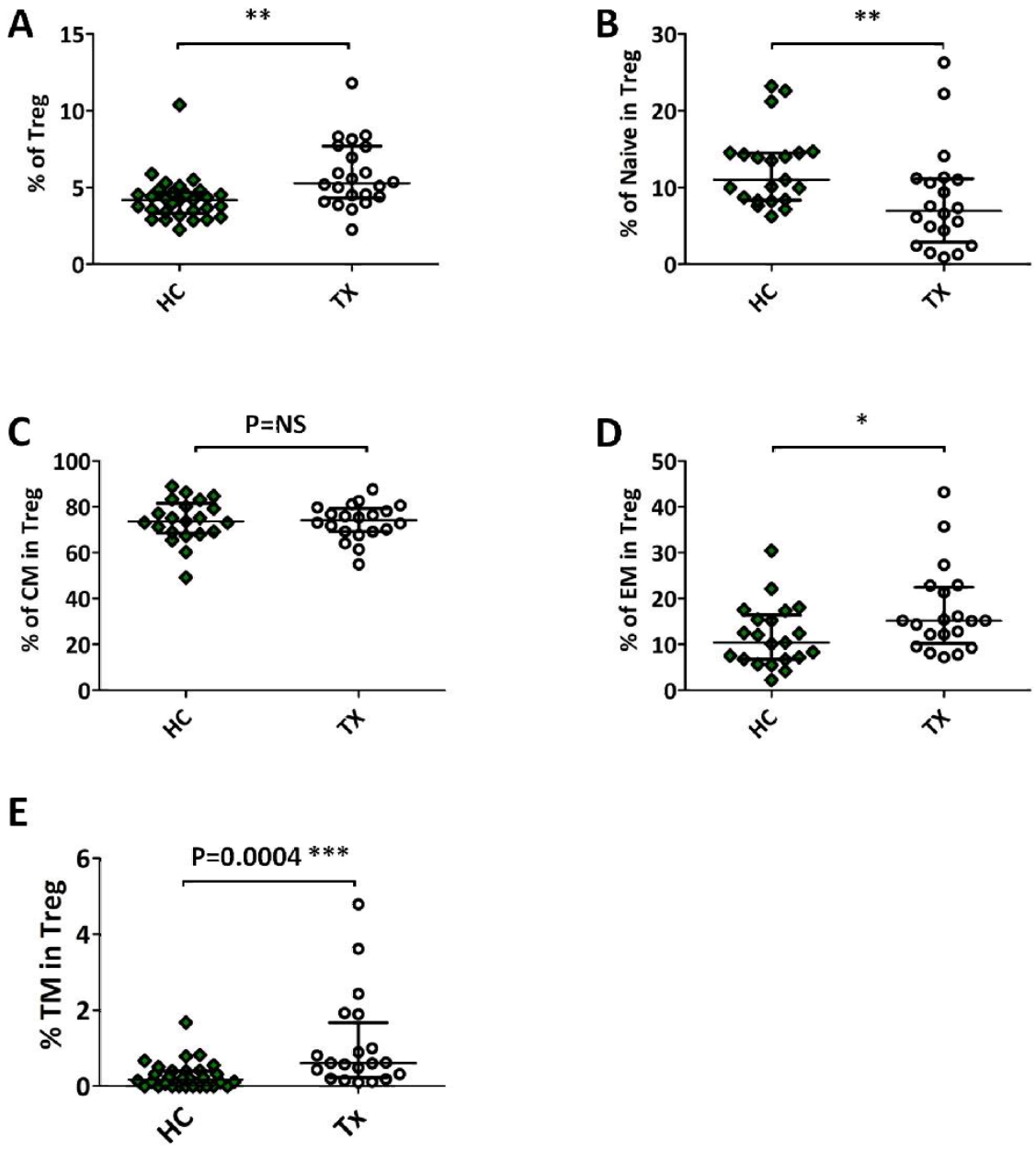

Figure 12: Tx patients contained more effector-memory and T_{EMRA} cells than healthy donors in Treg population with same age period. (A) Increased Treg frequency was found in stable patients above 4 years post

renal transplantation than it in healthy donors (HC). (B) Decreased proportion of naïve cells in total Treg in Tx patients than HC donors with same age period (> 40 years). (C) Similar frequency of central memory cells was found in these Tx patients and HC donors. (D) Tx patients contained increased number of effector memory cells than HC donors. (E) Higher frequency of T_{EMRA} ($CD45RA^+CD62L^-$) cells was found in some Tx patients. All HC donors and Tx patients compared here are above 40 years old. (N_HC donors=21， N_T_X patients=20, Mann Whitney test.) (Lei et al. in Preparation)

Due to the important role of Treg in immune tolerance, Treg frequency and phenotype in Tx patients was also compared with that in healthy donors. In Tx patients post renal transplantation more than 4 years, increased proportion of Treg has been observed. As the proportion of both naïve and memory cells in Treg were quite stable in healthy donors older than 40 years (Figure 7D, E, F), only probands above 40 years old were selected from both groups to make it comparable. Significantly less naïve Treg was found in Tx patients compared to healthy ones (Figure 12B); on the other hand, more TregEM were observed in Tx patients (Figure 12D) although there was no significant difference regarding TregCM proportion (Figure 12C). Interestingly, Small proportion (1.1%) of T_{EMRA} ($CD45RA^+CD62L^-$) cells were found from Tx patients while not at all was observed from healthy donors (Figure 12E).

4.1.3.2 Treg in Tx patients were pre-activated in periphery while Tconv were not without *in vitro* stimulation

Activation pattern of Treg and Tconv from Tx patients were analyzed the same way as we did for healthy donors. Interestingly, some pre-activation pattern was observed in Treg (Figure 13A) from Tx patients, rather than in Tconv (Figure 13B) without any TCR stimulation. In un-stimulated samples from Tx patients, >20% of $CD137^+CD154^-$ Treg were observed while almost no positive Treg were found in healthy donors, indicating some pre-activation in Tx patients, e.g. by allo-antigens exposure. However, we didn't see this pre-activation in Tconv from the same patients (Figure 13C). Regarding the specific Tconv marker (CD154) expression, we did see up-regulation of CD154 expression in Tconv upon *in vitro* stimulation (Figure 13D). CD137 expression in Tconv was also up-regulated in Tconv after stimulation (Figure 13C), suggesting that our *in vitro* stimulation and FACS staining themselves worked well. This early activation in Treg rather than Tconv may relate to the active suppression to allo-reactivity of Treg in these patients more than 4 years post renal transplantation.

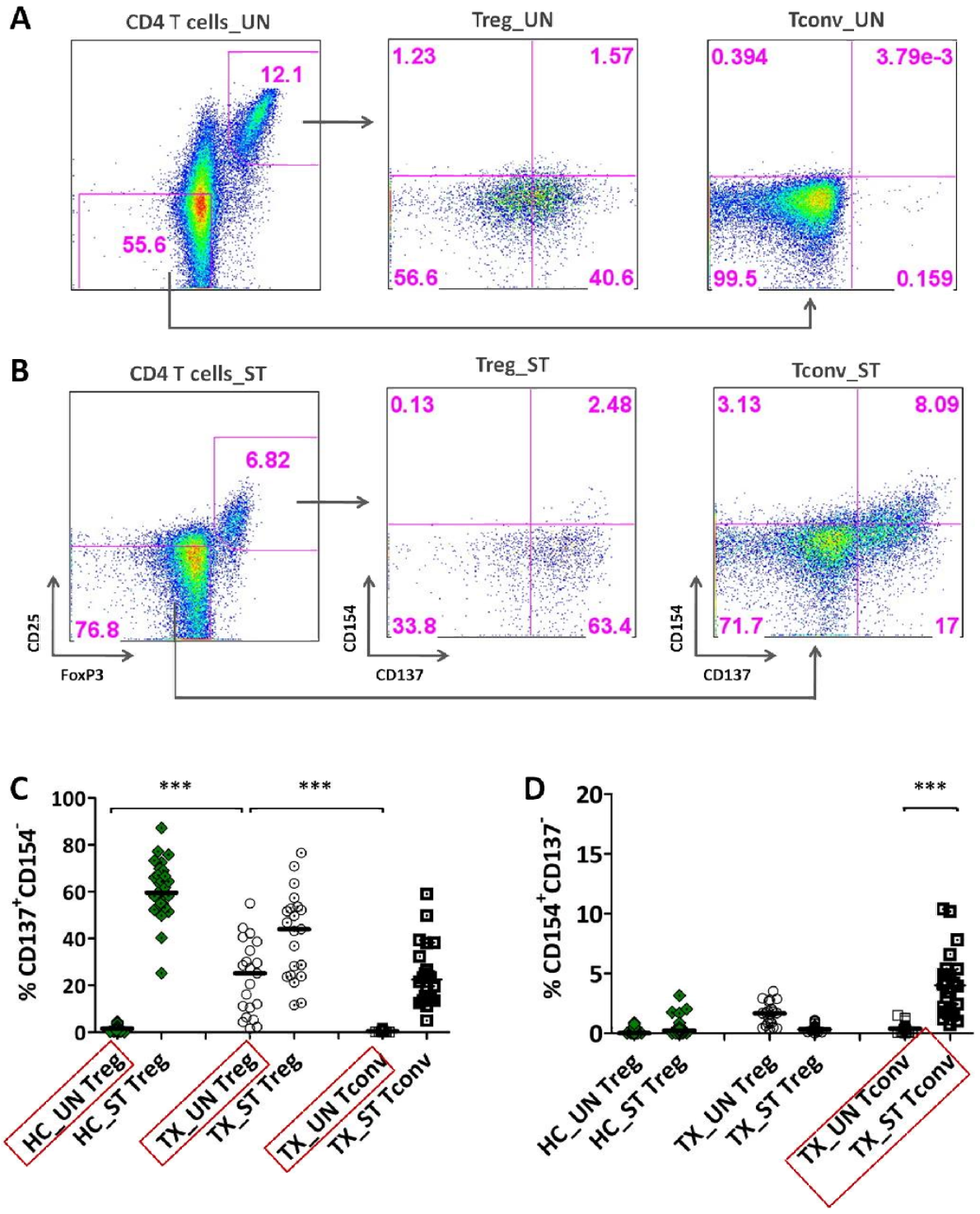

Figure 13: Peripheral Treg in Tx patients rather than healthy donors were pre-activated. (A) CD137 and CD154 expression in Treg and Tconv in one representative unstimulated Tx patient. (B) CD137 and CD154 expression in same stimulated patient. (C) Tx patients have significantly higher $CD137^{+}CD154^{-}$ cells in Treg population than healthy donors without in-vitro stimulation. (D) Tonv cells in Tx patients up-regulate CD154 expression upon in-vitro stimulation. (N_Healthy=30， N_Tx patients=22, Mann Whitney test was used for "HC_UN Treg" and "Tx_UN Treg"; Wilcoxon matched pairs test was for "Tx_UN Treg" versus "Tx_UN Tconv" and "Tx_UN Tconv" versus "T_X_ST Tconv". "UN" means un-stimulated and "ST" means stimulated.

4.1.3.3 Peripheral Treg in Tx patients are more susceptible to activation-induced cell death

Besides the pre-activation of Treg in Tx patients, total Treg proportion in CD4 T cells was compared before and after *in vitro* stimulation. Surprisingly, a dramatic decrease of Treg proportion was found in Tx patients after TCR stimulation (Figure 14A). However, this phenomenon was not observed before and after same stimulation in healthy donors (Figure 14B). Together with pre-activation and more effector-memory cells in Treg from Tx patients, these data suggest that Treg in Tx patients are more pre-activated *in vivo,* representing a sensitive balance of activation and regulation.

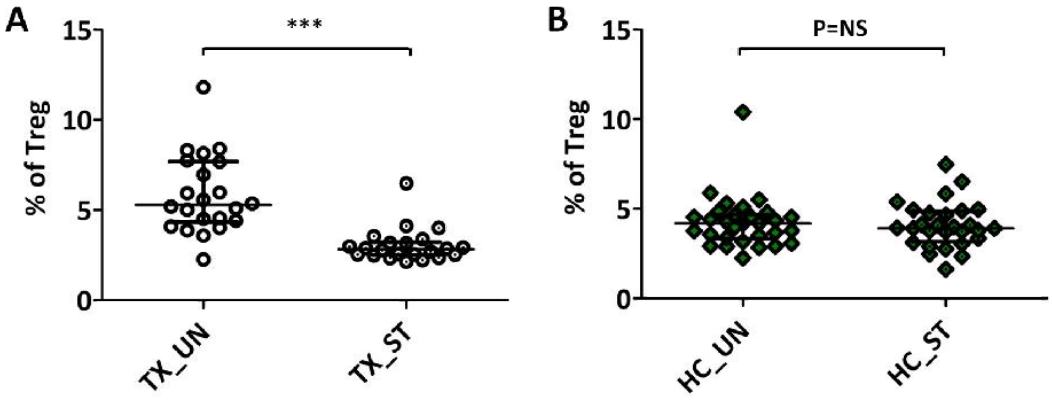

Figure 14: Peripheral Treg in Tx patients are more susceptible to activation-induced cell death. (A) Treg frequency in Tx patients dropped dramatically after in-vitro poly clonal stimulation. (B) Treg frequency in healthy donors did not change significantly before and after same stimulation. (N_HC=30, N_Tx=22, Wilcoxon matched pairs test)

Taken together, we have studied Treg frequency and heterogeneity in both healthy donors and Tx patients. Similar subsets composition of naïve, CM and EM cells as Tconv was found in Treg population, but Treg contained more CM cells than respective Tconv in healthy donors. Based on different age decades, stable frequency of total Treg in $CD4^+$ T cells was found without significant gender and age differences in healthy donors. Proportion of TregN decreased while TregCM increased with age, however, above 40 years old, the frequency change upon age decades was not significant any more.

Treg and Tconv have different activation patterns upon poly-clonal stimulation: Treg showed mainly $CD137^+CD154^-$ pattern while Tconv showed mainly $CD154^+CD137^-$ or $CD154^+CD137^+$ pattern. However, both memory Treg and memory Tconv have more CD137

expression than their naïve counterpart, which may be important for Treg and Tconv memory functionality and survival.

In Tx patients expressing > 4 years stable renal graft function, increased Treg frequency indicated dominate regulation to some extent. May be due to the allo-antigens experience, less naïve cells and more EM cells were observed compared with healthy donors with same age period. Interestingly, a pre-activation status in Treg was also observed only from Tx patients rather than healthy donors, which may be related to active suppression to allo-reactivity of Treg in these Tx patients with stable renal graft function. Furthermore, dramatic decreased Treg frequency upon TCR stimulation may indicate high susceptibility of these cells in Tx patients to activation-induced cell death.

4.2 Functional analysis of Treg subsets: central memory Treg show enhanced suppression efficiency than naïve Treg

The counterpart of Treg, Tconv were also composed of N, CM and EM subsets (124), whose contributions to recall response have been intensively discussed for decades. In CD4 Tconv, T_{CM} proliferate extensively whereas T_{EM} show less proliferation in response to recall antigens and have shorter telomeres (125). In CD8 T cells, human T_{EM} cells also display immediate cytotoxic activity at early memory phase of infection while T_{CM} populations acquire effector function after short-term stimulation and make a markedly greater contribution to recall responses from later memory time points because of their increased proliferative potential (124, 126-131). However, the contribution of the subsets in Treg is still rarely known up to now. Therefore, further studies on the suppressive functionality of the three Treg subsets were conducted in this part.

4.2.1 Freshly isolated TregCM showed enhanced suppression activity to early activation of both CD4 Tconv and CD8 Tconv

4.2.1.1 TregCM contained higher FoxP3 demethylation level than TregN

Treg subsets as Naive, CM and EM were sorted by MACS and FACS as described in the method 3.2.3. Gating for FACS sorting was showed in Figure 15. $CD25^{hi}CD45RA^{+}$ cells were

sorted as naïve Treg; $CD25^{hi}CD45RA^{-}$ cells in P5 were further gated into $CD62L^{+}$ cells as TregCM; $CD62L^{-}$ cells as TregEM.

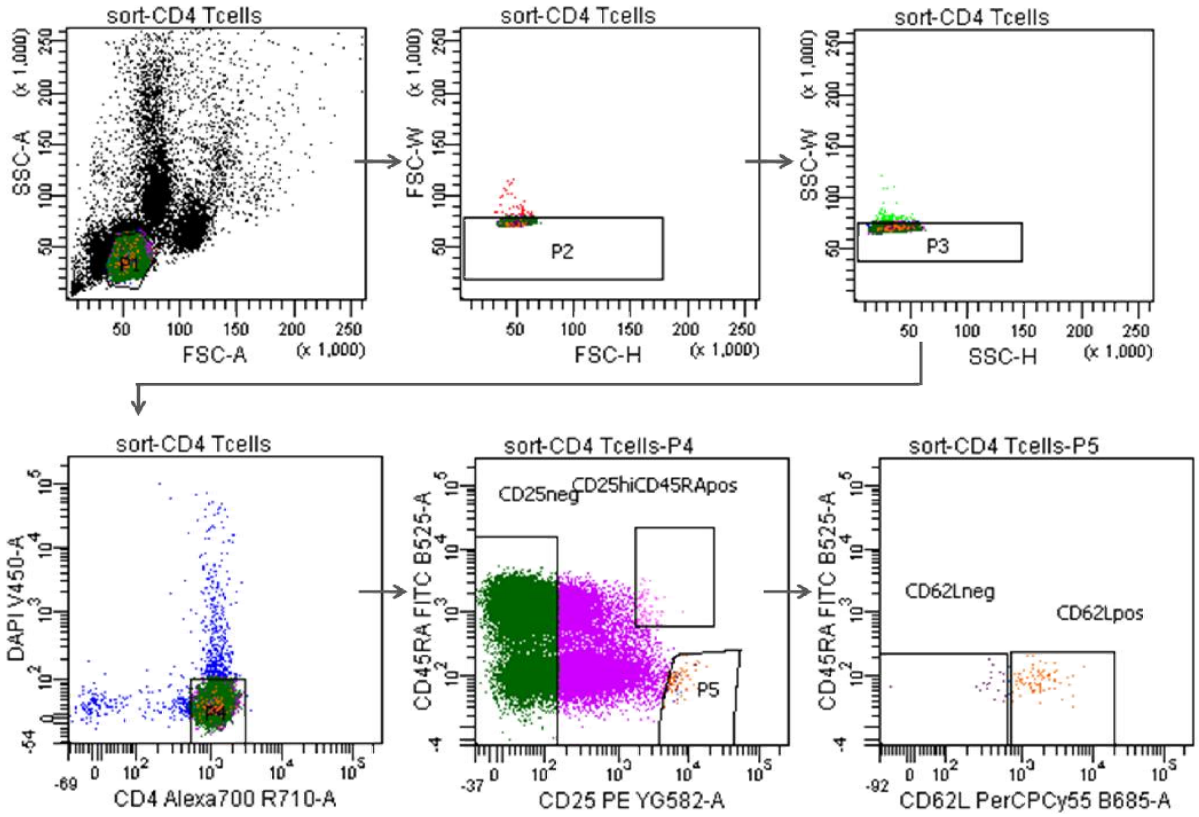

Figure 15: FACS sorting strategy was showed for Treg N, CM and EM subpopulations.

TregN, CM and EM were sorted, the purity of which were controlled by phenotype evaluation with FACS staining and demethylation of FoxP3 locus (TSDR). The TSDR experiment was performed by Katrin Vogt (Institute of Medical Immunology, Charité University Medicine Berlin).

The main phenotype evaluation of the freshly sorted Treg subsets was shown in Figure 16A, B by expressing both FoxP3 and Helios, an Ikaros transcription factor family member that might up-regulate FoxP3 by binding to the FoxP3 promoter (33). From the FoxP3 demethylation of TSDR in Treg subsets (Figure 16C), TregCM revealed a higher FoxP3 demethylation of TSDR than TregN, reaching almost 100% (Figure 16C).

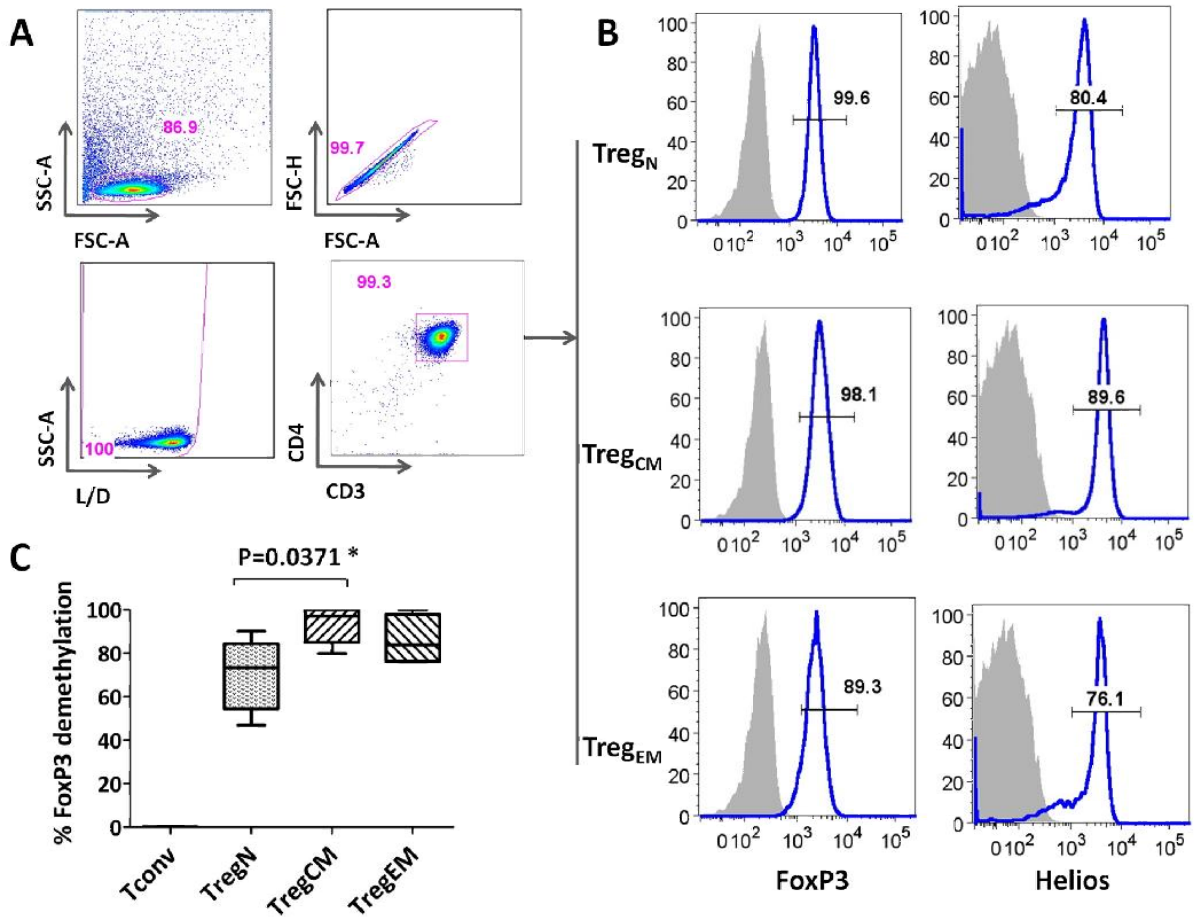

Figure 16: Central-memory Treg contained higher FoxP3 demethylation level of TSDR than naïve Treg. (A) Flow cytometric gating for sorted Treg from a representative donor. (B) > 80% of all $Treg_N$, $Treg_{CM}$ and $Treg_{EM}$ express FoxP3 and Helios. Gray area represents isotype control. (C) Higher FoxP3 demethylation status was observed in TregCM than TregN subset. Results are form 6 donors and data of Treg EM are from 4 of them. Statistics between $Treg_N$ and $Treg_{CM}$ was done with paired t test.

4.2.1.2 TregCM showed enhanced suppression activity to early activation of both CD4 Tconv and CD8 Tconv

Suppression activity of Treg subsets was performed by using suppression of activation markers (CD69, CD154) expression on stimulated Tconv, which could predict the suppressive ability for cells proliferation and cytokines production at 4 days (118). Gating strategy of the whole analysis was described in chapter 3.2.5.1 of the methods part (Figure 5). One representative example of CD69 and CD154 expression on responder cells with or without Treg subset is shown in Figure 17A. Suppression of CD69 expression on CD4 T cells (expression range: 72.2%-89.2%) by TregN, TregCM and TregEM is shown in Figure 17B. Suppression of CD154 expression on CD4 T cells (expression range: 52.10%-78.70%) is

shown in Figure 17C. Interestingly, in this early activation stage, TregCM showed enhanced suppression of CD69 and CD154 expression compared to TregN on CD4 T cells, similar suppression tendency was also observed on CD8 T cells (CD69 expression range in $CD8^+$ T cells: 46.6%-77.6%) (Figure 17D), but no significant difference was found between TregCM and TregEM in all cases. We did not analyze suppression of CD154 on CD8 T cells because the expression was very low even without Treg (< 2%).

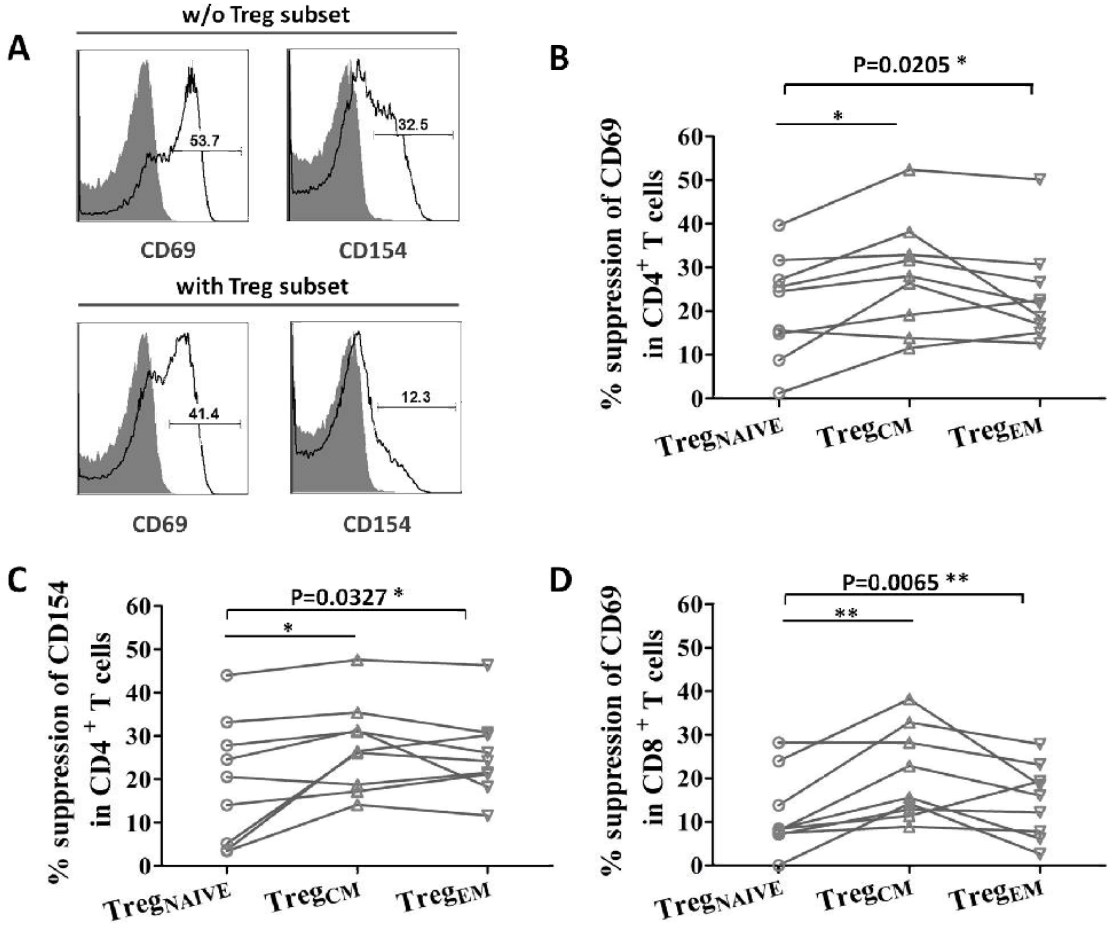

Figure 17: TregCM showed enhanced suppression of early activation in CD4 and CD8 Tconv than TregN. (A) Representative examples of CD69 and CD154 expression on CD4 T cells with or without Treg subset, gray area indicated un-stimulated cells. (B, C) Suppression of CD69 and CD154 expression on CD4 T cells by different Treg subsets. (D) Suppression of CD69 expression on CD8 T cells. Data were from 9 donors with 2-3 repetitions for every subset in each donor. Mean values of repetitions were showed and analyzed for statistics (Repeated measures ANOVA with Tukey correction). (Lei et al. in Preparation)

4.2.1.3 Suppression of Tconv by TregCM was dose-dependent and mainly focused on T cells, rather than CD3$^-$ cells

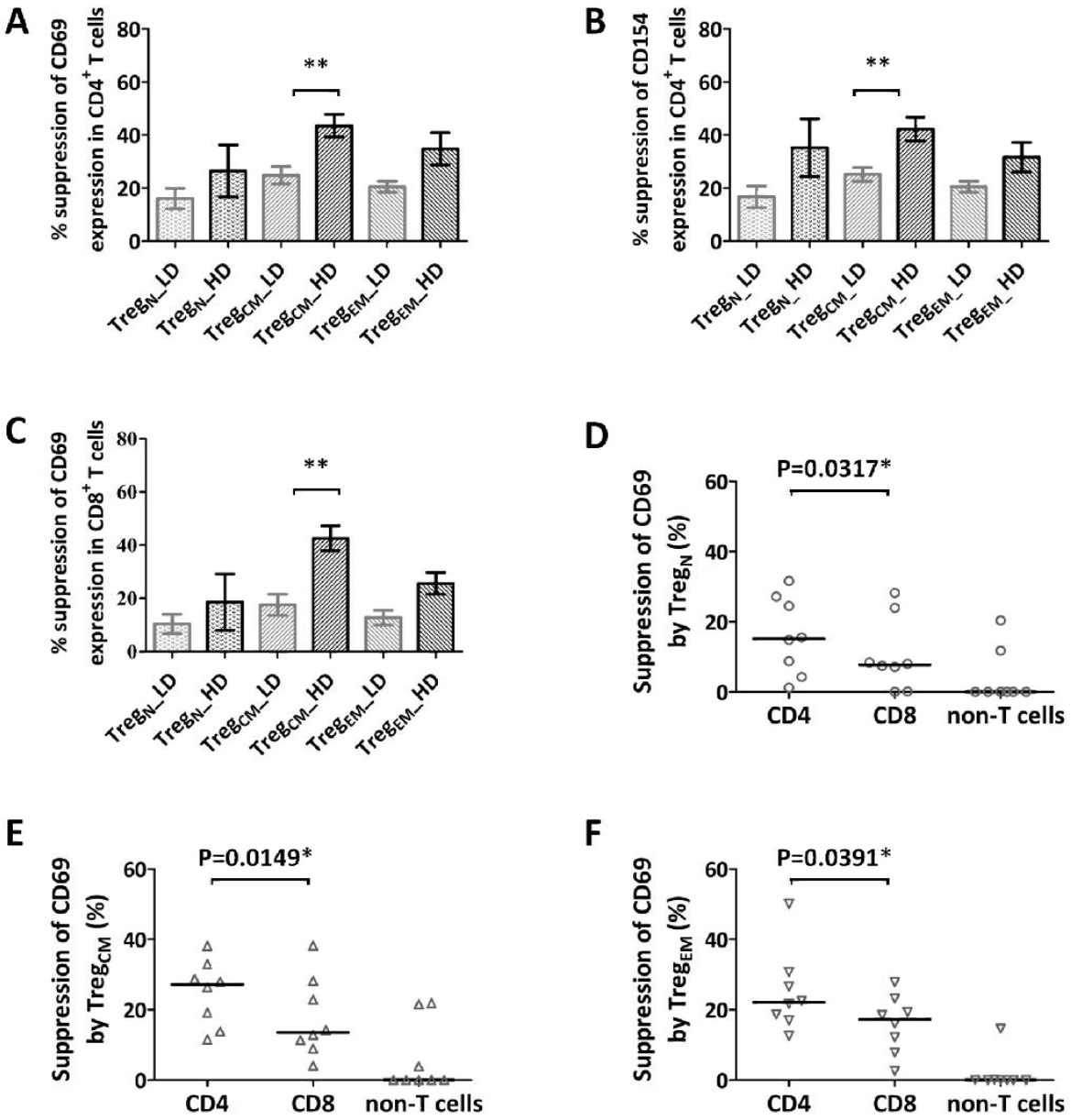

Figure 18: Suppression of early activation by TregCM was dose-dependent and mainly seen on T cells. (A-B) Stronger suppression of CD69 and CD154 expression in CD4 Tconv by high dose (HD) TregCM was observed compared to low dose (LD) Treg. (C) Dose dependent suppression of CD69 by TregCM was also found in CD8 Tconv. (D) Suppression by TregN was stronger in CD4 Tconv compared to respective CD8 Tconv, but not in CD3$^-$ cells. (E, F) Same suppression tendency to T cells was also observed by TregCM and EM. (n=8, Paired t test). (Lei et al. in Preparation)

Dose dependency by Treg subsets to the suppression of early activation in Tconv was observed (Figure 18A-C). Interestingly, slightly higher suppression of early activation was

found in CD4 T cells than in CD8 T cells by all 3 Treg subsets (Figure 18D, E, F). However, no suppression was observed regarding CD69 expression on non-T cells.

Due to similar subsets composition of Treg and Tconv, Tconv are also divided into naïve and memory subpopulation by gating in the analysis. However, all Treg subsets (N, CM, EM) showed comparable suppression to the activation of both naïve and memory Tconv via TCR stimulation (Figure 19).

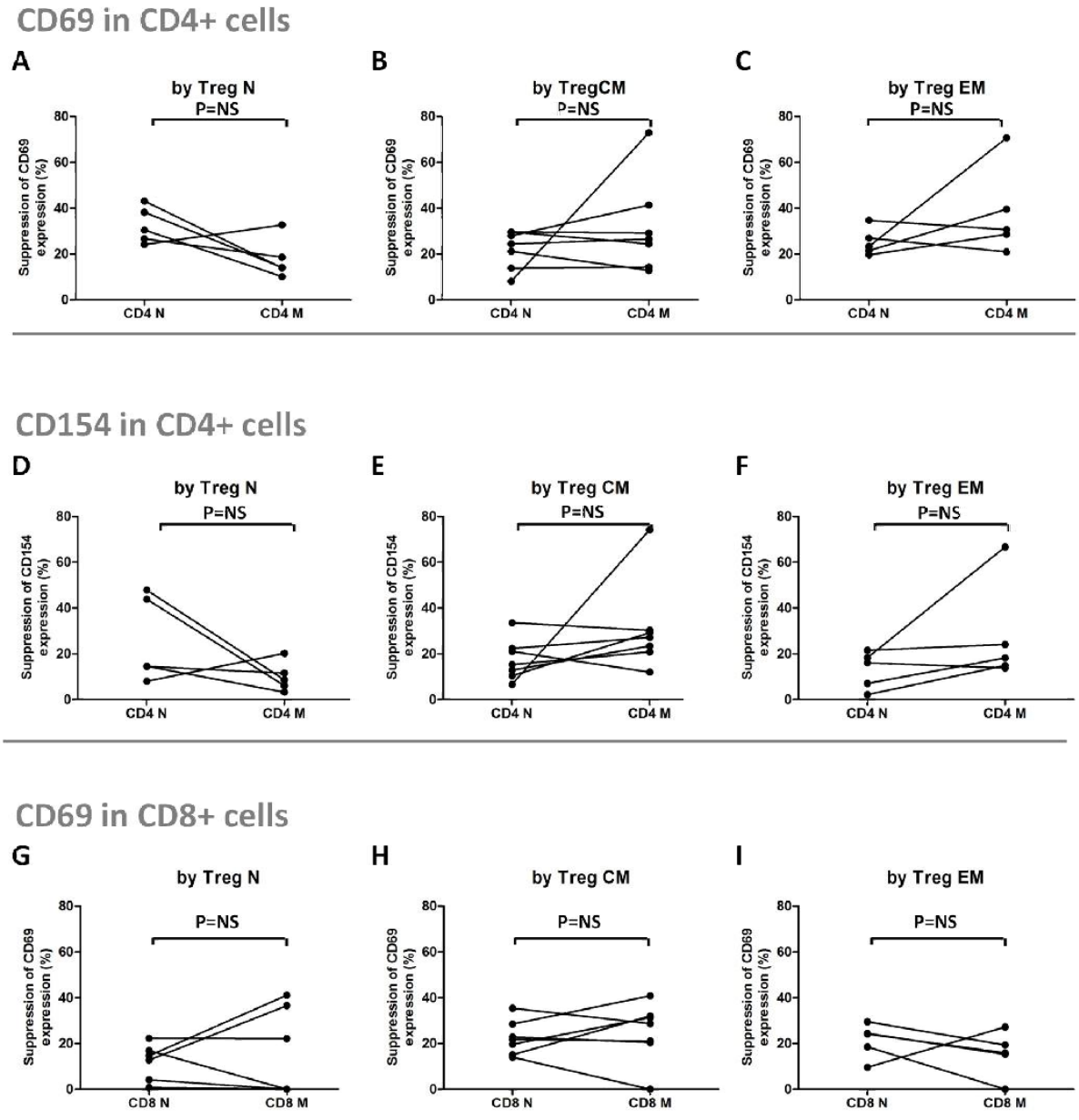

Figure 19: Treg subsets showed comparable suppression to activation of naïve and memory Tconv. (A-C) Suppression of CD69 expression on naïve and memory CD4 Tconv by Treg N, CM, EM were shown separately. (D-F) Comparable suppression of CD154 expression was observed on CD4 Tconv subsets. (G-I) Comparable suppression of CD69 expression was also observed on CD8 Tconv subsets (Wilcoxon matched pairs test).

4.2.2 Treg subsets showed similar suppression activity to both T cells and non-T cells regarding proliferation of non-apoptotic responder cells

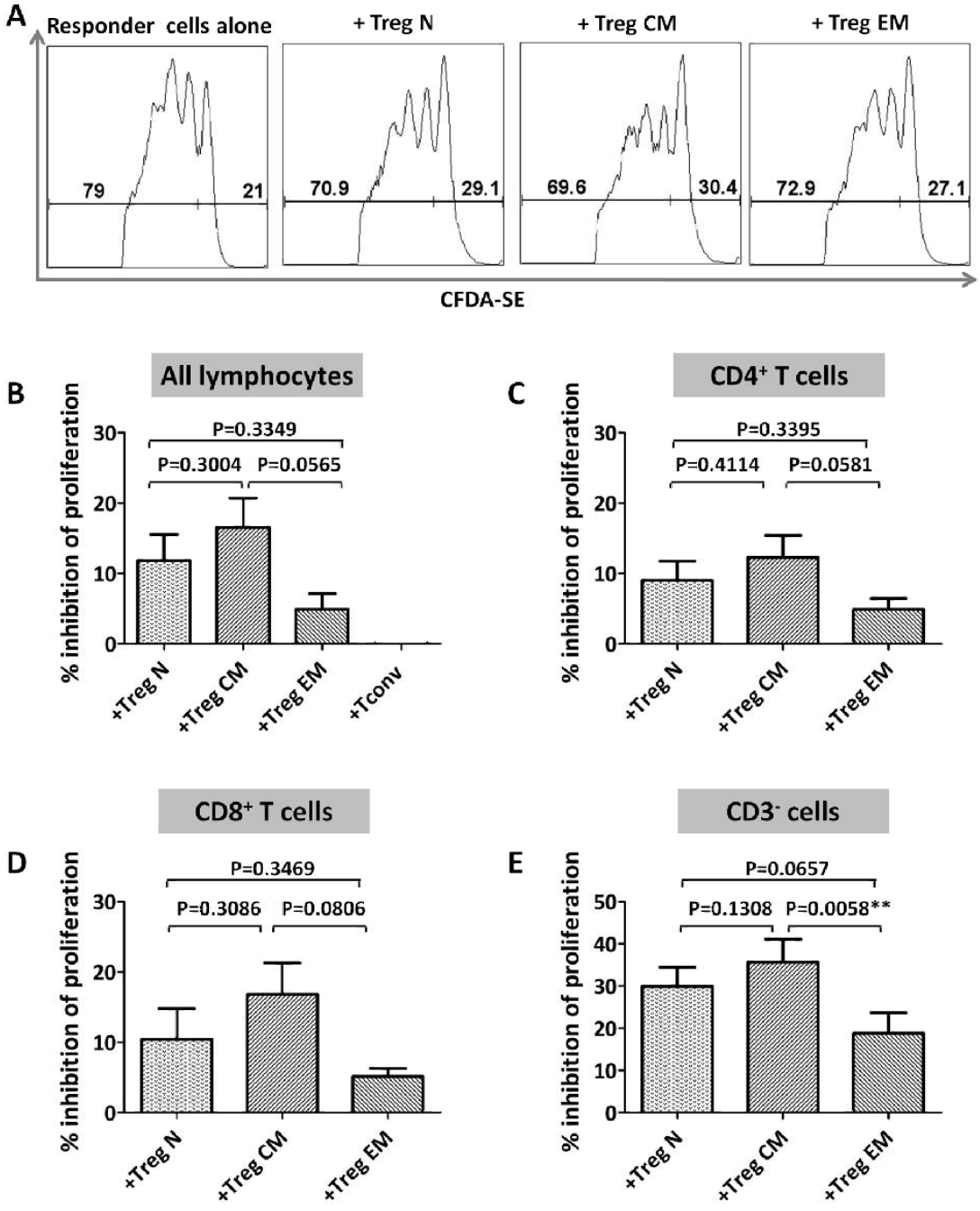

Figure 20: No Significant difference was found among Treg subsets regarding inhibition of the proliferation of non-apoptotic responder cells. (A) Proliferation of responder cells was showed in responder cells alone, or co-cultured with Treg subsets respectively from one representative donor. (B-D) No significant difference was found between any pair of Treg subsets regarding proliferation of CD4 T cells or CD8 T cells. (E) Treg subsets also inhibit proliferation of $CD3^-$ cells and TregCM showed stronger suppression than TregEM. Data were from 6 donors and Treg EM was from 5 of them with 2-3 repetitions for each donor (Paired -t-test).

Proliferation and apoptosis of responder cells co-cultured with Treg were examined as described in chapter 3.2.5.2 of the methods part. Regarding inhibition of the proliferation of non-apoptotic cells, no significant difference was found among TregN, CM, EM (Figure 20A, B). Similar phenomenon was observed in CD4 and CD8 T cells population (Figure 20C, D) as well. In addition, suppression of proliferation was also found here in non-T cells population, which should be mainly B cells and NK cells as lymphocytes were gated first (Figure 20E).

4.2.3 Treg CM induced apoptosis to CD3$^-$ cells while TregN did not.

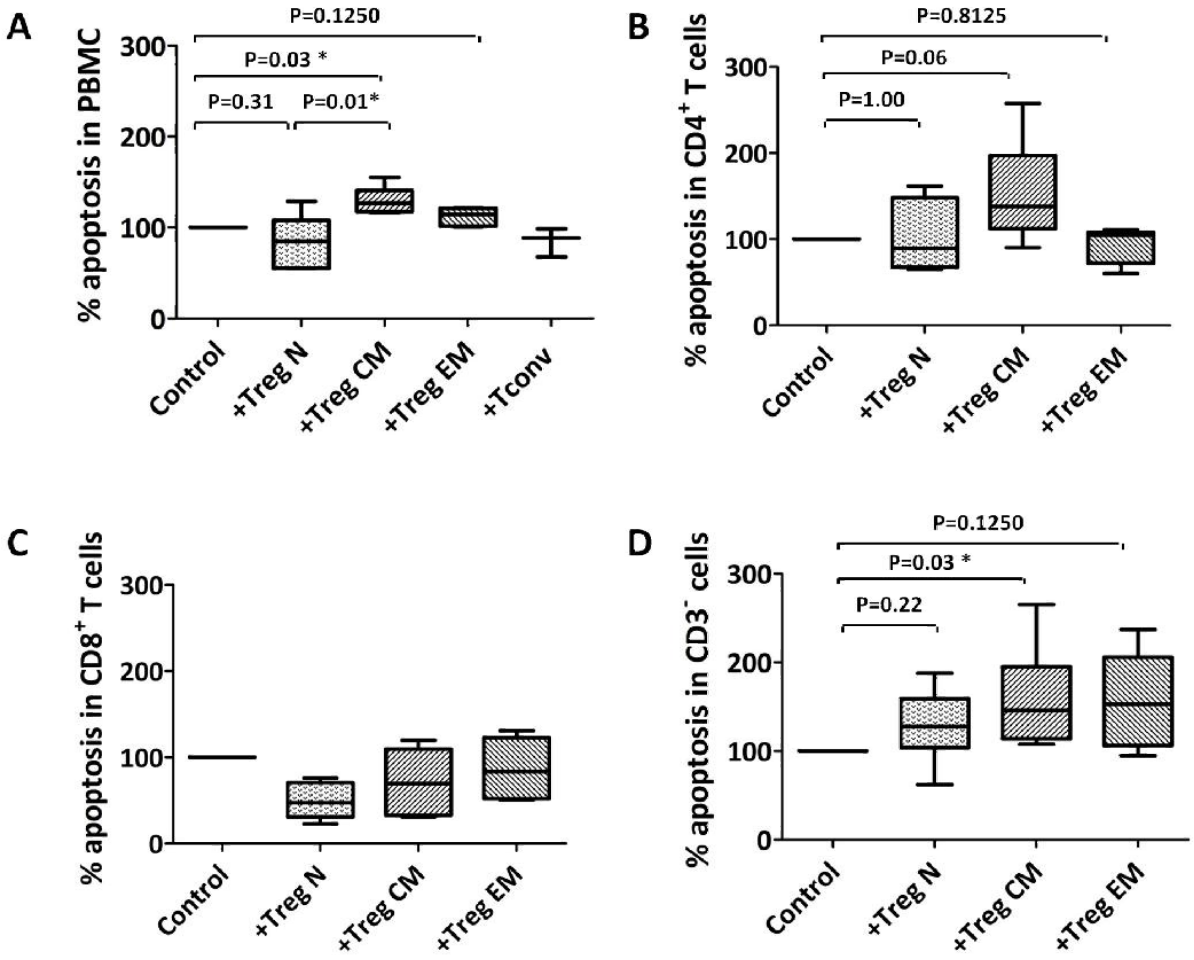

Figure 21: Apoptosis of total PBMC induced by Treg CM was mainly seen on CD3$^-$ cells. (A) TregCM could induce apoptosis to total PBMC while TregN or Treg EM could not. (B, C) No apoptosis induction was found on either CD4 T cells or CD8 T cells by any Treg subset. (D) Only TregCM induced apoptosis in CD3$^-$ cells. "Control" was responder cells alone. (n=6, Treg EM are from 5 of the total 6 donors. Wilcoxon signed rank test was for comparison of any subset and "control"; paired-t-test was for comparison of TregN and TregCM.)

The apoptosis induction of responder cells by different Treg subsets was studied. First of all, apoptosis in total PBMC was caused only by TregCM rather than TregN (Figure 21A). When

total lymphocytes were divided into CD4 T cells, CD8 T cells and non-T cells ($CD3^-$) by gating, no significant apoptosis induction was found by any Treg subset in CD4 and CD8 T cells (Figure 21B, C), only apoptosis of $CD3^-$ cells was observed by TregCM rather than TregN (Figure 21D), indicating that apoptosis of total PBMC induced by TregCM was mainly focused on $CD3^-$ cells.

Furthermore, when apoptotic cells during proliferating process and un-proliferating process were separated, the apoptosis induction by TregCM was mainly observed in un-proliferating process (Figure 22).

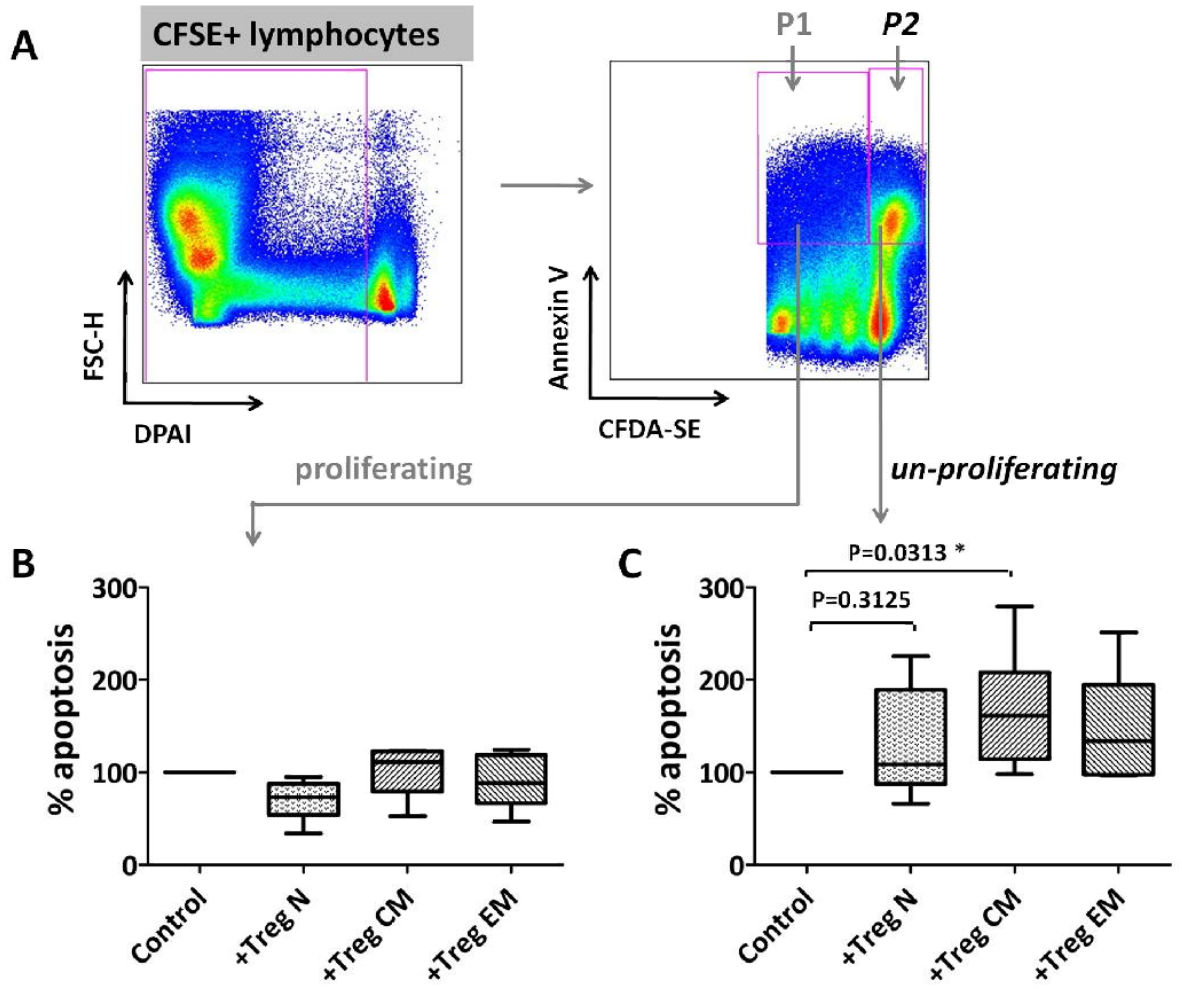

Figure 22: Apoptosis induced by Treg CM mainly occurred in non-proliferating cells. (A) $DAPI^-$ responder cells were further analyzed for Annexin V expression versus proliferation. Apoptosis in proliferating cells were showed in P1 and in un-proliferating cells were showed in P2. Regarding apoptosis induction in proliferating cells, mean % of P1 in responders alone was regarded as A'; mean % of P1 in responders with Treg was B'; B'/A' ×100 generated final "% apoptosis" in proliferating cells as Y-axis indicated in Figure B. Same calculation in P2 was performed to show "% apoptosis" in un-proliferating cells in Figure C (n=6, Treg EM are from 5 of the total 6 donors). (Lei et al. in Preparation)

4.2.4 Enhanced suppression activity of TregCM may be related to particular cell-cell interaction due to higher CTLA-4 expression on TregM than TregN

Based on the results from the enhanced suppression of early activation and apoptosis induction by TregCM than TregN, the mechanism behind was also explored partly. CTLA-4 expression on memory Treg and naïve Treg were compared because of its highly important role in cell-cell contact suppression mechanism. As what we expected, memory Treg did express higher level of CTLA-4 than naïve Treg (Figure 23).

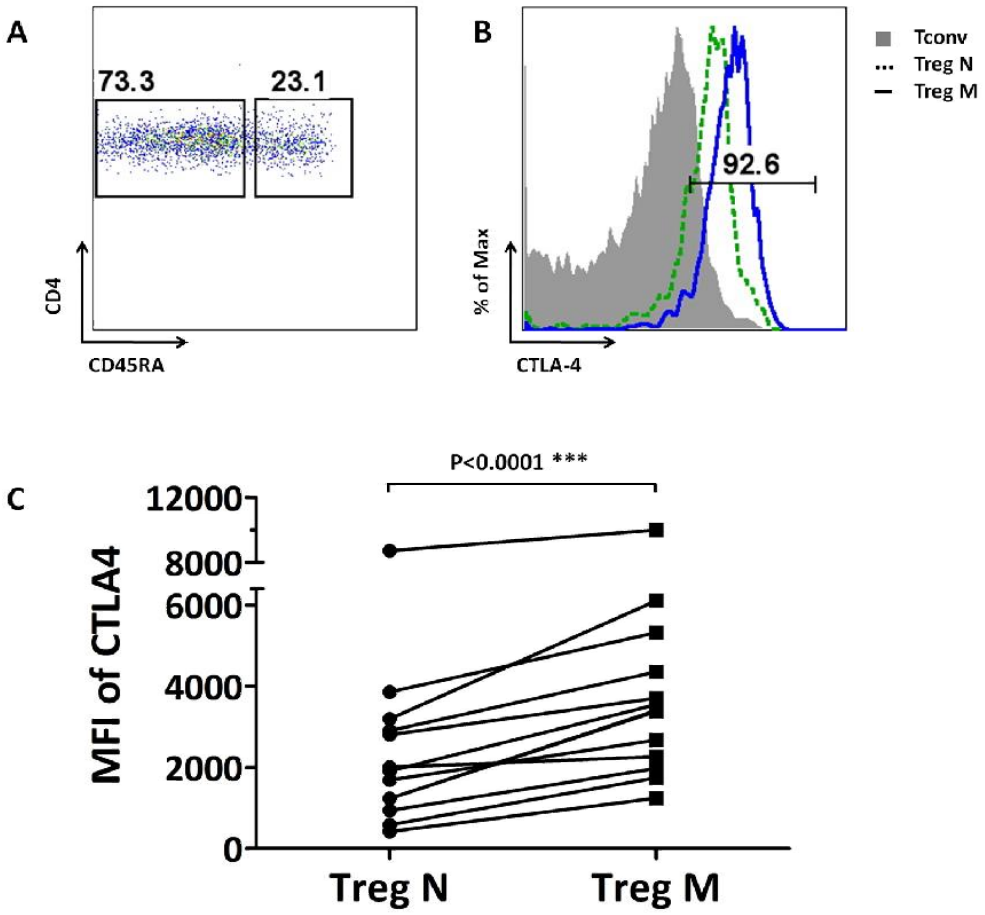

Figure 23: Memory Treg express higher CTLA-4 than naïve Treg. (A) Treg in peripheral blood were separated into naïve and memory sub-populations by CD45RA expression, shown from one representative donor. (B) CTLA-4 expression was compared in naïve and memory Treg. Gray area represents expression in Tconv. (C) Significantly higher geometric mean fluorescence intensity (MFI) of CTLA-4 was observed in memory Treg. (n=12, paired-t-test).

In summary, TregCM showed a stronger expression of FoxP3 and their demethylation level of the FoxP3 TSDR was even higher than the one of TregN. Most importantly, they expressed significantly stronger suppressive capacity to prevent early activation of Tconv compared to TregN and similar ability to inhibit proliferation of Tconv. Moreover, TregCM rather than

TregN were able to induce apoptosis of responder cells; the apoptosis was mainly seen on $CD3^-$ cells (mainly B cells and NK cells). Lastly, the enhanced regulation of memory Treg may be related to CTLA-4 dependent cell-cell contact mechanism. Thus, Treg could not only suppress activation of T cells, but also inhibit proliferation of both T cells and non-T cells and induce apoptosis in non-T cells, in parallel. Treg CM showed significantly stronger capacity than TregN in these processes.

4.3 Expansion of Treg and subsets indicated promising clinical translation for Treg therapy

4.3.1 Expansion with rapamycin could keep FoxP3 expression and suppression function of Treg

Due to the low frequency of Treg in peripheral blood, expansion of them *in vitro* without losing their suppression activity is a big issue for clinical translation of adoptive Treg therapy. Rapamycin, a mTOR inhibitor, plays a key role in achieving this. After 3-wk expansion with rapamycin, the expansion rate of Treg was less effective (Figure 24A). However, expanded Treg with rapamycin did keep most of FoxP3 and Helios expression while cells without rapamycin lost them dramatically (Figure 24B). In contrast to Tconv, almost no IL2, IL17 and IFNg production were found in expanded Treg with rapamycin after PMA/Ionomycin stimulation, although small amount of TNFα was detected. By contrast, in expanded Treg without rapamycin, >20% IL2 and IFNg producing cells and >50% TNFα producing cells were observed (Figure 24C).

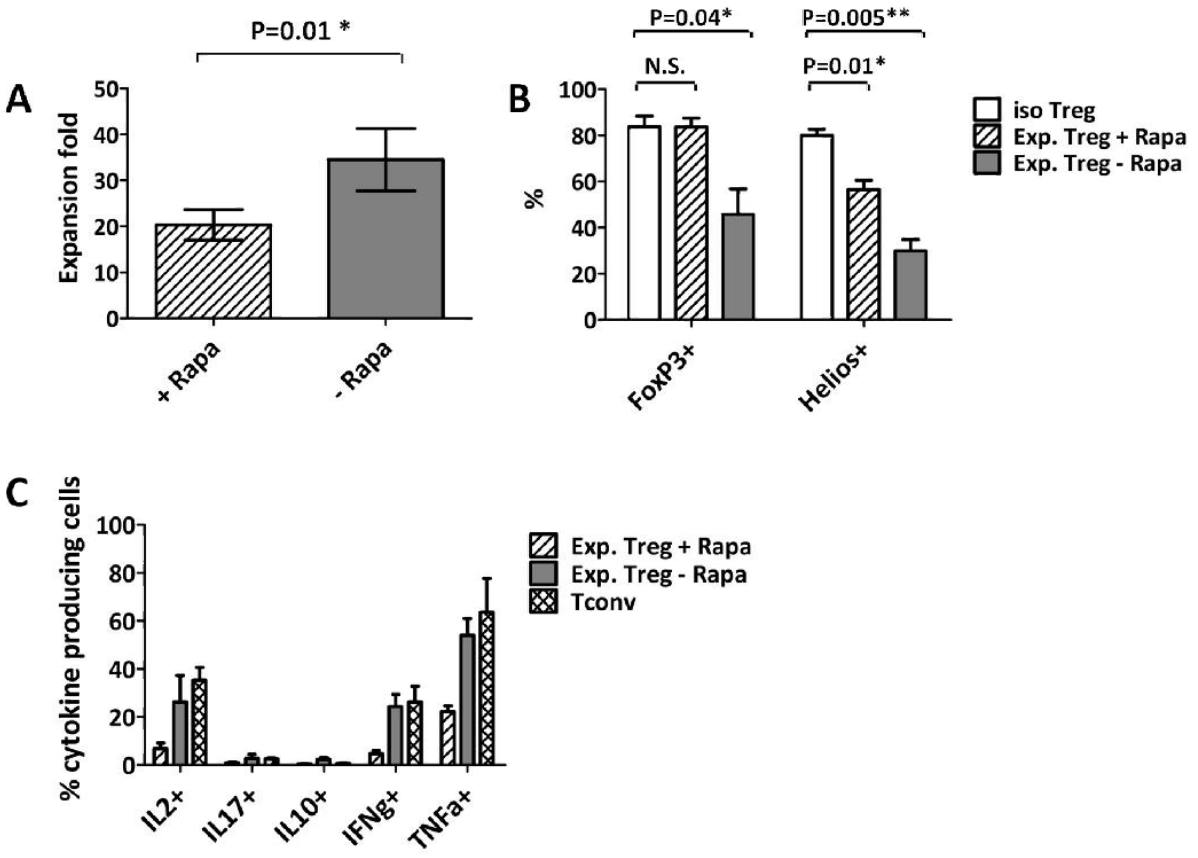

Figure 24: Expansion of Treg with rapamycin could keep Foxp3 expression and inhibit effecter cytokine production. (A) Expansion of Treg with rapamycin led to slightly lower expansion. (B) Expansion of Treg with rapamycin could keep Foxp3 expression. (C) Expansion with rapamycin could minimize regeneration of effector cytokine producing cells. (n=6, paired-t-test).

4.3.2 Enhanced expansion of Treg with G-Rex device, even closer to translation

Culturing Treg with poly-clonal stimulation in conventional cell culture plates could achieve around 20 fold of expansion stably. But this is still not enough for clinical translation as from preclinical data a >50 fold expansion of Treg from 50-100 ml blood is required. G-Rex device is a gas-permeable and GMP-compliant cell culture device. Expansion of Treg in this G-Rex10 device could enhance expansion to at least 50 fold (Figure 25A) and even higher with increased starting cell numbers. Regarding phenotype, Treg marker (FoxP3 and Helios) expression in expanded cells from plate, and G-Rex10 device were comparable, i.e. >70% of the cells expressed FoxP3 and half of them expressed Helios (Figure 25B, C).

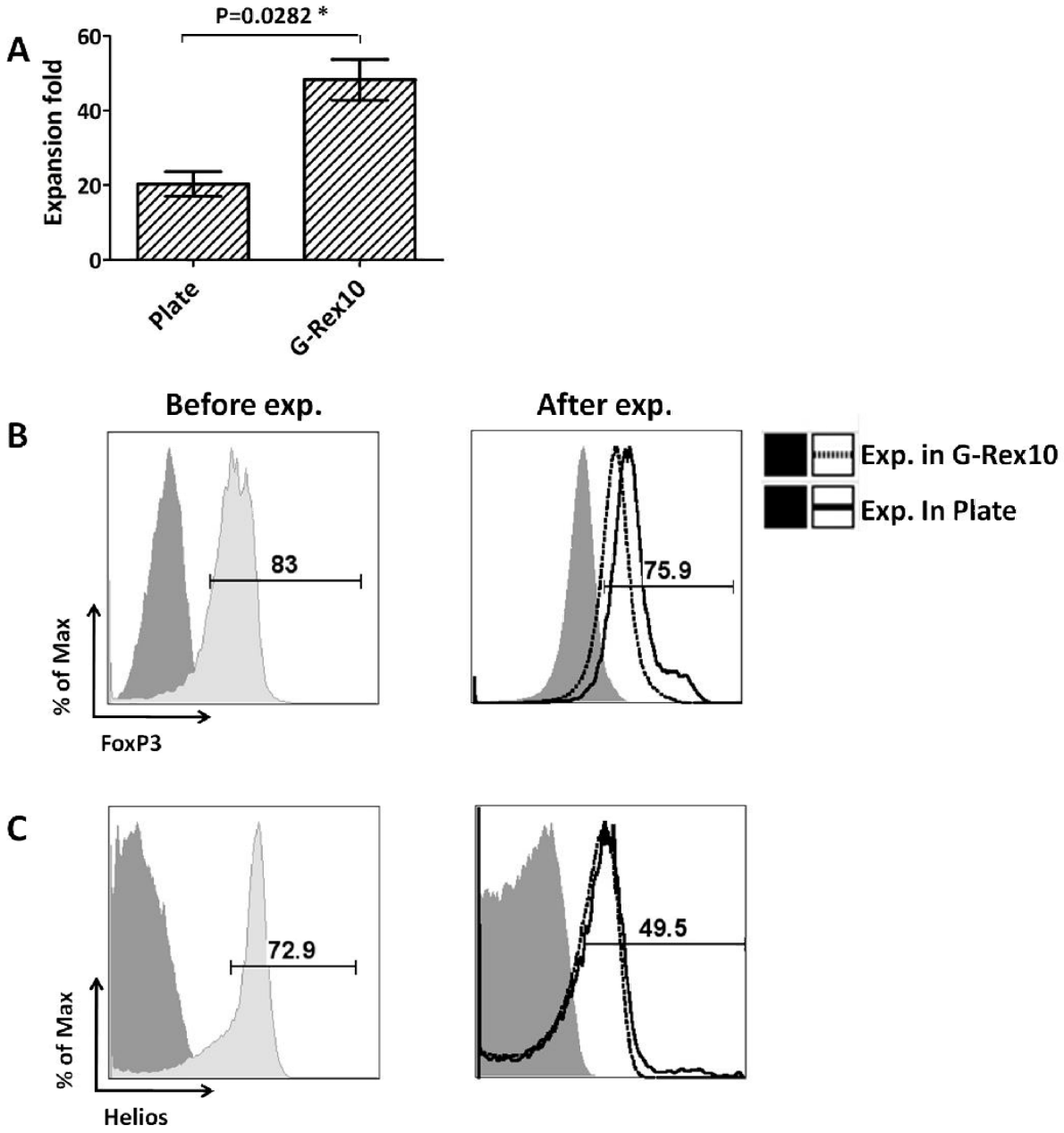

Figure 25: Expansion of Treg in G-Rex10 device could significantly increase the expansion without losing FoxP3 expression. (A) Higher expansion of Treg in G-Rex10 device. (B, C) Both of the expansion could keep FoxP3 expression and half amount of Helios expression. Expansion data in plate were from 6 donors and in G-Rex10 were from 4 of them (Paired-t-test).

Similar to the cells expanded in plates, small amounts of IFNg and TNFα but no IL2 and IL17 were detected in 3-wk expanded Treg in G-Rex 10 device upon in vitro PMA / Ionomycin stimulation (Figure 26). Therefore, expansion of Treg in the GMP-compliant G-Rex device contributes significantly to Treg expansion, thus promotes the clinical translation of Treg therapy moving forward.

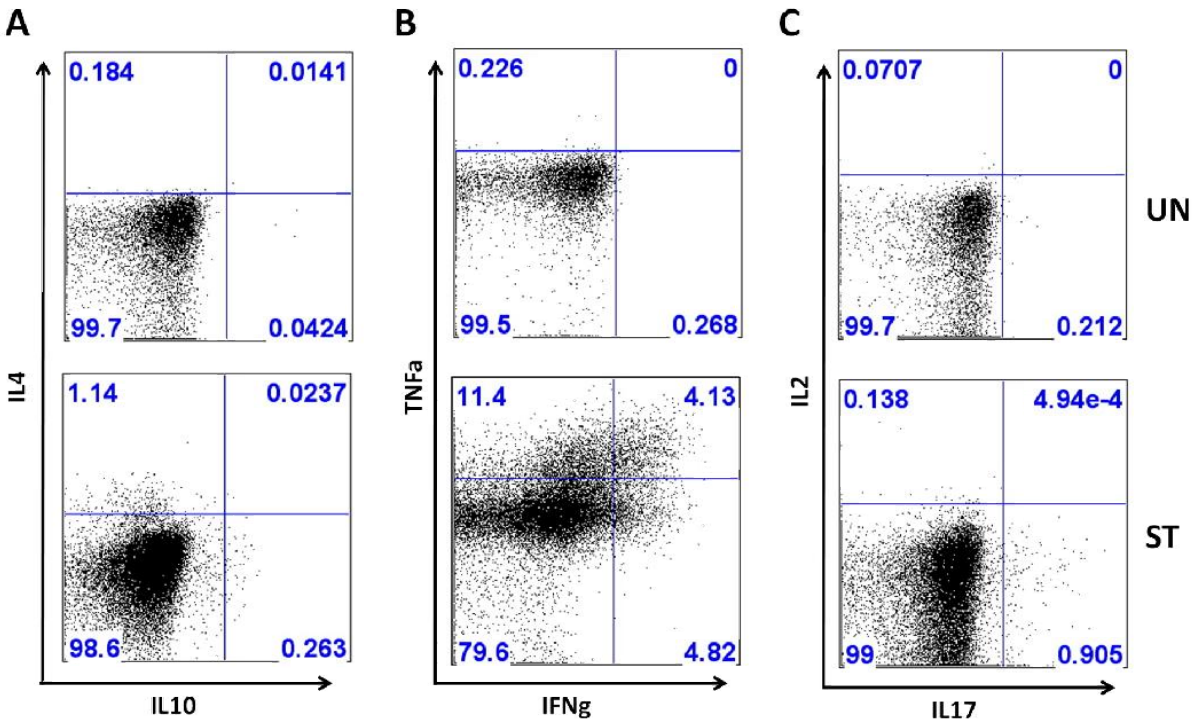

Figure 26: Prevention of effector cytokines production after expansion in G-Rex10 device was similar to expansion in plates. Cytokine production of expanded Treg in G-Rex10 from one representative donor was shown. Unstimulated cells (UN) were used as the control for gating on stimulated ones (ST).

4.3.3 Shift from naïve to memory Treg phenotype after expansion is associated with enhanced suppressive capacity

Freshly isolated TregCM show a more enhanced suppressive capacity than TregN regarding activation, proliferation and apoptosis induction of responder cells (Results 4.2). Functional analysis of Treg subsets were further performed after expansion with polyclonal stimulation in presence of rapamycin and IL-2 for 3 weeks. Firstly, no significant difference was found between TregN and TregCM regarding expansion, but proliferation of TregEM was very poor (Figure 27A), which was in agreement with a previous report (132). Therefore, we only focused on the comparison of TregN and TregCM. After 3-wk expansion, naïve Treg lost most of the CD45RA expression (Figure 27B) and diverted into CM phenotype (Figure 27C); Most TregCM were still in CM phase although few of them diverted into TregEM (Figure26C, D). These subsets could keep more than 70% of FoxP3 expression after expansion (Figure 27E). Zooming into these FoxP3$^+$ cells in each cell type, naïve Treg kept all their Helios expression as compared with freshly sorted cells; however, TregCM lost >50% Helios expression (Figure 27F). Interestingly, expanded TregN obtained enhanced suppressive activity to the level of isolated and expanded TregCM.

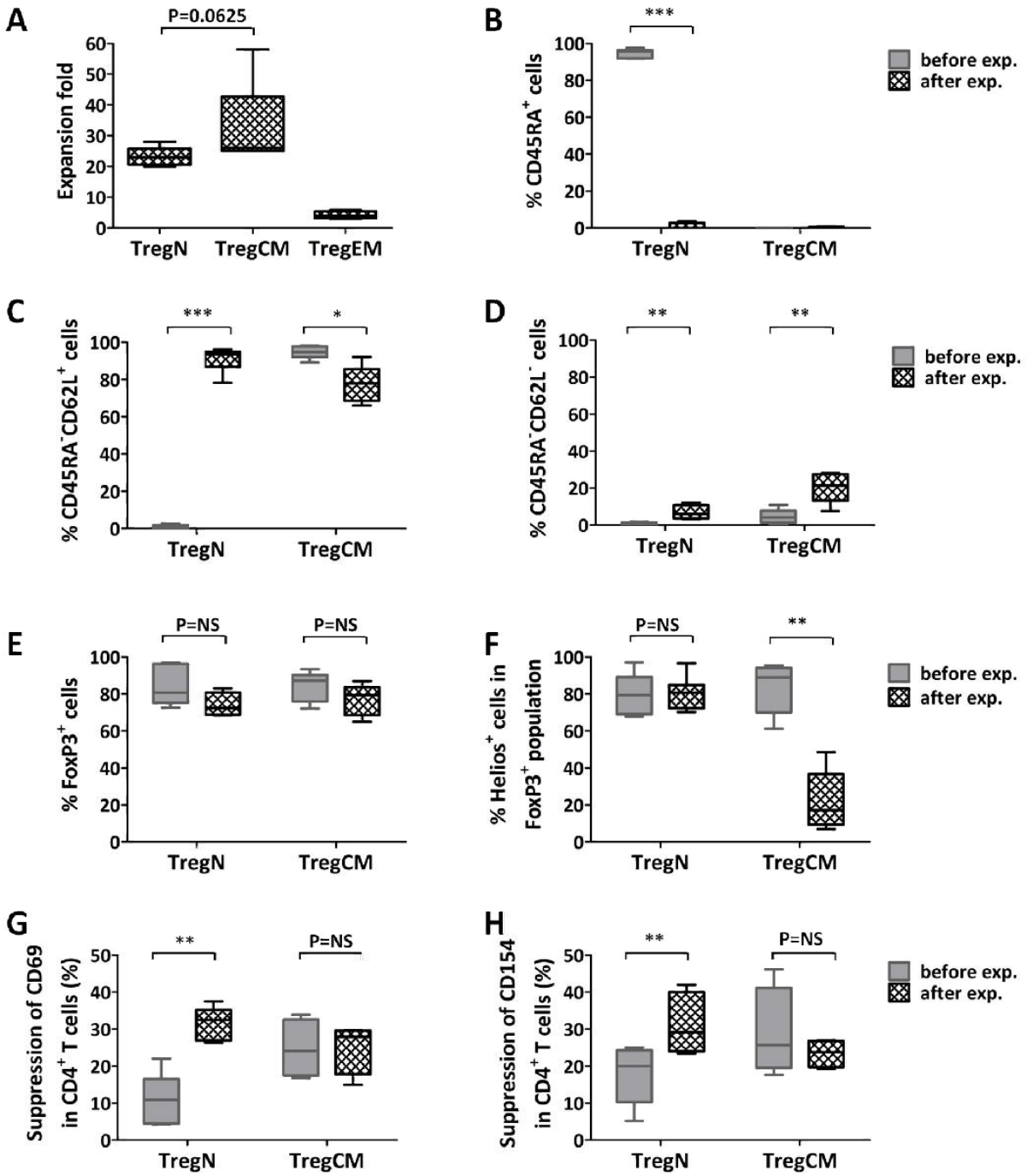

Figure 27: Naïve Treg converted into TregCM show enhanced suppression activity after expansion with rapamycin, while TregEM proliferate poorly. (A) Similar expansion of TregN and TregCM is observed after 3-wk expansion with rapamycin and proleukin, but proliferation of TregEM is very poor. (B) TregN lost naïve phenotype dramatically after expansion. (C) >80% expanded TregN expressed CM phenotype while majority of TregCM still kept CM phenotype. (D) Small proportion of expanded TregN and TregCM converted into EM phenotype. (E) TregN and TregCM kept >70% FoxP3 expression after expansion. (F) Expanded TregCM lost > 50% Helios expression while TregN did not. (G, H) Expanded TregN acquired enhanced suppression activity compared to freshly isolated ones. Data are form 5 donors while expansion of Treg EM is from 4 of them (paired-t-test). (Lei et al. in Preparation)

In short, expansion of Treg with rapamycin could keep FoxP3 expression and their suppression activity; moreover, expansion in gas-permeable and GMP-compliant device G-Rex 10 could enhance significantly expansion to at least 50 fold using poly clonal stimulation without losing FoxP3 expression. As for Treg subset, expanded naïve Treg converted mainly into TregCM keeping FoxP3 and Helios expression with enhanced suppression activity while expanded TregCM kept FoxP3 expression but lost some Helios expression, nevertheless, they still showed equivalent suppressive activity before and after expansion, but proliferation of TregEM was very poor.

Together with the previous data, we concluded that the majority of Treg in peripheral blood from healthy donors and Tx patients stayed in central-memory phase, showing most efficient suppression regarding early activation, proliferation and apoptosis induction of other immune cells (mainly T cells, B cells, NK cells). Consequently, TregN converted into TregCM and did more efficiently the regulation job. Cell-cell contact suppression was one of the mechanisms behind this enhanced suppression efficiency, due to higher expression of CTLA-4 on memory Treg than naïve ones. However, TregEM might be the terminal differentiation phase as they proliferated very poorly. This hypothesis was strengthened once more by subsets distribution in Tx patients that they were pre-activated expressing more EM phenotype and more susceptible to activation-induced cell death.

4.4 TCR repertoire analysis indicated memory Treg derived from naïve ones rather than Tconv

Enormous T cell receptor (TCR) repertoire clonal diversity is essential for T cells to recognize millions of antigens to exert immune responses. Thus, apart from playing a significant role in monitoring infections and diseases, analysis of TCR repertoire is also an important method to study the relationship between different cell lineages or cell types in the process of differentiation.

Based on the third aim of this project, we would figure out whether Treg might be derived from naïve ones in an antigen-driven process or induced from Tconv, as shown for TGFβ (109). In order to clarify the differentiation relationship between Treg subsets and Tconv, Treg (N, CM, EM) and Tconv from 6 healthy donors and Treg (N, M) and Tconv (N, M) from additional 4 healthy donors were prepared for DNA extraction, subsequently applied to TCRβ-chain repertoire analysis in this study using next-generation sequencing (NGS) and bioinformatic tools. The bioinformatics computing of TCR repertoire data with Shannon entropy and the Morisita-Horn similarity (MH) index has been done by Leon Kuchenbecker and Prof. Avidan U. Neumann (Berlin-Brandenburg Center for Regenerative Therapies, Charité University Medicine Berlin) for collaboration.

4.4.1 Next-Generation Sequencing (NGS) is a much powerful tool for TCR repertoire analysis

Third complementarity-determining region (CDR3) is the most variable region of TCRβ-chain, thus analysis of TCRβ-chain repertoire is always focused on CDR3, which contain sequences encoded by V, D, J gene segments. The conventional and classic way is to use TCRβ-chain spectratyping for measuring CDR3 length.

The distribution of CDR3 length in every V-Family of TCR β-chain from 3 representative donors is shown in Figure 28. Normal distribution in TregN indicates polyclonal composition of the repertoire; in contrast, abnormal distribution in TregCM especially TregEM suggest clonal expansion in those Vβ families, e.g. Vβ4 and Vβ9 in TregEM. However, CDR3 length distribution could not provide direct sequence information at clonal level. Consequently, a

new technology for TCR repertoire analysis is essentially required to visualize clonal relationship of cell subsets.

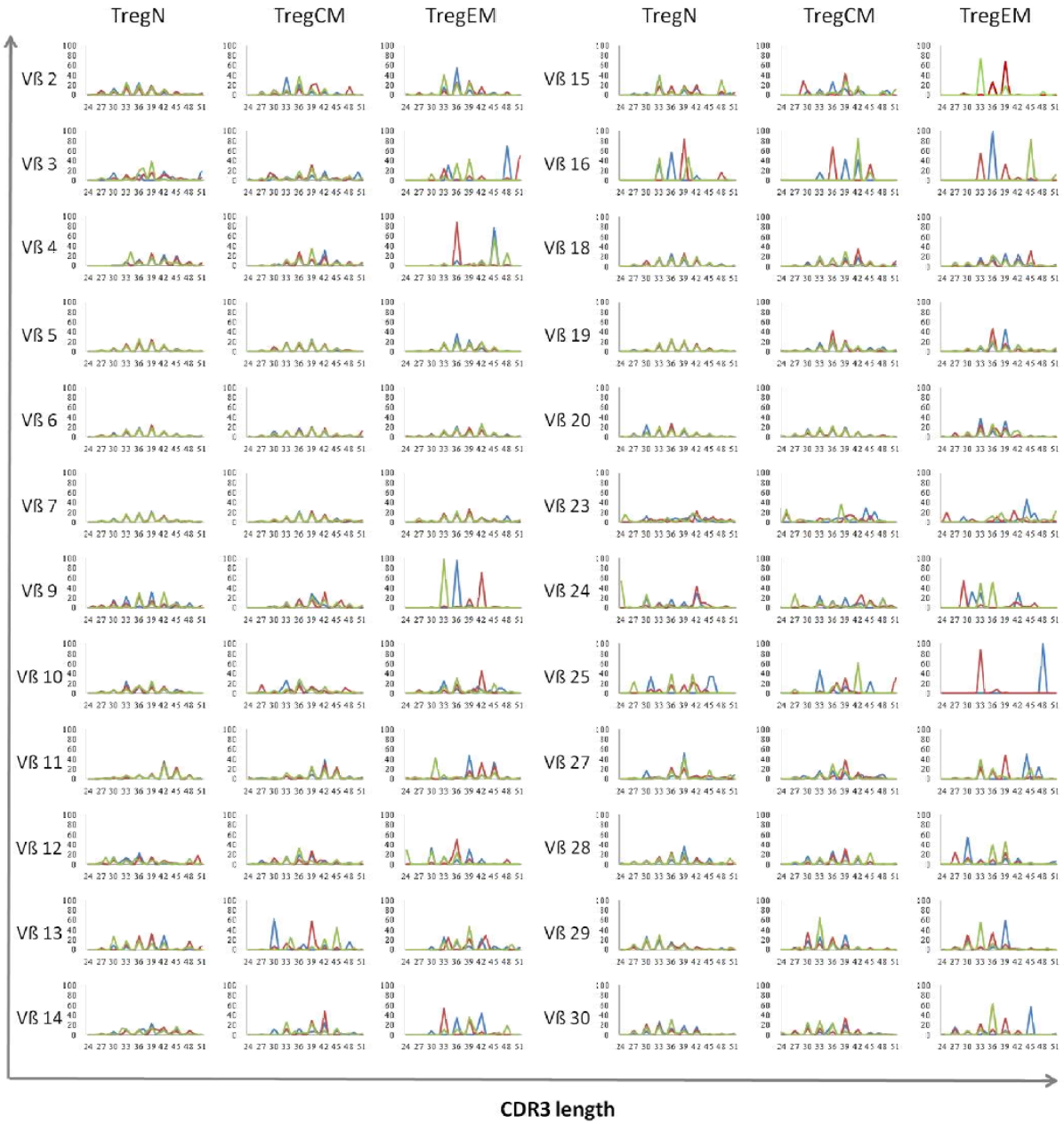

Figure 28: CDR3 length distortion of Vβ families of Treg subsets indicates indirectly clonal expansion of memory Treg. CDR3 length distribution in all V-families of TregN, TregCM, TregEM from 3 donors were shown separately. Different color represents different donor.

Next generation sequencing (NGS) is the powerful new technology to analysis TCR repertoire at a sequence level with V-gene and J-gene composition. Treg subsets and Tconv from total 10 donors were analyzed for their TCR repertoire by this high-through put sequencing at Adaptive Biotechnologies (USA). The average obtained total reads from each

cell type were 660,000 with average unique sequences of 198,000. Sequence composition of naïve Treg from one representative donor was shown as a 3D image in Figure 29 showing all V and J-genes. Poly clonal distribution was observed with highest frequency of a single clone as low as 1.1%.

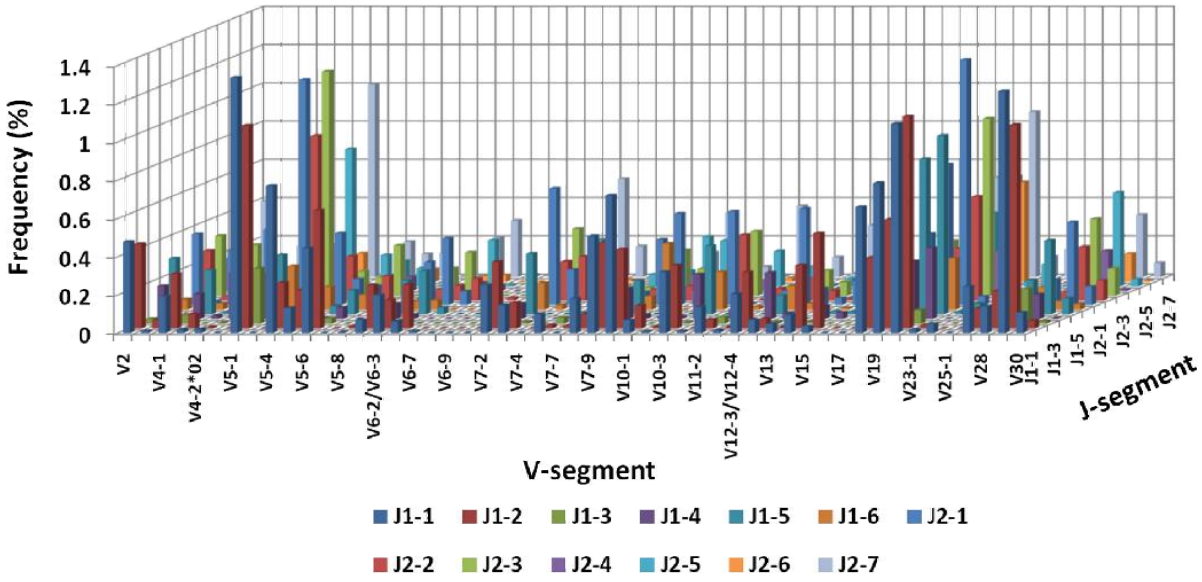

Figure 29: Poly clonal distribution of TCR repertoire from TregN was observed by using NGS with frequencies of single clones lower than 1.1%.

4.4.2 Memory Treg seems to be derived from naïve Treg rather than Tconv suggesting an antigen-driven process

4.4.2.1 Clonal expansion in memory Treg compared to naïve ones

The frequencies of the top 1,000 clones (defined by unique V, J and CDR3 sequence) of each cell type from one representative donor is shown in Figure 30A according to the rank. Clones with much higher frequencies are observed in TregCM or EM compared to TregN, indicating clonal expansion in the memory Treg repertoire. Additionally, the Shannon entropy (SE) is one of the most common and robust tools for measuring diversity and complexity of a data set, thus widely used in analyzing immunoglobulin repertoire diversity (35). A reduced SE in memory Treg confirms clonal expansion with statistical significance (Figure 30B).

To determine the source of expanded memory Treg clones – from Tconv (induced Treg) or from TregN in an antigen-driven process, we analyzed all the obtained distinct sequences

from Treg subsets, e.g., all the unique sequences of TregN and TregCM from one representative donor are shown in Figure 30C with their frequencies in each subset. As an example, the clone highlighted in Figure 30C has a frequency of 0.12% in TregN while in TregCM it has a frequency of 4.17%, suggesting approximately 35-fold expansion of this specific clone in TregCM. The same clonal expansion was analyzed in all memory-naïve pairs from all 10 donors, as shown in Figure 30D. It reached up to 200-fold clonal expansion in the memory Treg subset (CM or EM) and up to 500-fold in total memory Treg repertoire compared to the TregN pool.

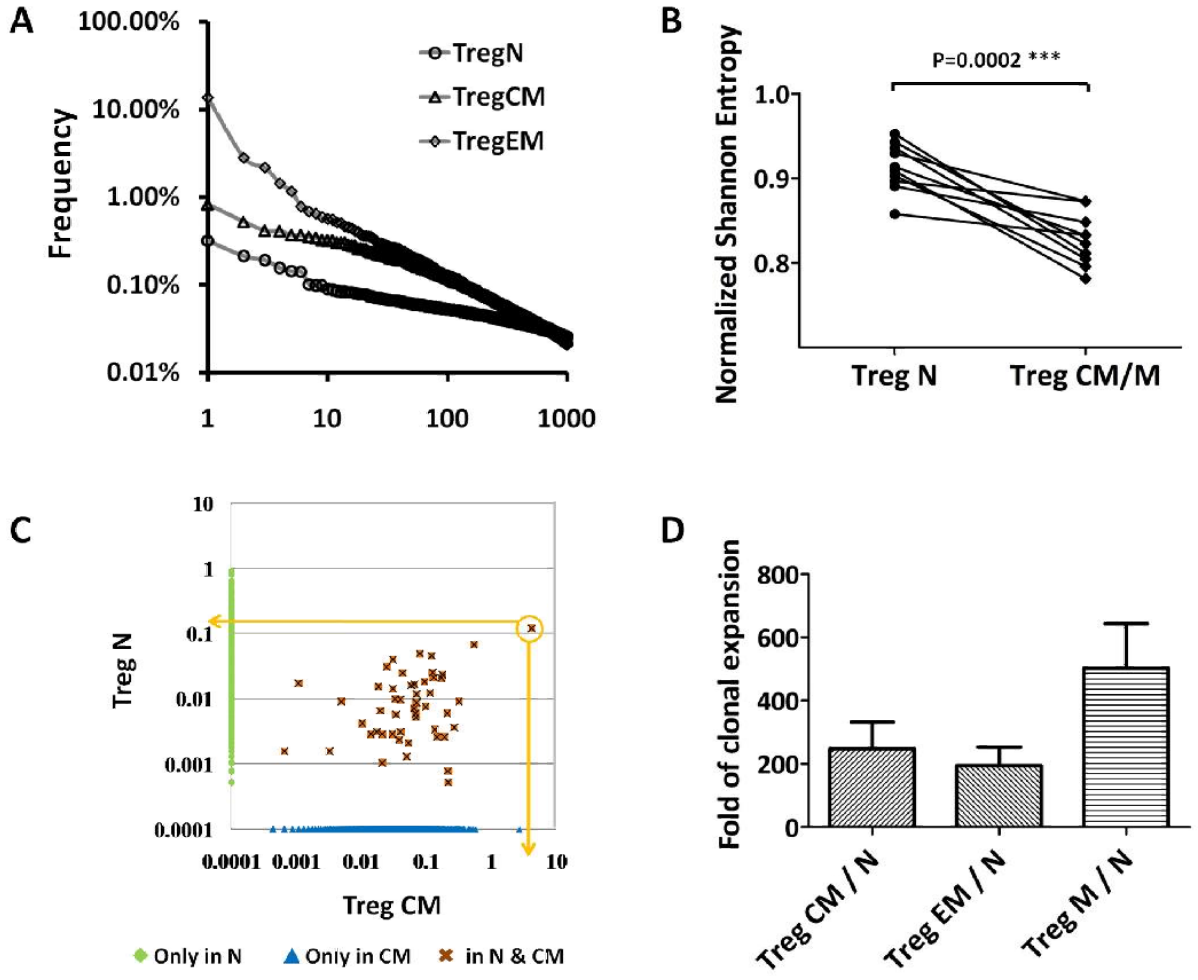

Figure 30: Visible clonal expansion is observed in memory Treg compared to naïve ones by NGS. (A) Frequency of the top 1,000 clones of the TCR repertoires in each cell type is shown according to the rank from one representative donor. (B) Significantly decreased normalized Shannon Entropy (SE) in memory Treg than naïve ones indicate strongly clonal expansion in memory phase. (C) Clones in TregN and TregCM were plotted according to their frequency in TregN and TregCM. Green rhombus represents the clone detected only in TregN; blue triangle represents the clone detected only in TregCM and cross represents shared clone between TregN and CM. (D) Highest clonal expansion from naïve Treg repertoires are shown both in memory Treg subset (CM or EM) and total TregM population from 10 donors. (Lei et al. in Preparation)

4.4.2.2 Memory Treg seem to be derived from naïve ones rather than Tconv with highest similarity within CM/EM

In addition to the shared clones with highest expansion rates from naïve to memory Treg repertoire, the total number of overlapping clones from all sequences in our groups were compared, including TregCM with EM, memory Treg subsets (CM or EM) with TregN, and memory Treg subsets with Tconv from same donors as shown in Figure 31A; the percentage of overlapping clones was calculated as fraction of clones in the total number of clones in the latter cell population for each pair (Figure 31A). Interestingly, there is an overlap of more than 25% between TregCM and TregEM. Although the TCR repertoire of TregN is very diverse and the individual clones therefore very rare, we observed an overlap of more than 2.5% between TregN and the memory Treg repertoire (CM or EM) at the applied sequencing depth. Only a very small fraction of clones can be found both in the Tconv repertoire as well as in the memory Treg (TregCM or TregEM) repertoire (Figure 31A). Furthermore, when we search for triple overlapping clones detected in all three Treg subsets (N, CM, EM), there is an overlap of around 1.5% between TregN, CM and EM, but almost no triple overlap is found in the groups of TregCM, EM and Tconv (Figure 31B).

The Morisita-Horn similarity (MH) index is another method to compare two TCR repertoires (36, 37). The MH index of the same groups in Figure 31A reveals a significant similarity of memory Treg subsets (CM or EM) with TregN, while almost no similarity with Tconv is observed (Figure 31C). Alike to the comparison by overlap, the highest similarity is observed between TregCM and EM. This data suggests that memory Treg seem to be derived from TregN, rather than Tconv.

Regarding the highest similarity of TregCM and EM, rank of overlapping clones in the repertoires of both populations from one representative donor is shown in Figure 31D. Interestingly, these clones are not just top rank ones; instead, they are distributed evenly with both high and low frequencies, indicating shared and similar memory development in TregCM and EM differentiation phases. Moreover, when we look at the rank of the triple overlapping clones in TregN, CM and EM in the CM and EM TCR repertoires, the majority of these clones show up around the diagonal, suggesting a similar expansion level in both memory phases. However, a small proportion of triplets present in the top ranks of EM, rather than CM repertoires, indicating a potentially advanced clonal expansion in EM. However, no

significant decreased Shannon entropy was obtained in TregEM compared to TregCM, although the tendency was observed (Figure 31E).

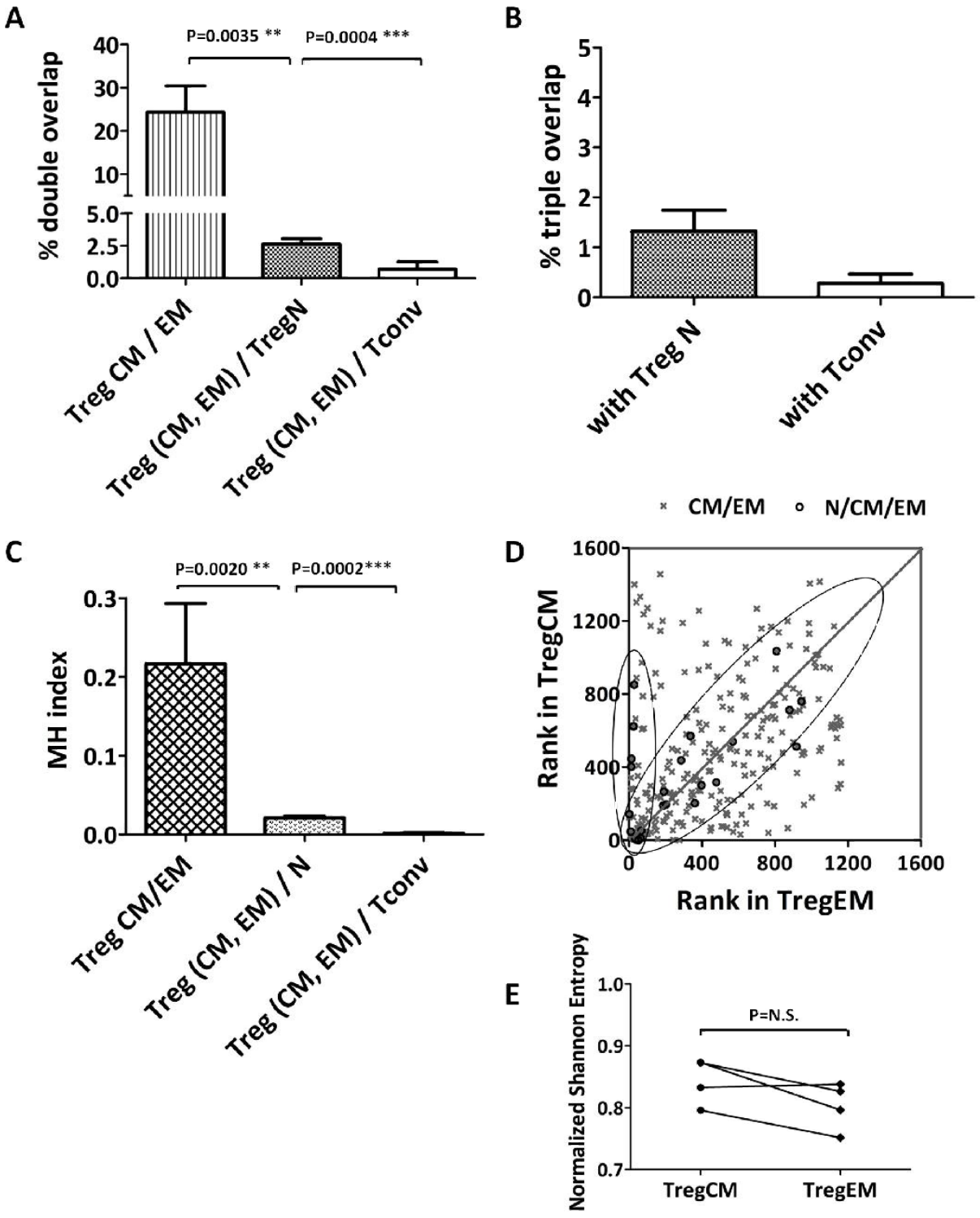

Figure 31: Memory Treg seem to be derived from naïve ones rather than Tconv with highest similarity between TregCM and TregEM. (A) Intersections of clones between TregCM and EM, memory Treg subset (CM or EM) and TregN or Tconv are shown separately as % double overlap in total clones of the latter cell type. (B) Triple overlapping clones are still observed in 3 Treg subsets (TregCM / EM / N), but not in the group of

"TregCM / EM / Tconv". % triple overlap is proportion of shared clones in total clones of the last T cell type. (C) Morisita-Horn index of "TregCM / EM", "Treg (CM, EM) / TregN" and "Treg (CM, EM) / Tconv" reveal highest similarity of repertoire of CM and EM, almost no similarity is shown between the repertoires of memoryTreg and Tconv. (D) Distribution of double overlapping clones between "TregCM / EM" and triple overlapping ones in "TregN / CM / EM" are shown according to their rank in TregCM and TregEM from a representative donor. Cross represents shared clones between TregCM and TregEM; cross with a circle represents triply the overlapping clone in TregN, CM and EM. (E) Similar SE values of TregCM/EM. (Lei et al. in Preparation)

Although no similarity was observed between TregM and Tconv TCR repertoire, small similarity of the repertoire between TregN and Tconv is found in Figure 32A. Similarly, very small amount of overlapped clones (< 0.3%) is also found between TregN and Tconv; however, no triple overlap among TregN, TregM and Tconv could be observed (Figure 32B), i.e. potential overlap of clones between naïve Treg and Tconv are not detectable in memory Treg repertoire, suggesting possible contamination of highly diverse naïve Treg with Tconv by sorting. Nevertheless, expanded clones in memory Treg are totally different with Tconv repertoire, thus "self-reactive". In addition, memory Treg repertoire is much more clonal expanded than respective memory Tconv due to lower Shannon entropy (Figure 32C).

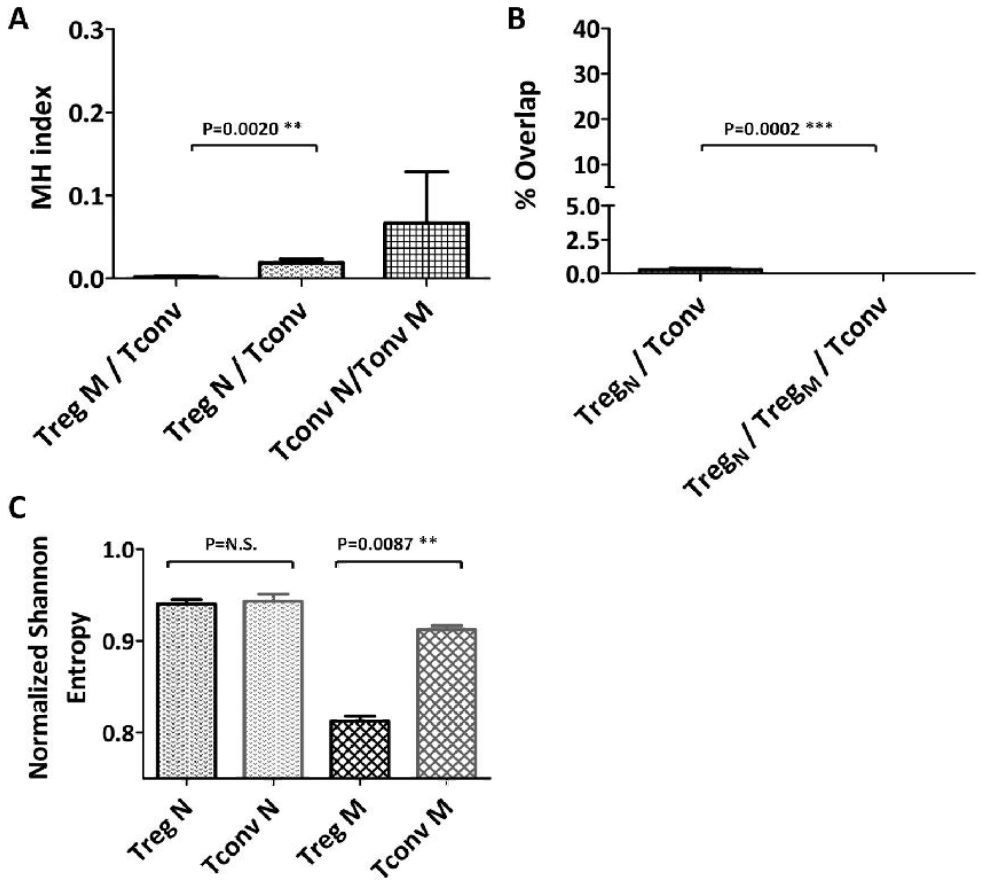

Figure 32: Small overlapped clones between TregN and Tconv were not detectable in more clonal expanded TregM. (A) Small similarity of TregN and Tconv was observed. (B) Small double overlapped clones were found between TregN and Tconv, but no triple overlap among TregN, TregM and Tconv could be observed. (C) Similar clonal diversity of TregN and TconvN, but more clonal expansion in TregM compared to Tconv M. (n=8, Wilcoxon matched pairs test).

To sum up, the NGS data on TCR repertoires sequences suggest a distinct usage of TCR between Treg and Tconv in human. Similar to TconvM, clonal expansion was also observed among TregM while TregN showed a polyclonal repertoire (top clones with frequency <1%). The strong overlap between TregN and TregCM and even more between TregCM and TregEM as well as the absence of overlap between TregCM/EM and Tconv suggest that TregM are more likely derived from TregN rather than Tconv (e.g. as so called induced Treg). Additionally, diversity of the TCR repertoire of TregM seems to be lower than the respective TconvM in human although both naïve repertoires are equally diverse.

5. DISCUSSION

According to the first aim of this study, heterogeneous composition and activation pattern of Treg in healthy donors and Tx patients were analyzed. In both cases, Treg population consist of naïve ($CD45RA^+CD62L^+$), central-memory (CM, $CD45RA^-CD62L^+$) and effector-memory (EM, $CD45RA^-CD62L^-$) sub types although 1.1% of T_{EMRA} ($CD45RA^+CD62L^-$) cells are found in Treg form Tx patients. Central memory cells are the majority of Treg in both cases. Age and gender have no significant influence on total Treg frequency; however, proportion of naïve and CM subsets are affected reversely by age. Furthermore, this subset composition in Treg is the same as their counterpart, Tconv; however, their activation patterns are distinct: Treg up-regulate only CD137 expression whereas Tconv up-regulate both CD137 and CD154 upon *in vitro* upon TCR stimulation. Interestingly, due to long exposure to allo-antigens, Treg from Tx patients are pre-activated with CD137 expression in absence of in-vitro stimulation and more susceptible to activation induced cell death; together with other observations that Treg in Tx patients contain less naïve but more EM cells than age-matched healthy donors; these data indicate that Treg in Tx patients are further differentiated and effector-memory might be the terminal phase.

To the second aim of this work, functional analysis of Treg subsets were performed from different aspects and CM was the most efficient phase for Treg to do regulation job. Firstly, TregCM showed significantly higher suppression activity in controlling early activation of CD4 and CD8 T cells than naïve Treg. In a longer (4 days) proliferation assay, TregN and TregCM showed equivalent inhibition to proliferation of non-apoptotic PBMC, but only TregCM could induce apoptosis to autologous PBMC whereas TregN could not. Moreover, cross-talk between Treg and other immune cells was found as inhibition of proliferation was observed from both T cells and non-T cells (mainly NK cells and B cells) and significant apoptosis induction was mainly seen on non-T cells. After 3-wk expansion of Treg subsets with rapamycin, TregN converted into CM phenotypically with enhanced suppression activity whereas most TregCM still stay in CM phase with similar suppression activity and small proportion of it diverted into EM phase. Poor expansion of TregEM indicates again the possible terminal differentiation phase for EM. These data imply that expansion with total Treg is optimal for adoptive Treg therapy as the majority of them belong to highly potent CM phenotype; additionally, Treg expansion in a newly developed gas-permeable and GMP-

compliant rapid culture device, G-Rex10, provides promising prospect for adoptive Treg therapy as >50 fold of expansion could be achieved stably by that.

To the third aim of this work, the NGS data on TCR repertoires sequences suggest a distinct usage of TCR between Treg and Tconv. Similar to TconvM, clonal expansion was also observed among TregM while TregN showed a polyclonal repertoire (top clones with frequency <1%). The strong overlap between TregN and TregCM and even more between TregCM and TregEM as well as the absence of overlap between TregCM/EM and Tconv suggest that TregM are more likely derived from TregN rather than Tconv (e.g. as so called induced Treg).

5.1 Phenotypic analysis of Treg from healthy donors and Tx patients

5.1.1 Similar subsets composition, but more memory cells in Treg than in Tconv

Regarding Treg subsets composition in peripheral blood, similar to CD4 Tconv, Treg also consist of naïve, central-memory and effector memory subsets (10, 11, 38); moreover, each subset in Treg was weakly but positively correlated with the same subset in donor-matched Tconv, indicating their possible in parallel development, which have been proposed recently by Duhen et al. (138). But unlike Tconv (particularly CD8 but also some CD4) (39), no T_{EMRA} cells are found in Treg populations from healthy adults as shown in Figure 7G. Furthermore, we could show that the majority of memory Treg belongs to the CM phenotype. Although we did not find an impact of age or gender on total Treg counts, there is a significant inverse relation between loss of TregN and increase of TregM with age as recently described (12, 40, 41). We could show for the first time that TregCM but not TregEM significantly increase with age. Enrichment of TregCM reaches a plateau at the age >40 years, which is similar to the very recent publication on total memory Treg by Schlossberger et al. (41). Moreover, compared to the respective Tconv, Treg contain much higher frequencies of CM cells. This data suggests that memory Treg population development might be the result of (auto) antigen exposure during the first decades of age that is obviously stronger than exposure for non-self antigens to Tconv. Interestingly, autoimmunity rarely manifests after the age of 50 years, suggesting that auto-regulatory processes are decided within the first half of life. In contrast to Treg; however, memory Tconv further expand in elderly (>50 years) donors as the result of chronic/repetitive non-self antigens exposure.

5.1.2 Distinct activation patterns of Treg and Tconv subsets via TCR stimulation

Regarding the activation pattern, different activation patterns are found in Treg with $CD137^+CD154^-$ cells and Tconv with $CD137^+CD154^+$ or $CD154^+CD137^-$ cells as others have reported with antigens stimulation (122). Furthermore, when Treg subsets are included in the analysis, more $CD137^+CD154^-$ cells and less $CD137^+CD154^+$ cells are found in TregM than TregN. According to the recent publication, $CD137^+CD154^-$ cells represent an antigen-reactive Treg population with higher purity due to higher demethylation level than $CD154^+$ cells (122). Additionally, CD154 was important for naïve T cells polarization (133) and was also regarded as an specific marker for antigen specific Tconv (121). Thus, TregM with higher CD137 but no CD154 expression may indicate a pure Treg population while TregN contain small proportion of double positive cells, which could be contaminated with some Tconv as CD154 works more as an activation marker for Tconv rather than Treg; however, in mice, up-regulation of CD154 expression and its transduced signals promote $FoxP3^+$ Treg generation and development in thymus (134). Therefore, it is possible that the CD154 expression in peripheral naïve Treg may have similar effect for FoxP3 induction. Higher expression of CD137 in TconvM and TregM might relate with its important role in cell survival, proliferation and function in CD4 cells (120). Higher expression of both CD137 and CD154 in TconvM than TconvN may also indicate the faster and stronger reactivity of TconvM upon infections. Moreover, according to what Choi et al. have found, CD137 signaling is required to neutralize suppressive function of activated (memory) rather than resting (naïve) Treg (135), thus higher expression of this molecule on TregM may relate with their stronger suppression as we will discuss later.

Helios is another important Treg marker as the transcription factor that binds to FoxP3 promoter and up-regulate its expression (123). In this study, $FoxP3^+Helios^+$ cells show no CD154 expression, whereas $FoxP3^+Helios^-$ cells have around 10% $CD154^+$ cells, indicating again they might be “contaminated” with Tconv. These data support that $FoxP3^+Helios^+$ cells define rather pure Treg population as a recent publication (136). However, Helios expression might not be very stable for Treg as we found later that expanded Treg could lose some of the expression, but still keep their suppressive capacity.

5.1.3 Treg subsets distribution in Tx patients

The final goal of this research is to translate Treg "from bench to bedside" especially into SOT patients. Therefore, it is important to first study the distribution and characteristics of Treg subsets in Tx patients. Our data have shown that Tx patients post above 4 years transplantation have slightly increased total Treg proportion than healthy donors, indicating tolerance induction to some extent. Less naïve but more EM cells are found in Tx patients than age-matched healthy donors with even 1.1% of T_{EMRA} cells ($CD45RA^{+}CD62L^{-}$), which were only found before in CD8 T cells as the terminal differentiated memory cells and very susceptible to apoptosis with high levels of perforin and Fas ligand (137); the increase of T_{EMRA} cells in CD8 T cells in Tx patients were even associated with acute kidney rejection (138). Therefore, increase of EM cells, even T_{EMRA} cells suggest a further differentiate status of Treg in Tx patients due to long term exposure to allo-antigens.

Activation patterns of Treg subsets in Tx patients showed that frequency of total Treg decreased dramatically upon *in vitro* stimulation (Figure 14A); while that is not the case in healthy donors. When CD137 expression was studied, a pre-activation pattern was found in only Treg population rather than respective Tconv population in Tx patients. The early activation of Treg in these patients should be result of exposure to allo-antigens; and this pre-activation may also be required for providing more efficient suppression functionality as we find later that Treg do the regulation job more efficiently in the CM phase. Nevertheless, together with increased EM cells and presence of few T_{EMRA} cells, pre-activation pattern and susceptibility to activation induced cell death of Treg in Tx patients all reveal that these Treg are pre-activated, thus prone to exhaustion and apoptosis. These data suggest some potential limitations of the Treg from Tx patients in keeping their immune tolerance after transplantations.

In short, the age-dependent shift to memory Treg from both healthy donors and Tx patients raises at least three questions that are of interest for both basic knowledge on Treg biology and adoptive Treg therapy in elderly patients as it might be impacted: i) are memory Treg (particularly the major fraction of TregCM) comparable to TregN regarding their regulatory capacity?; ii) are memory Treg stable after *in vitro* expansion – a prerequisite for adoptive T cell therapy?; iii) are memory Treg derived from TregN or converted Tconv, so called induced Treg? Data from this study gives at least partial answers to all three questions in the following three parts.

5.2 Functional analysis of Treg subsets with potential mechanism

5.2.1 Enhanced suppression by TregCM and possible differentiation of Treg subsets

CD69 is one of the earliest activation markers for CD4 and CD8 T cells upon stimulation. Once expressed, it works also as a co-stimulatory factor for further activation and proliferation. Additionally, CD69 is also expressed by other immune cells like NK cells and B cells. Thus, it is an important molecule to study the activation and differentiation status of immune cells (139, 140). CD154, another important T cells activation marker (121, 141), works as a co-stimulatory molecule of the tumor necrosis factor (TNF) family. It is involved in immune responses regarding B cells activation and germinal centre formation; development of specific or lineage-committed T cells is also reported to critically depend on CD154 expression (133). Therefore, monitoring CD69 and CD154 expression can be used as a new method to study the early activation status of immune cells (118).

The majority of Treg in adult healthy donors belong to the TregCM subset, increasing with age up to 40 years but only few cells expressing the TregEM phenotype. Therefore, we focused on TregCM functionality but found similarities between the two TregM subsets. TregCM showed a stronger expression of FoxP3 and their demethylation level of the FoxP3 locus (TSDR) was even higher than the one of TregN. Most importantly, they expressed significantly stronger suppressive capacity to prevent early activation of Tconv regarding CD69 and CD154 expression compared to TregN and similar ability to inhibit proliferation of Tconv. Moreover, TregCM but not TregN were able to induce apoptosis of responder cells; and the apoptosis mainly occurred in un-proliferating cells rather than proliferating ones, indicating that suppression of proliferation and induction of apoptosis in responder cells could be two separate suppression mechanisms for Treg and occur in parallel in keeping immune homeostasis.

Interestingly, similar advantages of $DR^{hi}CD45RA^{-}$ Treg and antigen-specific memory Treg suppression were also shown by others very recently (142-144), suggesting memory Treg containing stronger suppression activity than naïve ones at least in some aspects.

5.2.2 Potential mechanism of enhanced suppression activity by memory Treg

Since central memory is the most efficient phase for Treg to exert their suppression, what is the mechanism behind? To this end, cytotoxic T-lymphocyte–associated protein-4 (CTLA-4)

expression on naïve and memory Treg was compared and more expression was found on TregM. As CTLA-4 competes for B7 family molecules CD80/CD86 and inhibits co-stimulation of neighboring T cells (145), higher expression of CTLA-4 on TregM should contribute partly to the enhanced suppression activity. Besides this, Booth et al. also found TregM contained higher expression of CD39 than naïve cells indicating more production of adenosine (146). Galectin-1, another suppression mechanism, belongs to galactoside–binding proteins family and can function as a key regulator for Treg suppression; expression of this molecule on Treg is up-regulated upon TCR stimulation (50). Therefore, expression of galectin-1 in TregM should also be higher than TregN, thus contributing to the apoptosis induction by TregCM rather than TregN.

5.2.3 Cross talk of Treg and other immune cells

Besides inhibition of T cells proliferation, suppression to proliferation of non-T cells is also observed in this study, indicating cross talk of Treg and other immune cell as others have reported before (71). As the gating in this study was first set mainly on lymphocytes, the non-T cells ($CD3^-$ population) should be mainly NK cells and B cells. In the 6-hour stimulation assay, suppression of activation by CD69 expression on these cells is not observed, but in the 4-day proliferation assay, suppression to the proliferation of $CD3^-$ population (NK cells and B cells) is observed, which is even stronger than suppression to T cell proliferation. Moreover, apoptosis induced by TregCM to total PBMC is mainly seen on this non-T cell part, indicating indirectly that NK cells and B cells might be more susceptible than T cells to Treg suppression regarding cell proliferation and apoptosis. To confirm this, co-culture of Treg and B cells or NK cells directly could be performed with assessment of proliferation and apoptosis induction after wards.

5.3 Expansion of Treg in G-Rex10: New options for clinical translation

5.3.1 Expansion of Treg subsets *in vitro*

To get sufficient number of Treg for adoptive therapy, *in vitro* expansion is a prerequisite for adoptive Treg therapy. Recently, it could be shown by several groups that expansion of human nTreg is feasible by cross-linking CD3/CD28 and IL-2 in the presence of the mTOR inhibitor to prevent conversion and the loss of regulatory activity (147, 148). Here we could show that

TregCM are comparably expanded as TregN. Both subsets kept their regulatory potency and FoxP3 expression. Remarkably, almost all TregN switched to the TregCM phenotype by expansion and reached comparable regulatory potency, supporting the advantage of the TregCM phenotype. Total peripheral naïve T cells were reported to divert completely into memory cells after 10 days upon poly-clonal stimulation(124). Additionally, Chai et al. have reported before that expanded Treg showed higher suppression than freshly isolated ones (149), which could also be explained by enhanced suppression activity of originally naïve Treg upon TCR stimulation during expansion. Interestingly, regarding the FoxP3 and Helios expression in expanded Treg subsets, naïve Treg kept following both expansion and the switch to the TregCM phenotype while TregCM kept the TregCM phenotype as well as the FoxP3 expression, but lost >50% of the Helios expression, suggesting a rather moderate stability of TregCM upon stimulation since Helios binds to the FoxP3 promoter and up-regulates its expression (123). However, these expanded TregCM still showed equivalent suppressive activity as Du et al.. have also reported recently (150), suggesting Helios expression for Treg suppressive capacity might not be as important as it is for FoxP3 up-regulation and Treg stability (123, 151).

A smaller proportion of TregN and TregCM also switch to $CD45RA^{-}CD62L^{-}$(EM) after 3-wk expansion. Due to poor proliferative capacity and inefficiency of $CD4^{+}CD25^{hi}CD62L^{-}$ cells in protecting from lethal acute graft-versus-host disease (GvHD) *in vivo* (152), EM might be the terminal differentiation stage for Treg. More discussion on the subsets differentiation will continue in next part together with TCR repertoire data.

5.3.2 Expansion of Treg in G-Rex10 device

Expansion of Treg with rapamycin could keep most of the Treg phenotype and suppression function in our hands. However, limited expansion fold is always a big challenge for Treg therapy. Using the newly developed cell culture device, G-Rex10, could enhance the expansion to at least 50 fold stably without losing phenotype and suppression function. Chakraborty et al. have also reported recently that expanded Treg with G-Rex device could suppress division of CD8 T cells *in vitro* and GvHD *in vivo* in a mouse model (153). Together with the GMP compliancy of this air permeable cell culture device, expansion of Treg in G-Rex will facilitate clinical translation of Treg therapy without doubt.

5.4 TCR repertoire analysis

To answer the third question if memory Treg derived from TregN or converted from Tconv like induced Treg, TCR repertoire analysis of Treg and Tconv was performed. Conventional tools to do this are mainly focused on CDR3 length distribution rather than real sequence analysis. Development of next-generation sequencing (NGS) technology makes the repertoire analysis at sequence level achievable. In this study, analysis of repertoire from Treg and Tconv subsets from 10 donors shed some light on differentiation relation of these cells during thymus and peripheral development.

5.4.1 Treg M derived from naïve Treg with clonal expansion

First of all, an average of 660,000 reads was obtained from each sample by NGS technology. These data revealed further clonal expansion in memory Treg compared to naïve ones with enhanced frequencies of the clones. Due to the influence of different sample size, Shannon Entropy (SE) was used to access the diversity of the repertoires in sense of how flat versus oligoclonal the clone type distribution was. Significantly decreased SE value in TregM than TregN reveals oligo-clonal distribution in TregM at a statistical level. However, to focus on each specific clone defined by same CDR3 sequence and V, J genes, clonal expansion of same clone in both naïve and memory populations was showed and the highest expansion of one clone from naïve to memory Treg reached 500 fold in peripheral blood, which was showed for the first time in human.

Besides highest expanded clones in the overlap between two population repertoires, proportion of total overlap between different populations also support that TregM contain significantly higher overlap of the repertoire with TregN than Tconv. However, this is only proportion of absolute overlap; taking the possible influence from different sample sizes into consideration, the MH index [6] was applied with more statistical relevance. Similarly significant higher similarity was found in the group of “TregM / TregN” than the group of “TregM / Tconv”, indicating once more memory Treg should derive from naïve ones rather than Tconv.

[6] MH index: Morisita-Horn similarity index

5.4.2 Highest similarity of TCR repertoire between TregCM/EM

However, in both comparison of overlap and MH index, highest overlap and similarity was found between Treg CM and EM. Although higher clonal expansion in TregEM was found than TregCM in Figure 30A, when we tried to analyze that in a more statistical way with Shannon entropy, no significant difference was found although reduced SE was observed in 3 of the total 4 donors (Figure 31E). However, it should be noted that we calculate the SE value by strictly defining each clone containing exactly same V-gene, J-gene and a.a. sequence[7]; significantly reduced SE in TregEM repertoire would be observed compared to TregCM (data not shown) if we used the SE value provided by Adaptive Biotechnologies (USA), which was computed based on all detected clones without excluding those sequences containing "undefined" V-gene or J-gene. To be on the safe side, the former computation was applied in this study.

In addition, rank of shared clones in Treg CM and EM are evenly distributed with both high and low frequencies. However, rank of the triple overlap of TregN, CM and EM show that they are not only clones with similar rank in both CM and EM (clones around the diagonal in Figure 31D) , but also clones with high rank in TregEM. All of these indicate Treg EM could be the possible terminal phase for Treg differentiation with highest clone expansion; however, due to the largely distributed shared clones between TregCM and EM, we still could not exclude the possible turnover between them.

5.4.3 TCR Repertoire of human Treg and Tconv

As the distinct TCR repertoire between TregM and Tconv in human have been clarified before, the TregN repertoire was also compared with Tconv and very slight overlap was found, i.e. < 0.3% Tconv repertoire were overlapped in TregN repertoire (Figure 32); however, these overlapped clones were not expanded or detected in TregM repertoire (Figure 32B). Thus, the small overlap between TregN and Tconv could be explained to potential sorting contaminations as TregN have lower CD25 expression or to the very high diversity of TregN repertoire and sensitivity of NGS technology. Nevertheless, as majority of human Treg population express memory phenotype, these NGS data suggest a distinct usage of TCR

[7] a.a. sequence: amino acid sequence

between Treg and Tconv in human, which is in agreement with the similar data in mice (106, 107, 154) and in human by other methods (110).

Regarding clonal diversity revealed by Shannon entropy, similar diversity was found between TregN and TconvN repertoire as other authors have speculated and showed before (155-157). However, when the TCR repertoire of TregM and TconvM were compared, significantly reduced diversity was found in TregM repertoire, indicating their advanced clonal expansion than donor-matched TconvM repertoire. To our knowledge, this is revealed in human for the first time. However, the limited repertoire of TregM might have an impact on the efficacy of polyclonal Treg therapy, particularly in elderly patients, as target specificities (allo, auto) might be missing

From the methodological aspect, the adoption of Shannon entropy and MH index in the TCR repertoire analysis shed light on the relevance at a statistical level. The analysis of overlapping clonotypes as function of ranks can provide even more hints on relations between different sub-population.

To sum up, from the first part of the results, heterogeneous composition of Treg with N, CM, EM subsets were found in both healthy donors and Tx patients. The age-dependent shift from TregN to TregM was mainly occurring into CM rather than EM phase before 40 years old and Treg contained significantly higher CM than respective Tconv, indicating memory Treg development might be the result of (auto) antigen exposure during the first decades of age that was obviously stronger than exposure for non-self antigens to Tconv. Treg from Tx patients were even more activated with expressing CD137 and contained more EM cells; furthermore, they were susceptible to activation induced cell death, suggesting the limitations of these cells in keeping immune tolerance.

The second part of the data indicated that TregCM contained enhanced suppression activity than TregN in controlling an early activation of T cells and inducing apoptosis to both T cells and non-T cells, which may relate with cell-cell contact suppression. For clinical translation, expansion of Treg with rapamycin in the air permeable and GMP compliant device, G-Rex10, significantly increased the expansion fold to 50 stably without losing Treg phenotype and function. Following *in vitro* expansion of subsets, the majority of TregN switch to TregCM phenotype associated with enhanced suppressive capacity to the level of TregCM, indicating

expansion of total Treg are optimal for Treg therapy as majority of them express CM phenotype. Poor proliferation of TregEM hinted the terminal differentiation stage.

Lastly, TCR repertoire data from NGS suggest that TregM derived from naïve one rather than Tconv with highest clonal expansion of 500 fold in an antigen-driven process. Highest similarity was found between TregCM and EM. However, the limited repertoire of TregM might have an impact on the efficacy of polyclonal Treg therapy, particularly in elderly patients, as target specificities (allo, auto) might be missing.

In short, these data shed some light on human Treg subsets distribution, functionality and differentiation. The enhanced suppressive capacity of freshly isolated TregCM and the stability after expansion have significant importance for clinical translation of adoptive Treg therapy, implying that expansion with total Treg is optimal for adoptive Treg therapy since the majority of them belong to CM phenotype. From the basic immunological aspect, these data shows for the first time that peripheral human Treg M derive from naïve ones rather than Tconv with the highest similarity found between CM and EM repertoires and TregEM might be the terminal differential phase.

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7. APPENDIX

Publications list

Lei H., Kuchenbecker L., Vogt K., Sawitzki B., Streitz M., Landwehr-Kenzel S., Juelke J., Babel N., Neumann A., Reinke P., Volk H.D. (2013) Human central memory regulatory T cells – antigen-driven increase with age and enhanced regulatory efficiency
(In submission)

Landwehr-Kenzel S., Issa F., Luu S.H., Schmück M., Lei H**.**, Zobel A., Thiel A., Babel N., Wood K., Volk H.D., Reinke P. Novel GMP-compliant Protocol Employing Allogeneic B Cell Bank to Induce Clonal Expansion of even Low-Abundance Alloantigen-Specific Natural Regulatory T cells, Am J Transplant. 2014 Mar;14 (3):594-606

Selbstständigkeitserklärung

Hiermit erkläre ich, Hong Lei, dass ich die vorliegende Arbeit “Human natural regulatory T cells subsets: Functional characterization and T cell receptor repertoire analysis” selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde.

Hong Lei

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