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Habilitationsschrift

Regulatorische T-Zellen als neues Therapiekonzept in der Transplantationsmedizin

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Was wir wissen ist ein Tropfen;
was wir nicht wissen, ein Ozean.
Isaac Newton (1643-1727)

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Abkürzungsverzeichnis

AMP	Adenosinmonophosphat
ATG	Anti-Thymozyten-Globulin
ATMP	Advanced Therapy Medicinal Products, Arzneimittel für neuartige Therapien
ATP	Adenosintriphosphat
CD	Cluster of Differentiation
CNI	Calcineurin-Inhibitor
CSA	Cyclosporin A
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
FoxP3	Forkhead-Box-Protein P3
GMP	Good-Manufacturing-Practice
GvHD	Graft-versus-Host Disease
HSCT	Hämatopoetische Stammzelltransplantation (Hematopoietic Stem Cell Transplantation)
HvGD	Host-versus-Graft Disease
IDO	Indolamin-2,3-Dioxygenase
IFN	Interferon
IL	Interleukin
MACS	Bead-basierte Magnetisolation
MHC	Major Histocompatibility Complex
MMF	Mycophenolat-Mofetil
nTreg	natürliche regulatorische T-Zellen (identisch mit tTregs)
mTOR	Mechanistic Target of Rapamycin
pTreg	peripher induzierte regulatorische T-Zellen
TGF	Transforming Growth Factor
TNF	Tumornekrosefaktor
Treg	regulatorische T-Zellen
TregCM	central memory Tregs
TregEM	effector memory Tregs
TregEMRA	terminally differentiated RA^+ Tregs
TregN	naive Tregs
TSDR	Treg-specific demethylated region
tTreg	im Tymus gereifte Tregs
TZR	T-Zell-Rezeptor

1. Einleitung

Ohne unser Immunsystem, das uns durch fein justierte Sensoren vor Autoimmunphänomenen, Infektionen und Malignomen schützt, wäre ein Überleben unmöglich. In ständiger Alarmbereitschaft klassifiziert unser Immunsystem erkannte Strukturen als „ungefährlich“ oder „gefährlich“. Hierbei ist das Zusammenspiel zahlreicher zellulärer und humoraler Komponenten des angeborenen und adaptiven Immunsystems essentiell. Kleine Fehlinterpretationen des Immunsystems können zu klinisch relevanten, teils lebensbedrohlichen Krankheitsbildern führen. Beispielsweise kann die Erkennung von körpereigenen Strukturen auf Tumorzellen ein Toleranzsignal („selbst“) darstellen und fälschlicherweise als „ungefährlich“ interpretiert werden. Im Falle von Autoimmunerkrankungen hingegen führt die Erkennung spezifischer körpereigener Moleküle zur Aktivierung der Signalkaskade „fremd“ oder „gefährlich“ und Mechanismen zur Beseitigung dieser Strukturen werden aktiviert. Zusätzlich können funktionelle Mechanismen oder die Immunhomöostase fehlerbehaftet sein und zu einer nicht ausreichenden Effektivität (Immundefizienz) oder überschießender bzw. nicht abklingender Reaktion (Hyperinflammation) führen. Im Falle einer soliden Organtransplantation wird das Spenderorgan durch das Immunsystem des Empfängers als „fremd“ bzw. „gefährlich“ erkannt und eine Entzündungs- bzw. Abstoßungsreaktion, die Host-versus-Graft-Erkrankung, entsteht. Im Gegensatz dazu erkennt nach einer hämatopoetischen Stammzelltransplantation das Immunsystem des Spenders das Gewebe des Empfängers als „fremd“ und führt zu einer gegen den Empfänger gerichteten Abstoßungsreaktion, der sogenannten Graft-versus-Host-Erkrankung (engl. Graft-versus-Host Disease, GvHD). Die Aktivierung primär protektiver pro-inflammatorischer Mechanismen resultiert schließlich in einer Schädigung und Abstoßung des Gewebes. Die verbesserten Möglichkeiten der HLA-Typisierung sowie der Einsatz moderner Therapieansätze, die überschießende Immunreaktionen hemmen (sollen), hat uns in den letzten Jahren einen wesentlichen Fortschritt in der Behandlung der betroffenen Patienten beschert und die Mortalität ist deutlich gesunken; leider sind die Ergebnisse insgesamt bei weitem nicht zufriedenstellend und noch immer führen Abstoßungsreaktionen zu einer hohen Morbiditätsrate mit signifikanter transplantationsassoziierter Mortalität. Medikamentenassoziierte Nebenwirkungen stellen ein zusätzliches Problem dar.

Die medizinische Notwendigkeit, neue Therapieansätze zu entwickeln, die zielgerichteter als bisherige und damit sowohl effektiver als auch nebenwirkungsärmer unser Immunsystem modulieren erscheint daher offensichtlich; ein genaues Verständnis der pathophysiologischen immunologischen Prozesse ist hierfür essentiell. Die in der vorliegenden Arbeit adressierten Fragestellungen verfolgen daher das gemeinsame Ziel, spezifische immunregulatorische Mechanismen genau zu analysieren und diese für eine zielgerichtete Therapie nutzbar zu machen.

1.1. Unser Immunsystem: Der Schutz vor „fremden“ Strukturen

Etymologisch stammt der Begriff des Immunsystems vom lateinischen *„immunitas“, der* „*Freiheit von etwas*“ ab. Im ursprünglichen Kontext wurde der Begriff für die Steuerfreiheit oder die Freiheit von öffentlichen Pflichten verwendet. Im politischen und biologischen Kontext prägte der Begriff den Schutz der Gesellschaft vor Fremden oder vor in der Gesellschaft grassierenden Krankheiten. Die Zusammenfassung der körpereigenen Mechanismen zum Schutz von Krankheiten entspricht damit dem Bild eines Schutzschilds, das uns gegenüber eindringenden Schädlingen verteidigt. In höher entwickelten Organismen setzt sich das Immunsystem aus zwei wesentlichen Bausteinen, dem ortständigen (häufig als passiv bezeichneten) sowie dem systemisch zirkulierenden (häufig als aktiv bezeichneten) Immunsystem zusammen. Während das lokale Immunsystem eine physische und chemische Barriere bildet und unseren Körper vor dem Eindringen fremder, potentiell gefährlicher Strukturen schützt, dient die systemische Immunabwehr der Elimination von Strukturen, die diese Barrieren bereits überwunden haben. Die komplex aufgebaute systemische Immunität setzt sich ebenfalls aus zwei Komponenten zusammen, dem angeborenen und dem adaptiven Immunsystem. Das angeborene, phylogenetisch ältere Immunsystem zeichnet sich durch eine ligandenspezifische Erkennung von Gefahrensignalen aus. Neben zellulären Bestandteilen, den Granulozyten, Makrophagen, dendritische Zellen und natürlichen Killerzellen zählen auch das Komplementsystem und Interleukine (IL) zu den Komponenten des angeborenen Immunsystems. Die Struktur der involvierten Proteine ist genomisch festgelegt und unterliegt keiner Adaptation. Wesentliches Merkmal des angeborenen Immunsystems ist eine schnelle und effiziente Aktivierung, die innerhalb von Sekunden bis Minuten zur Erkennung und Elimination von Erregern führt. Im Gegensatz dazu zeichnet sich das adaptive Immunsystem

durch spezifische Rezeptoren aus, die nicht nur antigen- sondern auch pathogenspezifisch Muster erkennen. Zu den Komponenten des adaptiven Immunsystems zählen T- und B-Lymphozyten sowie die von B-Zellen produzierten Antikörper. Im Rahmen des Entstehungs- und Reifungsprozesses entwickeln Lymphozyten durch somatische Mutationen, klonale Expansion und antigenspezifische Selektion eine klonale Diversität, die es erlaubt 10^{11} - 10^{12} unterschiedliche Antigene spezifisch zu erkennen und „selbst“ von „fremd“ zu unterschieden. Da T-Zell-Rezeptoren (TZR) nur an prozessierte Antigene binden können, die ihnen von antigen-präsentierenden Zellen (beispielsweise Makrophagen und dendritische Zellen) auf MHC-Molekülen präsentiert werden, ist für diesen Reifungsprozess eine enge Interaktion mit dem angeborenen Immunsystem essentiell. Nach Aktivierung des TZR werden Mitogen-aktivierte Proteinkinase-abhängige Signalwege aktiviert, die einen komplexen Prozess in Gang setzten, der zur Expansion, Differenzierung und Reifung aktivierter T-Zell-Klone führt. Ein immunologisches Gedächtnis entsteht. Funktionell sind T-Zellen, die mit Hilfe des TZR-Korezeptors CD3 präsentierte Peptidantigene erkennen, dafür verantwortlich die Zellen unseres Körpers auf ihre Integrität hin zu überprüfen und infizierte oder „fremde“ Zellen zu eliminieren. Dafür setzt sich das T-Zell-Kompartiment aus einer heterogenen Gruppe von Zellen zusammen, die jeweils spezifische Aufgaben übernehmen, insgesamt jedoch einer gewissen Wandlungs- und Anpassungsfähigkeit unterliegen und je nach umgebendem Milieu und Aktivierungszustand zwischen Subgruppen wechseln oder weiter differenzieren können. Entsprechend der Expression spezifischer Proteine, die gemeinsam mit dem TZR einen sog. T-Zellrezeptorkomplex bilden, können T-Zellen in $CD4^+$ Helfer-Zellen und $CD8^+$ zytotoxische T-Zellen unterteilt werden. Die Expression weiterer Oberflächenproteine erlaubt eine Bestimmung des Reifungszustandes und die Einteilung in naïve T-Zellen (naive T-cells, T_N $CD45RA^+CCR7^+$), zentrale Gedächtnis-T-Zellen (central memory T-cells, T_{CM}, $CD45RA^-CCR7^+$), Effektor-Gedächtnis-T-Zellen (effector memory T-cells, T_{EM}, $CD45RA^-CCR7^-$) und CD45RA exprimierende, terminal differenzierte Gedächtnis-T-Zellen (CD45RA expressing terminally differentiated effector memory cells, T_{EMRA}, $CD45RA^+CCR7^-$) [1–4]. Zytotoxische $CD8^+$ T-Zellen erkennen “fremde” Strukturen wie Pathogene oder entartete körpereigene Zellen und zerstören diese. Die Aktivierung von $CD4^+$ Helferzellen hingegen führt zur Freisetzung von Botenstoffen, die Zell-Zell-Kontakt unabhängig weitere $CD8^+$-Zellen und natürliche Killerzellen rekrutieren und aktivieren. Über eine direkte und indirekte Interaktion werden zudem B-Zellen stimuliert spezifische Antikörper zu bilden und den Reifungsprozess zu durchlaufen. CD4+ T-

Zellen zeichnen sich durch eine hohe Plastizität aus. Diese ermöglicht ihnen sich in Abhängigkeit von dem sie umgebenden Milieu in verschiedene Subtypen, die pro- aber auch antiinflammatorische Funktionen ausüben zu differenzieren.

1.2. Abstoßungsreaktionen in der Transplantationsmedizin: Wenn zu viel Schutz krank macht

Die Transplantation von soliden Organen oder hämatopoetischen Blutstammzellen hat sich in den letzten Jahrzehnten bei zahlreichen malignen und nicht-malignen Indikationen zur einzigen kurativen Therapieoption und damit zum Goldstandard entwickelt. Nach Angaben der *Global Database on Donation and Transplantation (*www.transplant-observatory.org) wurden 2020 weltweit 125.482 Transplantationen durchgeführt, wobei Nierentransplantation mit 78.627 vor Leber- (31.044), Herz- (7.928), Lungen- (5.788), Pankreas- (1.938) und Dünndarmtransplantationen (157) die am häufigsten durchgeführte solide Organtransplantation war. Im Bereich der Hämatoonkologie wurden über das *Worldwide Network of Blood and Marrow Transplantation (WBMT)* 2016 insgesamt 82.718 hämatopoetische Stammzelltransplantationen (HSCT) registriert, wovon 46,5% von allogenen Spendern durchgeführt wurden [5]. Bei einer jährlichen Zunahme der weltweiten HSCT-Aktivität um etwa 10.000 erreichten wir 2019 damit die Marke von 1,5 Millionen HSCT [5].

Die immunologische Erkennung fremder Oberflächenstrukturen stellte die Transplantationsmedizin initial jedoch vor scheinbar kaum überwindbare Hürden. Im Falle einer soliden Organ- oder hämatopoetischen Stammzelltransplantation wird das fremde Gewebe attackiert und eine Entzündungsreaktion entsteht. Dieser Prozess spiegelt sich klinisch nach einer soliden Organtransplantation in einer Abstoßungsreaktion (Host-versus-Graft Disease) und nach einer hämatopoetischen Stammzelltransplantation in der Spender-gegen-Empfänger Erkrankung (Graft-versus-Host Disease, GvHD) wider. Die Identifikation des Histokompatibilitätskomplexes MHC ermöglichte seit den 1950er Jahren einen enormen Fortschritt in der Aufklärung der an Abstoßungsreaktionen beteiligten immunologischen Mechanismen [6] und ermöglichte die Auswahl von geeigneten Spender-Empfänger-Paaren. Für „ihre Entdeckungen bezüglich genetisch bestimmter Strukturen auf der Zelloberfläche, die immunologische Reaktionen regulieren" erhielten Baruj Benacerraf, Jean Dausset und George

Snell 1980 den Nobelpreis für Medizin oder Physiologe [7]. Die weitere Aufschlüsselung pathophysiologischer Prozesse und die Erkenntnis, dass Abstoßungsprozesse ein multifaktorielles Geschehen sind, die durch ein Zusammenspiel von zytotoxischen sowie Helfer-T-Zellen, B-Zellen, Antikörpern und Zytokinen initiiert und aufrechterhalten werden ebnete den Weg für pharmakologische Interventionen.

Eine wesentliche Komponente im Prozess der MHC-vermittelten Abstoßungsreaktion stellen T-Zellen dar, wenngleich ein enges Zusammenspiel mit humoralen Faktoren und dem angeborenen Immunsystem für die Initiierung und Aufrechterhaltung des Abstoßungsprozesses essentiell ist. „Fremde“ Strukturen werden von T-Zellen entweder direkt auf fremden APCs über den T-Zell-Rezeptor erkannt oder indirekt auf eigenen APC, die diese Strukturen auf MHCs präsentieren. Hierdurch wird die Ausschüttung von Zytokinen ausgelöst und es resultiert eine lokale Entzündungsreaktion, die in eine systemische übergehen kann. Initial führt dabei die Freisetzung pro-inflammatorischer Zytokine wie beispielsweise IFNɣ, IL-2, IL-4, IL5, IL-17 und TNFα [8] zur Rekrutierung von allogen-spezifischen $CD4^+$ T-Zellen und zytotoxischen $CD8^+$ T-Zellen aber auch von antikörperbildenden B-Zellen. Zusätzlich werden andere pro-inflammatorisch agierende Immunzellen rekrutiert, die ins Gewebe infiltrieren und dort im Zusammenspiel mit gewebeständigen Zellen des angeborenen und adaptiven Immunsystems den Abstoßungsprozess durch kostimulatorisch wirkende Moleküle weiter verstärken [9]. Nach spezifischer Aktivierung proliferieren und reifen $CD8^+$ T-Zellen zu hoch effektiven zytotoxischen Akteuren, die über eine Sekretion von Granzymen, Perforin und zytotoxisch wirksamen Zytokinen eine Gewebeschädigung verursachen. Gleichzeitig werden weitere Kaskaden des inflammatorischen Geschehens in Gang gesetzt und aus einem lokalen Geschehen entwickelt sich eine systemische Erkrankung.

Die Entdeckung der immunsuppressiven Wirkung von Glukokortikoiden im 1930er Jahren und die Einführung von synthetisch hergestelltem Prednison seit 1954 legten den Grundstein immunsuppressiver Therapieansätze der modernen Medizin. In 1950-1960er Jahren erfolgte die Entwicklung und Zulassung von Azathioprin und dem ersten immunmodulatorischen Medikament, Methotrexat [10]. Das rapide wachsende Verständnis über die tragende Rolle von Effektor-T-Zellen im Abstoßungsprozesse führte zur Entwicklung von Cyclosporin A (CSA) [11], Cyclophosphamid [12] und Tacrolimus [10], sowie Sirolimus und Mycophenolat-Mofetil (MMF)

welche um die Jahrhundertwende klinische Studien und die Zulassung zur Behandlung von auto- und alloimmunologisch bedingten Erkrankungen erreichten. Zusätzlich erbrachte der Einsatz von Anti-Thymozyten-Globulin (ATG) einen weiteren wesentlichen Fortschritt in der Prophylaxe und Therapie der chronischen GvHD [13–17]. Leider blieben die positiven Effekte moderner immunsuppressiver Regimes im Hinblick auf die Vorbeugung und Behandlung von Abstoßungsprozesse sowohl im Bereich der soliden als auch der hämatopoetischen Stammzelltransplantationen weit hinter den Erwartungen zurück. Therapiebedingte Nebenwirkungen wie direkte Organtoxizitäten (Nephrotoxizität [18]), Stoffwechselstörungen [19] und Herz-Kreislauf-Erkrankungen [20] erwiesen sich als ebenso lebensbedrohliche Komorbiditäten führen wie das erhöhte Risiko für schwere Infektionen [21–25] oder Malignome [26–29]. Zusätzlich erwiesen sich chronische, immunologisch schwer zu beeinflussende Abstoßungsreaktionen in den letzten Jahren als besonders therapierefraktär und die Verbesserungen des langfristigen Überlebens in den letzten 20 Jahren sind enttäuschend [30–35]. Aktuell entwickelt noch ca. 30-50% aller Patienten nach allogener Stammzelltransplantation eine akute GvHD, wovon etwa jeder siebte schwer erkrankt [36]. Die kumulative Inzidenz einer akuten GvHD Grad 2-4 liegt trotz des Einsatzes moderner Therapieregimes bei ca. 80%; innerhalb der ersten zwei Jahre nach Transplantation entwickeln zudem etwa 34% der Patienten eine chronische GvHD [37]. Der Übergang in bzw. die Entstehung einer chronischen GvHD ist bei einem 6, 12, und 24-monatigen Überleben von 63%, 45% und 29% mit einem besonders hohen krankheitsbezogenem Mortalitätsrisiko assoziiert [34,37–39]. Ähnlich ernüchternd sind sich die Daten hinsichtlich des langfristigen Überlebens bei soliden Transplantationen: nach einer Nierentransplantation liegt das 5-Jahres-Transplantatüberleben aktuell bei 72% bis 91%, während die Rate 10 Jahre nach Transplantation zwischen 34% und 48% variiert [32]. Die jährliche Rate des Transplantatversagen liegt bei 3-5% [40].

1.3. Regulatorische T-Zellen: natürliche Vermittler der immunologischen Toleranz

Natürliche, im Thymus gereifte regulatorische $CD4^+CD25^{high}FoxP3^+$ T-Zellen (tTreg; früher: nTreg) stellen die physiologischen Gegenspieler zytotoxischer T-Lymphozyten dar. Sie sind insbesondere durch eine hohe Expression des IL-2-Rezeptors CD25 sowie stabile Expression des Transkriptionsfaktors FoxP3 [41] und eine niedrige Expression von CD127 [42] charakterisiert. Ihre Identifikation [43] brachte unser Verständnis über immunregulatorische Mechanismen

sprunghaft voran. Ein numerischer oder funktioneller Verlust an Tregs ist beim Menschen als auch in Mausmodellen mit teilweise fatal verlaufenden Autoimmunerkrankungen assoziiert [44,45]. Tregs gelten heute allgemein als Schlüsselmediatoren der Toleranz und ihre funktionelle Rolle bei Autoimmunerkrankungen [46,47], Infektionen [48,49], transplantationsbedingten Alloreaktionen [50,51] sowie in der Malignomentstehung [52] ist gut charakterisiert. Die immunologische und klinische Maximalform des Treg-Verlustes wird durch eine Mutation mit funktionellem Verlust des Transkiptionsfaktors FoxP3 verursacht und wurde beim Menschen durch Powell und Kollegen 1982 erstmalig beschrieben [53]. Auf Grund des klinischen Bildes, das mit Immundysregulation, Polyendokrinopathie sowie Enteropathie einher geht und X-chromosomal vererbt wird, ist diese Erkrankung auch als IPEX-Syndrom bekannt. Unbehandelt verläuft die Erkrankung meist in den ersten Lebensjahren tödlich [53–57]. Das klinische Bild des IPEX-Syndroms, das mit massiven Diarrhoen, ekzematöser Dermatitis und endorkinen Störungen wie beispielsweise Nahrungsmittelallergien oder Diabetes sowie immunologisch vermittelten Mono- bis Panzytopenien einher geht [58] zeigt damit eine große phänotypische Überlappung mit dem klinischen Bild der GvHD [59] welche im Sinne einer Multisystemerkrankung alle Organe betreffen kann und sich häufig primär an der Haut, der Leber sowie im Gastrointestinaltrakt manifestiert.

Funktionelle Phänotypanalysen führten zur Identifikation weiterer Zellpopulationen, die durch periphere Reifung regulatorische Funktionen erlangen können und gemeinsam eine große Familie regulatorisch wirkender Zellen bilden (Tabelle 1). Während tTregs im Thymus einen zentralen Reifungsprozess durchlaufen, der zu einer stabilen Expression von FoxP3 führt und die sichere Unterscheidung zwischen „selbst“ und „fremd“ ermöglicht, entstehen peripher induzierte regulatorische T-Zellen (pTregs) aus konventionellen FoxP3$^-$ T-Zellen, die die Fähigkeit, FoxP3 zu exprimieren und regulatorisch zu wirken, durch kontinuierliche Antigenstimulation in der Peripherie erwerben [60,61]. Letzteres ermöglicht, dass Autoimmunphänomene unterdrückt und Entzündungsprozesse nach einem Infektionsgeschehen terminiert werden, um eine chronische Inflammation zu unterbinden. Zusätzlich entsteht hierdurch eine Toleranz gegenüber natürlichen, beispielsweise intestinalen Kommensalen.

Die Identifikation funktioneller Marker, die die definitive Unterscheidung zwischen tTregs und anderen Tregs erlauben, ist für den Translationsprozess von wesentlicher Bedeutung und war daher das Ziel intensiver Untersuchungen. Leider erwies sich die alleinige Anwendung der klassischen Treg Marker $CD4^+CD25^{high}$ und $FoxP3^+$ bzw. $CD127^{low}$ zwar als notwendiges aber leider nicht hinreichendes Kriterium zur Bestimmung von tTregs [62–64]. Über lange Zeit waren daher die Expression des Transkriptionsfaktors Helios [65] sowie die Demethylierung der Treg-spezifischen Demethylierungsregion (TSDR) im FoxP3-Promotor [66] als zusätzliche Marker für tTregs anerkannt. Später konnte jedoch nachgewiesen werden, dass Helios auch in pTregs exprimiert wird während es in tTregs fehlen kann [67]. Gleichzeitig kann eine TSDR Demethlyierung auch in pTregs induziert werden [68]. Auf Grund dieser Plastizität können pTregs und andere Tregs zwischen pro- und anti-inflammatorisch wirkenden Phänotypen wechseln. Nach aktuellem Stand der Wissenschaft ist der stabile regulatorische Phänotyp von tTregs jedoch an die fehlende Induzierbarkeit proinflammatorischer Zytokine geknüpft und damit in Kombination mit der Expression von $CD4^+CD25^{high}Foxp3^+$ die sicherste Methode zur Identifikation von tTregs.

Tabelle 1: Regulatorische T-Zellpopulationen

Population	Auswahl charakteristischer Marker	Regulatoren	Zytokine	Ref.
tTregs	$CD25^{+/high}FoxP3^+CD127^{low}$	FoxP3	Keine; nach Aktivierung und Reifung: IL-10, TGF-ß	41,42,69–72
pTreg	$CD45RA^-CD25^+FOXP3^+$	FoxP3	IL-17, IFN-γ, IL-2, IL-10,IL-22, IL-4	72–75
Th1-like	$CXCR3^+$	FoxP3, t-bet	IL-10, IFN-γ	76–80
Th2-like	$CCR4^+CCR6^-CXCR3^-$	FoxP3, GATA3	IL-10	77,81,82
Th17-like	$CXCR3^-CCR6^+CCR4^+CCR10^-$	FoxP3, ROR γT	IL-10, IL-17	76–78,81–84
TH22-like	$CXCR3^-CCR6^+CCR4^+CCR10^+$	FoxP3	IL-10	78–80
Tfr	$CXCR5^+PD\text{-}1^+ICOS^+$	FoxP3, BCL6	IL-10	85–88
Tr1	$CD49b^+LAG3^+CD226^+CD25^{low}CTLA4^{low}$	$FoxP3^-$	IL-10, TGF-ß	89–93
Th3	$LAP^+CD69^+CD25^{low}CTLA4^{low}$	$FoxP3^-$	IL-10, TGF-ß, IL4	94–96

Nach Giganti *et al* Eur. J. Immunol. 2021 51:39-55 [97]

Die genaue Wirkmechanismen, die Tregs dazu befähigen, immunmodulatorisch zu wirken, sind derzeit (noch) nicht vollständig geklärt. Bisherige Erkenntnisse weisen darauf hin, das Tregs zur Ausübung ihrer immunmodulatorischen Wirkung ein Zusammenspiel aus verschiedenen Zell-Zell-Kontakt-abhängigen und -unabhängigen Mechanismen, die teils antigen-abhängig teils antigen-unabhängig initiiert werden, nutzen, um die Rekrutierung, Aktivierung und Proliferation von Effektor-T-Zellen effektiv zu hemmen [98]. Direkte Zell-Zell-Kontakte über eine Bindung von CTLA-4 an die kostimulatorischen Moleküle CD80/CD86

induzieren Tregs in antigenpräsentierenden Zellen die Expression von Indolamin-2,3-Dioxygenase (IDO) [99]. IDO führt zu einer Tryptophan-Depletion und damit sekundär zu einer Hemmung von Effektor-T-Zellen. Gleichzeitig verlieren antigenpräsentierende DCs die Fähigkeit der weiteren Antigen-Präsentation, so dass eine weitere Aktivierung von T-Zellen verhindert wird [98,100]. Unterstützt wird dieses negative Feedback durch aktive regenerative Mechanismen, die Tregs dazu befähigen, die Regeneration von Gewebe zu unterstützen [101]. Da das Überleben von tTregs über den IL-2 Rezeptor, CD25, sehr stark IL-2 abhängig ist, sind tTregs effektive IL-2-Konsumenten und hemmen über den Verbrauch von IL-2 die Aktivierung, Rekrutierung und Proliferation von Effektor-T-Zellen [43,102]. Neben der IL-2 Depletion scheint auch – wenn auch nicht tTreg-spezifisch – die CD39/CD73-vermittelte ATP-Depletion im Extrazellularraum eine wesentliche Rolle zu spielen. Hierfür bindet und dephosphoryliert CD39 Adenosintriphosphat (ATP) zu Adenosinmonophosphat (AMP); CD73 kann anschließend AMP binden und AMP zu Adenosin umwandeln [98,103]. Während ruhende und naive CD45RA+ tTregs keine Zytokine bilden, führt eine starke Aktivierung von tTregs zur Reifung zu CD45RA-Memory-tTregs, welche stark proliferieren und in der Lage sind, TGF-ß und IL-10 zu bilden [69]. Durch die Sekretion von TGF-ß und IL-10, aber auch cAMP schaffen tTregs zusätzlich ein „immuntolerantes“ Milieu, das die Aktivierung von Effektorzellen aktiv hemmt [104]. Über die Sekretion von Perforinen und Granzymen induzieren Tregs in Effektor-T-Zellen Apoptose, den programmierten Zelltod [105,106].

Die therapeutische Nutzung von Tregs und die Treg-vermittelte Induktion von immunologischer Toleranz entwickelte sich seit der Erstbeschreibung der Tregs zum heiligen Gral der Transplantationsmedizin. Einzelne, spezifische Treg-Funktionen für therapeutische Interventionen zu nutzen erschien in präklinischen Untersuchungen zunächst vielversprechend, erwies sich während früher klinischer Translationsschritte jedoch vielfach als schwierig. Lediglich Abatacept, ein rekombinantes Fusionsprotein, welches aus der extrazellulären Domäne des humanen CTLA4-Moleküls und dem Fc-Rezeptorteil des humanen Immunglobulins G1 besteht, wurde zur Drittlinien-Kombinationstherapie mit Methotrexat für die Behandlung der rheumatoiden Arthritis zugelassen. Dem gegenüber steht der Einsatz des CTLA-4-Inhibitors Ipilimumab, einem monoklonalen Antikörper, der CTLA-4 bindet, inaktiviert und damit die regulatorische Treg Funktion hemmt. Ipilimumab wurde 2011 als erster Checkpointinhibitor zur Therapie des metastasierten Melanoms zugelassen.

Vor dem Hintergrund zahlreicher tierexperimenteller [15] und klinischer Untersuchungen, die gute Hinweise darauf lieferten, dass tTregs eine wesentliche Rolle bei der Induktion und Aufrechterhaltung der Toleranz auch gegenüber Alloantigenen bzw. in der Steuerung der Transplantatabstoßung und Graft-versus-Host-Erkrankung (GvHD) [107–110] spielen, stellt der adoptive Trasnfer entsprechender Zellprodukte ein erfolgversprechendes, neues Konzept der immunmodulatorischen Therapie dar [111–113]. Entsprechend sind Tregs als therapeutisch einsetzbares Agens in den letzten Jahren stark in den Fokus translationaler Forschergruppen gerückt und die Etablierung sowie schlussendlich auch die Translation einer tTreg-Therapie zur Behandlung von Patienten mit unzureichend kontrollierten Auto- oder Alloimmunerkrankungen medizinisch erscheint längst überfällig. Aktuell sind bei www.clinicaltrials.gov (Stand 04/2022) insgesamt 241 interventionelle Studien, die Tregs als Zelltherapeutikum verwenden gelistet (Suche: *Intervention: Treg OR Regulatory T cell OR T-regulatory cell*), wovon 82 Studien aktiv (Filter: *Not yet recruiting OR Recruiting OR Enrolling by invitation OR Active, not recruiting*) und bereits 118 abgeschlossen (Filter: *Suspended OR Terminated OR Completed*) sind. Für den Einsatz von Tregs im Kontext solider Organtransplantationen (Suche: *Condition or disease: Transplantation AND Kidney OR Liver OR Lung OR Heart OR Intestine, Intervention: Treg OR Regulatory T cell OR T-regulatory cell*) sind aktuell 66 Studien gelistet, für die Indikation der GvHD (Suche: GvHD OR Graft vs Host Disease OR Graft Versus Host Disase or Graft Vs. Host Disease OR GvH disease; Intervention: Treg OR Regulatory T cell OR T-regulatory cell) 23 Studien, wovon lediglich 8 Studien einen adoptiven Treg Transfer bei chronischer GvHD untersuchen.

1.4. Ziele der Arbeit

Die Etablierung von Zellen als therapeutisches Agens nach dem Standard der Good-Manufacturing-Practice (GMP) bedarf größter Sorgfalt und translationale Entwicklungsschritte unterliegen einer strengen behördllichen Kontrolle. Um den Translationsprozess voran zu bringen, sind eine genaue Produktcharakteristik, die es uns erlaubt Rückschlüsse über die beste Produktzusammensetzung, eine Optimierung von Expansionsprotokollen sowie die Charakterisierung von Spender- und Empfänger-Konstellationen essentiell. Um den Weg der Translation schließlich beschreiten zu können, ist die Identifikation der Patientengruppen, die

unter einer sorgfältigen Risiko-Nutzen-Abwägung von einer Treg-Therapie profitieren können, zu identifizieren. Die Durchführung einer Treg-Therapie kann diesen Patienten dann im Rahmen einer klinischen Studie bzw. bei dringlicher klinischer Notwendigkeit im Rahmen eines individuellen Heilversuches angeboten werden. Im Rahmen der hier vorliegenden Arbeiten sind wir diese Schritte gegangen und wurden dabei von den folgenden drei Zielen geleitet:

I. Funktionelle und Phänotypische Charakteristika der tTregs von gesunder Probanden und Transplantationspatienten genau zu analysieren und in ihrer Bedeutung für die Treg-Therapie zu verstehen.
II. Produktionsprozesse zur Herstellung eines tTreg-Produktes, das für die klinische Anwendung geeignet ist, GMP-kompatibel zu optimieren und daraus resultierende tTreg-Produkte hinsichtlich ihrer phänotypischen und funktionellen Eigenschaften genau zu verstehen.
III. Treg-Produkte zugunsten von Transplantationspatienten in die klinische Anwendung zu bringen.

2. Eigene Arbeiten

Die im folgenden präsentierten Arbeiten fassen unsere Ergebnisse des Translationsprozesses von der Produktentwicklung bis in die klinische Anwendung am Patienten zusammen. Die vier Publikationen der Kapitel 2.1 – 2.4 zeigen unsere präklinischen Arbeiten, die für die Erlangung der Herstellungserlaubnis für *„natürliche regulatorische T-Lymphozyten (nTregs) als sterile, aseptisch hergestellte, kleinvolumige Parenteralia (flüssig) für die Modulation der Immunantwort von Patienten*“ beim Landesamt für Gesundheit und Soziales Berlin maßgeblich waren. In den Kapiteln 2.5 und 2.6 werden unsere ersten Erfahrungen im klinischen Einsatz von *ex vivo* expandierten und adoptiv transferierten Tregs bei Transplantationspatienten zusammengefasst.

Das erste Ziel unserer Arbeiten war, ein Protokoll zu entwickeln, das uns ermöglicht, mittels *ex vivo* Expansion ein sehr reines und funktionell hoch wirksames Treg-Produkt unter Berücksichtigung aller GMP-Standards herzustellen. Hierbei kamen sowohl polyklonale als auch allogenspezifische Stimulationsstrategien zum Einsatz. Dabei stand neben einer guten Qualität des Endproduktes die Entwicklung einer Strategie im Vordergrund, die ausreichende Zellzahlen aus 50 bis 100 ml peripher entnommenem Blut ohne die Notwendigkeit einer Leukapherese erreicht.

2.1. Neuartiges GMP-kompatibles Protokoll zur polyklonalen Expansion von allogen-spezifischen regulatorischen T-Zellen mit Hilfe einer allogenen B-Zell-Bank.

Der folgende Text wurde durch die Autorin übersetzt und entspricht im Wesentlichen dem Abstrakt der Arbeit

Landwehr-Kenzel S, Issa F, Luu SH, Schmück M, Lei H, Zobel A, Thiel A, Babel N, Wood K, Volk HD, Reinke P. Novel GMP-Compatible Protocol Employing an Allogeneic B Cell Bank for Clonal Expansion of Allospecific Natural Regulatory T Cells. American Journal of Transplantation, März 2014, Seiten 594-606, Band 14. [114]

Der adoptive Transfer natürlicher regulatorischer T-Zellen (nTreg) ist eine neue Möglichkeit, unerwünschte Immunreaktionen bei Autoimmunität und Transplantationen

in Richtung "Toleranz" zu verschieben. Erste klinische Studien, bei denen ein adoptiver Transfer von polyklonalen nTregs angewandt wurde, zeigten, dass dieser Therapieansatz sicher ist, und lieferten erste Hinweise zur Wirksamkeit. Die Sicherheit und Wirksamkeit dieses Ansatzes sind jedoch durch den geringen Anteil an antigenspezifischen Zellen innerhalb des polyklonalen nTreg-Kompartiments und deren breite, unspezifische Suppression limitiert. Seit kurzem können (allo)antigenspezifische nTregs mit Hilfe Treg-spezifischer Aktivierungsmarker isoliert und expandiert werden; die Ausbeute ist allerdings relativ gering. Hier beschreiben wir nun ein Good-Manufacturing-Practice (GMP)-kompatibles Expansionsprotokoll für Alloantigen-spezifische nTregs, das auf der Stimulation von nTregs durch allogen aktivierte B-Zellen beruht. Im Hinblick auf die Funktionalität und Spezifität sind diese nTregs polyklonal expandierten nTregs sowohl in vitro als auch in vivo überlegen. Der Einsatz einer allogenen B-Zell-Bank, die die meisten HLA-Typen umfasst, ermöglicht die schnelle, GMP-kompatible Herstellung Spender-spezifischer nTregs für klinische Anwendungen in Kontext von Organ- und Stammzelltransplantationen. Die Analyse des T-Zell-Rezeptor-Repertoires durch Next-Generation-Sequencing zeigte eine beachtliche Expansion um mehrere Log-Stufen von Klonen, die in nur sehr geringer Menge vorlagen. Diese neuartige Methode bietet daher einen einfachen Ansatz zur Expansion antigenspezifischer nTregs, zeichnet sich durch eine hohe Reproduzierbarkeit aus und ist einfach auf volle GMP-Standards zu transferieren.

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Novel GMP-Compatible Protocol Employing an Allogeneic B Cell Bank for Clonal Expansion of Allospecific Natural Regulatory T Cells

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The adoptive transfer of natural regulatory T cells (nTreg) is a new option to reshape undesired immune reactivity in autoimmunity and transplantation toward "tolerance." The first clinical trials using adoptive transfer of polyclonal nTreg demonstrated safety and hints of efficacy. However, the low frequencies of antigen-specific cells among the pool of polyclonal nTreg and their broad antigen nonspecific suppression are limitations of this approach regarding efficacy and safety. Recently, the isolation and expansion of (allo)antigen-specific nTreg have successfully been achieved by using Treg-specific activation markers but the yield is relatively low. Here, we describe a novel good manufacturing practice (GMP)-compatible expansion protocol of alloantigen-specific nTreg based on the stimulation of nTreg by allogeneic activated B cells. Their functionality and specificity are superior compared to polyclonal nTreg both *in vitro* and *in vivo*. Employing an allogeneic B cell bank, designed to cover the majority of HLA types, allows fast GMP-compliant manufacturing for donor-specific nTreg for clinical application in organ and stem cell transplantation. TCR repertoire analyses by next generation sequencing revealed impressive expansion by several log-steps of even very low-abundance alloantigen-specific nTreg clones. This novel method offers a simple approach for expanding antigen-specific nTreg and is characterized by high replicability and easy transferability to full GMP standards.

Keywords: Cell therapy, clinical application, regulatory T cells, tolerance, transplantation

Abbreviations: ABCB, allogeneic B cell bank; APCs, antigen-presenting cells; CDR3, complementary determining region 3; DCs, dendritic cells; EBV, Epstein Barr virus; FACS, fluorescence-activated cell sorting; FMO, fluorescence minus one; FoxP3, Forkhead-Box-Protein P3; GMP, good manufacturing practice; GvHD, graft versus host disease; MFI, mean fluorescence intensity; mTOR, mammalian target of rapamycin; NGS, next generation sequencing; nTreg, natural regulatory T cells; PBMCs, peripheral blood mononuclear cells; Tconv, conventional T cells; Tresp, responder T cells

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Introduction

Naturally arising regulatory $CD4^+CD25^{++}FoxP3^+$ T cells (nTreg) play an essential role in the induction and maintenance of immunological tolerance to self-antigens. Loss in nTreg numbers or functionality is associated with fatal autoimmune diseases in both murine models and humans (1,2). In addition to their protective role in autoimmunity, nTreg have been demonstrated in several mouse model studies to play a key role in the induction and maintenance of tolerance to alloantigens, thus controlling allograft rejection and graft versus host disease (GvHD) (3–7). Consequently, adoptive transfer of nTreg is considered a promising new treatment option to reshape the autoimmune or alloimmune response toward tolerance. We have recently demonstrated that the adoptive transfer of nTreg induces tolerance to renal allografts in a clinically relevant model using a high responder strain combination and enhanced frequencies of donor-reactive memory T cells in the graft recipients (8). Adoptive transfer of nTreg from healthy pregnant mice also reverses the high fetal abortion rate in an abortion-prone combination (9). Interestingly, expanded nTreg have been reported to be more effective in suppressing undesired immune reactions than freshly isolated nTreg, suggesting that preactivation via their TCRs and IL-2 receptors enhances functionality (10).

Despite promising results from nTreg in various preclinical models, the translation into clinical trials has been a challenge due to the substantial hurdles regulating good manufacturing practice (GMP)-compliant manufacturing processes and clinical trials using novel approaches. In addition, the low frequency of nTreg in peripheral blood makes their isolation and expansion challenging, with leukapheresis indispensable as an initial source for nTreg isolation (11). Recently, however, powerful expansion protocols using polyclonal stimulation in the presence of the mammalian target of rapamycin (mTOR) inhibitor have been developed and allow rapid and sufficient expansion of human nTreg while maintaining their functionality and Forkhead-Box-Protein P3 (FoxP3) promoter demethylation (12–15). Under optimal conditions the average yield of nTreg after 2–3 weeks of polyclonal expansion can be estimated at 1×10^8 cells/100 mL of peripherally collected blood (own unpublished data). While the absolute number of nTreg that is required to be adoptively transferred to induce tolerance in solid organ or hematopoietic stem cell transplantation is still under debate, preclinical and clinical data suggest that 1×10^8 cells ($1–50 \times 10^5$ cells/kg) could be sufficient for effective prevention of alloresponses by conventional T cells (Tconv) (8,16–19).

Based on these promising preclinical data and the recent availability of GMP-compliant isolation and expansion protocols for human nTreg, the first clinical trials have successfully been initiated to combat GvHD by adoptive transfer of freshly isolated or polyclonally expanded nTreg. These clinical pilot trials demonstrated safety and suggested some efficacy by successfully preventing GvHD in a significant number of patients by transfer of 2×10^6 to 5×10^6 Treg/kg (16,18,20).

However, as a result of the low frequency of antigen-specific cells within the physiologic pool of nTreg, the transfer of large numbers of polyclonal nTreg is required to achieve sufficient efficacy. To prevent GvHD in murine models (including in humanized mouse models), nTreg have to be adoptively transferred into the lymphopenic host at a ratio of about one nTreg to one Tconv. In a clinically relevant organ transplantation model, nTreg are only protective if combined with *in vivo* T cell depletion to induce an adequate balance between regulatory and effector cells (8). Transfer of high numbers of nonspecific nTreg might be detrimental for the control of infections and tumors. Since (allo)antigen-specific nTreg are more potent in suppressing a particular (allo)response (21–24), significantly lower numbers of antigen-specific nTreg would be needed and are assumed to carry a lower risk of nonspecific suppression. However, in order to establish a safe and efficient antigen-specific nTreg product for clinical applications, it will be a prerequisite to establish GMP-compliant methods that allow unequivocal isolation and expansion of antigen-specific nTreg. This is a particular challenge because of the low frequency of antigen-specific nTreg.

Very recently, we characterized specific activation markers on (allo)antigen-specific nTreg that allow precise discrimination of nTreg from Tconv and the isolation of (allo)antigen-specific nTreg clones (25). Compared to polyclonal nTreg pools, antigen-specific nTreg were 50- to 100-fold more potent in suppressing the respective antigen-triggered Tconv response. However, the yield of antigen-specific versus polyclonal nTreg is very low, even after expansion, which limits translation into clinical trials. In addition, Chen et al showed that allogeneic B cells are able to induce alloantigen-specific nTreg in the presence of additional co-stimulation by anti-CD28 mAb (26). This strategy is further supported by other groups that also suggest B cell stimulation to generate alloantigen-specific Treg (22,23).

For applicable allospecific nTreg expansion, a safe, standardized and easily accessible GMP-grade source of Treg stimulating agents is essential. Therefore, we aimed to establish a protocol that is geared to these needs. Here, we present a technology that employs an allogeneic B cell bank (ABCB) to alloantigen-specifically induce nTreg expansion. The ABCB is composed of B cell lines from a range of healthy donors selected to reach a coverage >90% of HLA types in our patient cohorts. This allows a fast and specific selection of the respective alloantigens required to generate stem cell recipient–specific or solid organ transplant donor–specific nTreg. We further demonstrate that alloantigen specificity is tightly linked to oligoclonal expansion even of initially very low-abundance allospecific nTreg clones by data from TCR repertoire analyses using next-generation sequencing (NGS). Finally, we confirm that alloantigen-specific nTreg are superior suppressors of alloimmune activation as compared to polyclonal nTreg both *in vitro* and *in vivo*.

Materials and Methods

Allogeneic B cell bank

The donors of the ABCB were previously described (27–29). Briefly, 19 healthy blood donors were identified whose HLA-A and -B and -DR antigens cover more than 90% of the German population (Table S1). T cell depleted (RosetteSep; StemCell Technologies, Inc., Vancouver, BC, Canada) buffy coats of the donors were aliquoted and cryopreserved. The generation of conditional immortalized B cell lines was adapted from a previously described study (30). Aliquots of the 19 donors were serially diluted from 2×10^5 to 1.2×10^4 cells and plated on feeder cell–coated 96-well plates. CD40L-expressing fibroblasts were used as feeder cells and irradiated (180 Gy). A CD40L-expressing cell line that allows xenoantigen-free expansion of B cells is also available at GMP grade (31). B cell cultures were transferred every 5–7 days onto fresh feeder cell-coated plates. Cells in mid-log growth phase were recovered, lethally gamma-irradiated (30 Gy), and aliquots of 10^7 cells per vial were cryopreserved in serum plus 10% DMSO.

nTreg isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly collected blood of healthy volunteers by Ficoll density-gradient centrifugation (PAA Laboratories, Pasching, Austria). Treg isolation was performed either from PBMCs employing the $CD4^+CD25^+$ Treg isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's

recommendations or from buffy coats (Deutsches Rotes Kreuz, Blutspendedienst, Berlin, Germany) by CliniMACS isolation (Miltenyi Biotec) performing CD8-depletion followed by $CD25^+$ enrichment. Cell purity was determined by fluorescence-activated cell sorting (FACS) analysis staining for CD4-A700 (Biolegend, San Diego, CA), CD25-PE (BD Pharmingen, San Diego, CA) and FoxP3-A488 (BD Pharmingen). The Charité University Medicine Berlin ethics committee (Institutional Review Board) approved the study, and all blood donors provided written informed consent.

nTreg expansion

Freshly isolated nTreg were suspended in complete medium (X-VIVO 15 medium; Lonza, Basel, Switzerland) containing 10% human AB serum, 500 U/mL human recombinant IL-2 (Chiron Behring, Marburg, Germany) and 100 nM rapamycin (Sigma–Aldrich, St. Louis, MO). After isolation, cells were rested overnight before stimulated either with (CD3/CD28) Treg expansion beads (Miltenyi Biotec) according to the manufacturer's protocol or with B cell lines generated as described above. T cells were restimulated every 4–6 days (in total three to four antigen restimulations) with freshly thawed B cells of the indicated HLA type or polyclonal Treg expansion beads.

TCR high throughput sequencing

Complementary determining region 3 (CDR3) sequencing was performed on the ImmunoSEQ platform at Adaptive Biotechnologies as previously described in Robins et al (32) and Sherwood et al (33). Sequences that did not match CDR3 sequences were removed from the analysis. For further analysis, the standard algorithm as developed by Adaptive Biotechnologies and previously described in Monod et al (34) was used.

Suppression analysis

Polyclonally or alloantigenspecifically expanded nTreg were labeled with CFSE or CellTrace Violet (Invitrogen Life Technologies, Darmstadt, Germany), rested overnight and cocultured with freshly isolated or cryopreserved autologous PBMCs responder T cells (Tresp) as indicated (constant Tresp number at 10^5/well/200 çL medium). Cells were stimulated with lethally irradiated B cells as used for nTreg expansion, with a thirdparty HLAtyped B cell line, polyclonal T cell activation beads or a vehicle control for 7 h. Cells were stained using the FastImmune Human Regulatory T cell Function Kit (BD Pharmigen, San Diego, CA) according to the manufacturer's recommendations. An adapted inhouse kit revealed similar results. FACS analysis was performed on a NaviosÛ flow cytometer (Beckman Coulter, Krefeld, Germany). Data analysis was performed using FlowJo software version 8 (Tree Star, San Carlos, CA) or FCS Express (De Novo Software, Los Angeles, CA). CFSE/CellTrace Violetpositive nTreg cells were excluded by hierarchical gating, and responder cells were analyzed for the expression of CD154 and CD69 (in Figure 4 expressed as geometric mean fluorescence intensity [MFI]). Further analysis and graph design were performed using Microsoft Excel 2010 (Redmond, WA) and GraphPad Prism 5 (La Jolla, CA).

Humanized mouse skin transplantation model in $Rag2^{-/-}c\gamma^{-/-}$ mice

Protocols were approved by the Committee on Animal Care and Ethical Review at the University of Oxford in accordance with the UK Animals (Scientific Procedures) Act 1986. Collection of human tissue samples was performed with fully informed written consent and ethical approval from the Oxfordshire Research Ethics Committee (REC B), study number 07/H0605/130.

BALB/c $Rag2^{-/-}c\gamma^{-/-}$ (H2d) mice were housed under specific pathogen-free conditions in the Biomedical Services Unit of the John Radcliffe Hospital (Oxford, UK). Skin transplantation was performed as previously described (35–37). Only mice displaying >1% splenic human leukocyte chimerism were included in analyses. Recipients of human skin grafts received 5×10^6 human PBMCs (allogeneic to the skin graft donor) in pure RPMI via intraperitoneal injection, with or without 1×10^6 expanded nTreg from the same donor as the PBMCs. Skin graft rejection was evaluated in a blinded fashion with two persons scoring the graft independently.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 5. Suppression data from *in vitro* experiments are expressed as % suppression within each condition calculated as 100-([(MFI in presence of Treg)/(MFI in absence of Treg)] × 100). Log-rank tests were applied to *in vivo* graft survival data.

Results

Alloantigen-specific nTreg expansion can be induced by conditional B cell blasts from a readily available HLA-typed ABCB

Stimulation of nTreg by allogeneic dendritic cells (DCs) triggers allospecific nTreg to express 4-1BB (CD137) activation antigen and to proliferate in presence of IL-2 (25). As isolation of DCs is complicated, we tested whether freshly isolated B cells are able to replace DCs as antigen-presenting cells (APCs). In fact, allogeneic B cells stimulate nTreg and induce clonal expansion in presence of IL-2 but the proliferation reaches a low-level plateau within few days associated with enhanced frequency of apoptotic cells (data not shown). Freshly isolated B cells express low levels of CD28 and other co-stimulatory molecules that are essential to deliver survival signals to nTreg (26,38). In fact, the co-stimulatory CD28 ligand, CD80, is strongly up-regulated following B cell stimulation by co-incubation with a CD40L-expressing cell line (Figure S1).

Therefore, we used CD40L expanded allogeneic B cell lines for stimulation of nTreg. In contrast to previous studies where nonirradiated or Epstein Barr virus (EBV)-immortalized B cells were used (22,23), we repetitively monitored the EBV status by polymerized chain reaction to confirm negativity and exclude infection of the final nTreg product. Freshly isolated nTreg (1×10^5 to 1×10^6/starting culture) were stimulated with allogeneic, 30 Gy-irradiated B cell lines. The total number of nTreg expanded by a mean factor of 80 and 120 within 2 and 3 weeks, respectively, if stimulated every 7 days at a ratio of 10 B cells per nTreg cell (Figure 1A, blue dots). Alternatively, nTreg were stimulated with anti-CD3/anti-CD28 Treg expansion beads for polyclonal activation. The polyclonally expanded nTreg yield was comparably high but not significantly superior as compared to allogeneic stimulation (Figure 1A, green dots). To exclude B cell contamination in the final nTreg product, B cell viability was determined in pure B cell cultures (Figure 1B) and B cell/T cell co-cultures (Figure 1C). Initial B cell viability of thawed and resuspended cells was around 90%. Over the next 3 days cell viability and cell count were rapidly decreasing and below the lower detection limit on day 4 (Figure 1B). FACS analysis of the nTreg product >4 days after last B cell restimulation revealed high CD3 purity without relevant contamination by $CD19^+$ B cells (<0.5%) (Figure 1C). The final nTreg products were >90%

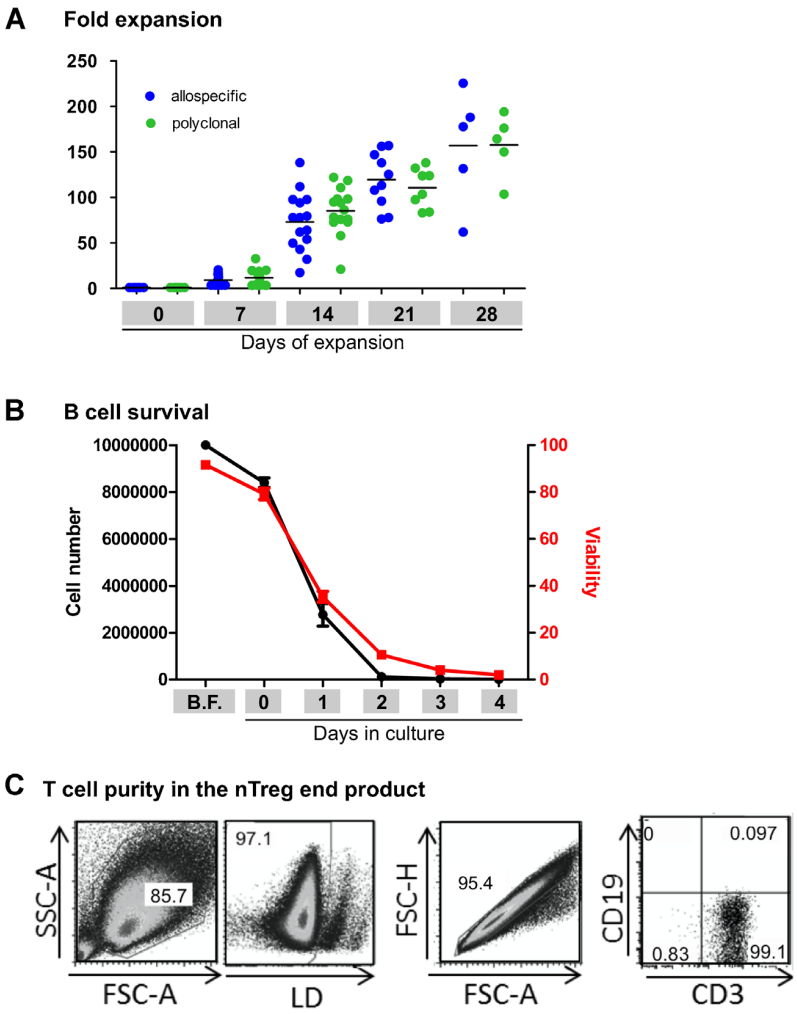


Figure 1: Alloantigen-specific expansion of natural regulatory T cells (nTreg) is highly efficient. (A) $CD4^+CD25^+$ nTreg cells were isolated by magnetic bead separation; 1×10^5 to 1×10^6 cells per starting culture were stimulated with Treg expansion beads (polyclonal) or lethally radiated HLA-mismatched B cells from our allogeneic B cell bank (allospecific). Depicted are fold expansion rates relative to the initial nTreg starting counts and arithmetic mean values. Blue dots, allogeneic B cell activation; green dots, polyclonal stimulation. (B) Depicted are B cell viability (red line) and B cell counts (living cells, black line) from three individual aliquots of radiated B cells (mean ± SD) before freezing (B.F.) and after freezing during 5 days of co-culture. (C) The final allospecific nTreg product was stained for CD3, CD19 and a live/dead cell dye to show purity. Gates were set on lymphocytes, living cells and singlets prior to analysis for CD3 (nTreg) and CD19 (B cells).

$CD4^+CD25^+FoxP3^+$ (Figure 2A) and did not contain significant amounts of effector cytokine producers following stimulation with PMA and Ionomycin (IL-2, TNF, IFNγ, IL-17A: all <5%) (Figure 2B and E). Similar purity data were observed for polyclonally expanded nTreg (Figure 2C–E).

For clinical therapy, it is important to have B cell lines as readily available ''off-the-shelf'' products since the GMP-compliant B cell expansion and release process takes time. Therefore, we established an ABCB from a panel of 19 healthy donors that represent the most frequent HLA antigens of the German population and reach a coverage of >90% (27,28). These cells can be isolated, expanded and released according to specific GMP requirements and, subsequently, be cryopreserved in small aliquots. From this ABCB particular B cell lines with the relevant HLA characteristics can be identified and used for alloantigen-specific nTreg stimulation. An example of this process is described in Table 1.

TCR analysis reveals clonal expansion of alloantigen-specific nTreg in response to HLA-mismatched B cell stimulation

We hypothesized that anti-CD3/anti-CD28 bead expansion results in a polyclonal TCR repertoire of expanded nTreg without significant clonal bias while the allogeneic B cell-

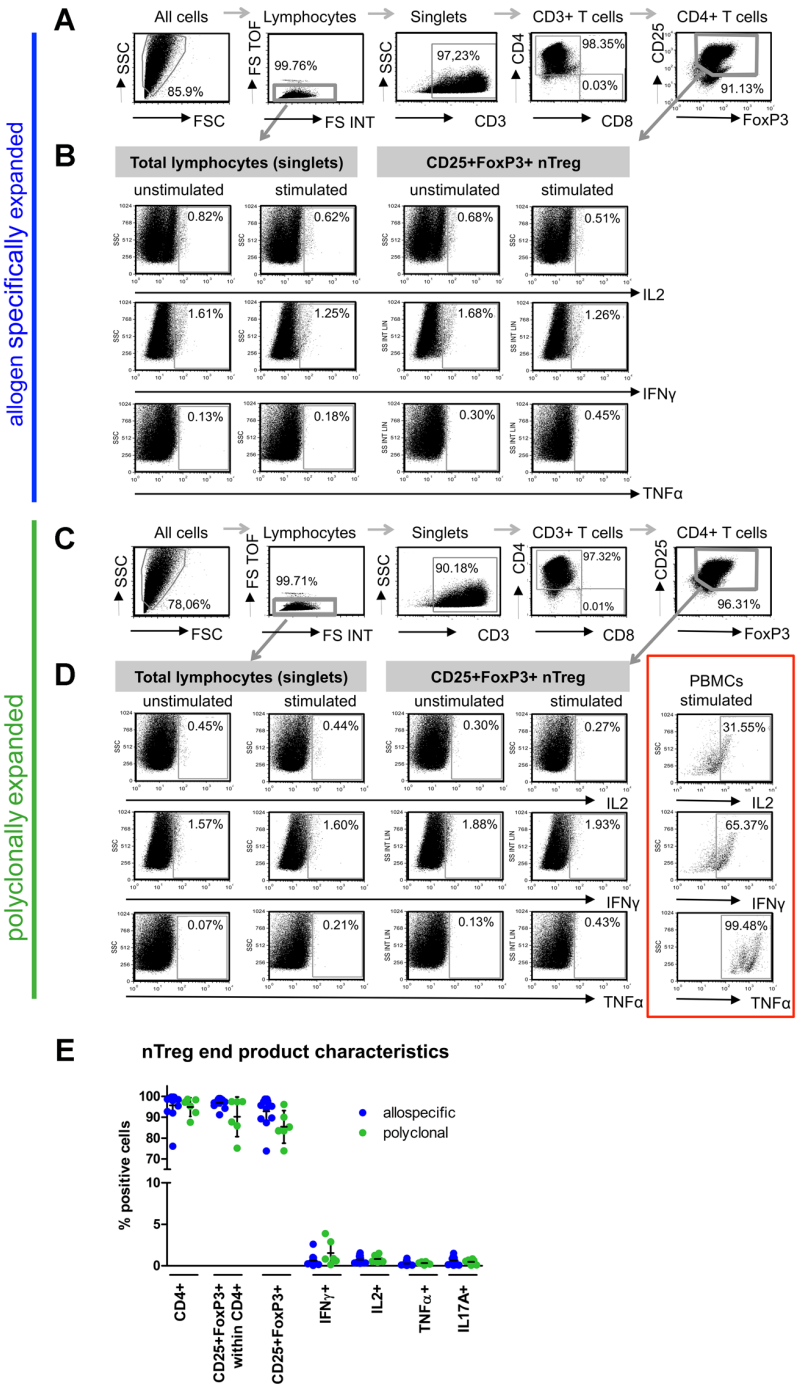


Figure 2: Alloantigen-specifically and polyclonally expanded nTreg products are phenotypically and functionally highly pure $CD4^{+}CD25^{+}FoxP3^{+}$ cells. Polyclonally and allogen-specifically expanded nTreg or freshly isolated PBMCs were stimulated with PMA/Ionomycin and analyzed for phenotypic nTreg markers and the formation of effector cytokines. Both alloantigen-specifically expanded (A and B) and polyclonally expanded (C and D) nTreg products show high purity (>90%) as defined by the expression of $CD4^{+}CD25^{++}FoxP3^{+}$ (A and C), while the frequency of contaminating effector cytokine-producing Tconv (impurity) remains <2% (B and D). PBMCs (red box) were used as positive control representing Tconv (D). Depicted are exemplary data from one out of multiple experiments. (E) Summary of phenotypic and functional nTreg end product characteristics after polyclonal (n = 6) or alloantigen-specific (n = 12) cell expansion. nTreg, natural regulatory T cells; PBMCs, peripheral blood mononuclear cells; Tconv, conventional T cells.

Table 1: Selection process for allogeneic B cell lines from allogeneic B cell bank (ABCB) for nTreg expansion

1. Comparison of PBMC (alloreactive T cell) and tissue (here skin graft) HLA-types

	HLA-I				HLA-II			
	A		B		DR		DQ	
T cell HLA	02	02	44	44	04	12	n.d.	n.d.
Tissue HLA	32	32	44	35	15	13	6	6

2. Identification of clinically relevant antigeneic HLA-mismatches presented on tissue cells which can be recognized as "nonself" by alloantigen-reactive T cells

	HLA-I				HLA-II			
	A		B		DR		DQ	
T cell HLA	02	02	44	44	04	12	n.d.	n.d.
Tissue HLA	**32**	**32**	44	**35**	**15**	**13**	**6**	**6**

3. Identification of B cell lines that cover antigeneic HLA-mismatches

	HLA-I				HLA-II			
	A		B		DR		DQ	
#1	29	**32**	7	44	7	**15**	202	602
#2	02	11	**35**	39	01	**13**	05	**06**
#3								
#4				**35**				
#5			**35**					
#6								
#7						**13**		
#8								
#9								
#10				**35**				
#11				**35**				
#12								
#13			**35**					
#14								
#15								
#16								
#17								
#18						**15**		
#19			**35**					

4. Use of appropiate B cell lines for nTreg expansion

To select B cell lines appropriate for generation of an allospecific nTreg cell product, (1) PBMCs (alloreactive T cells) and tissue (in this case, skin grafts) HLA types are compared. This allows (2) the identification of clinically relevant antigenic HLA mismatches presented on tissue cells, which can be recognized as nonself by alloantigen-reactive T cells and potentially trigger rejection/graft versus host disease processes. (3) Next, the ABCB is screened for B cell lines that carry HLA markers mismatched in a T cell to tissue direction. (4) B cell lines that allow a maximal coverage at minimal number of required cell lines are selected as stimulatory cells for the expansion process. nTreg, natural regulatory T cells; PBMCs, peripheral blood mononuclear cells.

driven expansion induces an oligoclonal expansion of alloantigen-specific nTreg clones. To verify this hypothesis, the TCR repertoire of different nTreg products was analyzed by NGS.

The process of variable (V), joining (J) and diversity (D) segments (V(D)J recombination) within CDRs provides an extremely high combinatorial diversity that can include up to 10^{15} distinct T cell clonotypes, with about 5×10^7 per human (39). NGS can be used as sensitive monitoring tool to track clonal expansion of specific T cell subsets for which it has been introduced for treatment monitoring (33,40). In line with these data, we used NGS to assess three cell products of five different donors: (i) freshly isolated nTreg, (ii) polyclonally expanded nTreg and (iii) alloantigen-specifically expanded nTreg. Genomic DNA was isolated from at

least 250 000 cells of each product. On average, about 3.5×10^6, 1.3×10^6 and 0.8×10^6 productive sequences were identified with 0.82%, 0.67% and 0.4% unique clones in freshly isolated, polyclonally expanded and alloantigen-specifically expanded nTreg, respectively. Within these, we found the average percentage of the most frequent clone being 2.09% (freshly isolated), 4.49% (polyclonally expanded) and 32.65% (alloantigen-specifically expanded) suggesting a strong clonal bias in the latter (Table 2). To visualize the clonal bias after alloantigen-driven expansion, Figure 3 shows the 200 most frequent clones in each cell product of two (Figure 3A and B) and three (Figure 3C–E) donors. Frequency analysis revealed massive expansion of individual clones in nTreg activated by allogeneic B cell lines (Figure 3A–E). The charts impressively demonstrate the clonal bias by alloantigen stimulation while the repertoire remained diverse after polyclonal nTreg expansion (Figure 3A and B). The frequency curves of allospecific nTreg further illustrate high numbers of the top 30–150 clones but significantly reduced frequencies of the following clones compared to freshly isolated nTreg (Figure 3C–E). If frequencies of >1% are defined as clear clonal expansion, we observed 2–12 individual clones expanded within the alloantigen-driven nTreg products, reaching peak frequencies of >1% to >80%. By contrast, freshly isolated nTreg displayed a broad polyclonal TCR repertoire with individual clonal frequencies commonly of the order <0.1% and usually fewer one or two clones with frequencies of >1% (Table 2). Similarly, unbiased TCR distribution is seen within polyclonally expanded nTreg (Figure 3A and B).

Interestingly, the most dominant clones (>1%) present in the alloantigen-specific nTreg products were detectable only at very low frequencies (<0.005%) in polyclonally expanded or freshly isolated nTreg (Figure 3C–E). These data demonstrate the dramatic expansion of low-abundance clones following alloantigen stimulation by up to >10 000-fold.

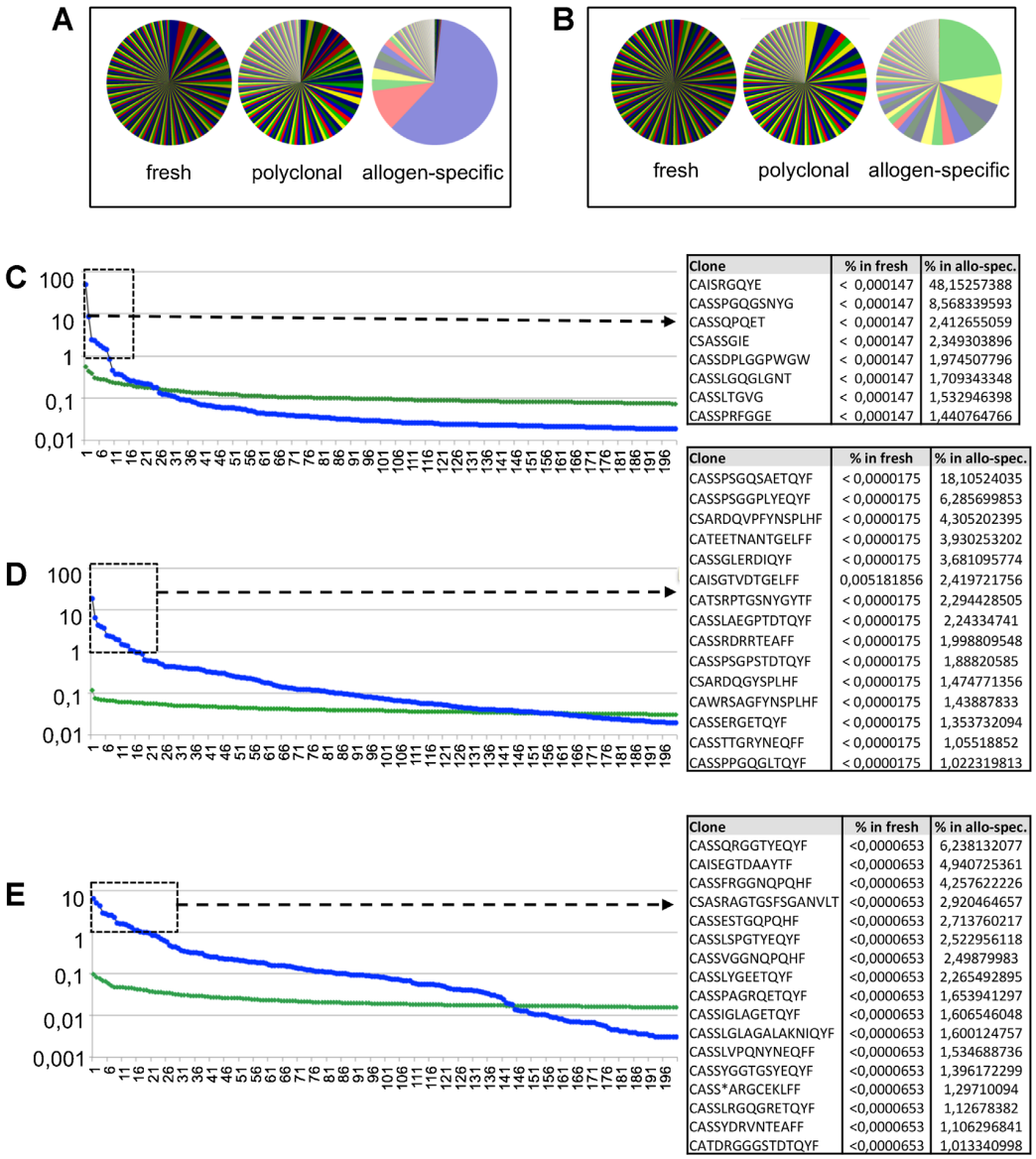
Alloantigen-specifically expanded nTreg are superior suppressors of alloantigen-activated conventional CD4$^+$ and CD8$^+$ T cells (Tconv) in vitro

Next, we studied the functionality of our nTreg products. Their capacity to suppress proliferation of CD4$^+$CD25$^-$ and CD8 Tconv is the traditional gold standard to analyze the functionality of Treg (41). However, the test has a high variability and is time-consuming. Recently, we have shown that nTreg, in contrast to CD4$^+$ Tconv, do not up-regulate CD154 following activation (25). Based on these differences, a novel functional nTreg test has recently been developed and is now commercially available. It is based on the quantification of stimulation-induced up-regulation of CD154 and CD69 as surrogate activation markers on CD4$^+$ and CD8$^+$ Tconv and the suppression of both markers in the presence of nTreg (e.g. [42], own unpublished data). This test is very robust, shows low inter-assay variability, and can be performed within 1 day. To determine whether alloantigen-specifically expanded nTreg are superior suppressors of respective allogeneic Tconv activation as compared to polyclonal nTreg pools, suppression experiments were performed with autologous PBMCs as responders in presence/absence of fluorescently labeled, resting nTreg lines. nTreg/Tconv co-cultures were specifically (re)-activated with allogeneic B cells from the ABCB of the same line used for nTreg expansion. As control, a third-party B cell line was chosen from ABCB to test specificity (Figures 4 and 5). Polyclonally expanded nTreg were used as gold standard. We found that polyclonal nTreg dose-dependently inhibited activation marker expression of Tconv with maximal suppression at Treg:Tconv ratios of 1:1 to 1:4. Similar results were obtained when alloantigen-specifically expanded nTreg were tested for their capacity to suppress third-party allo-activation. In contrast, alloantigen-specific nTreg were significantly more potent (>5-fold) in suppressing Tconv activation if the target-specific alloantigen was used for stimulation (Figures 4 and 5). In our test system, this suppressive superiority was

Table 2: TCR repertoire analysis of nTreg using high throughput TCR CDR3 sequencing

Cell product	Total productive sequences	% Unique	% Top 10	# Clones >1%	% Frequency of most frequent clone
Freshly isolated					
n	5	5	5	5	5
Mean	3 589 475.20	0.82	4.37	0.40	2.09
SEM	1 488 369.31	0.19	2.44	0.36	1.59
Polyclonally expanded					
n	4	4	4	4	4
Mean	1 352 157.25	0.67	15.62	3.00	4.49
SEM	450 324.88	0.31	11.96	3.00	3.97
Alloantigen-specifically expanded					
n	5	5	5	5	5
Mean	881 851.00	0.40	50.92	9.00	32.65
SEM	373 817.97	0.14	13.21	2.94	14.65

Genomic DNA was extracted from freshly isolated and either polyclonally or alloantigen-specifically expanded nTreg. Next generation sequencing was performed for TCR (CDR3) repertoire analysis. Depicted are summarized data showing the total number of productive sequences and percent unique sequences. The cumulative percentage of the 10 and 200 most frequent clones and the number of clones present at a frequency of >1% are also summarized. CDR3, complementary determining region 3; nTreg, natural regulatory T cells.



Clone	% in fresh	% in allo-spec.
CAISRGQYE	< 0,000147	48,15257388
CASSPGQGSNYG	< 0,000147	8,568339593
CASSQPQET	< 0,000147	2,412655059
CSASSGIE	< 0,000147	2,349303896
CASSDPLGGPWGW	< 0,000147	1,974507796
CASSLGQGLGNT	< 0,000147	1,709343348
CASSLTGVG	< 0,000147	1,532946398
CASSPRFGGE	< 0,000147	1,440764766

Clone	% in fresh	% in allo-spec.
CASSPSGQSAETQYF	< 0,0000175	18,10524035
CASSPSGGPLYEQYF	< 0,0000175	6,285699853
CSARDQVPFYNSPLHF	< 0,0000175	4,305202395
CATEETNANTGELFF	< 0,0000175	3,930253202
CASSGLERDIQYF	< 0,0000175	3,681095774
CAISGTVDTGELFF	0,005181856	2,419721756
CATSRPTGSNYGYTF	< 0,0000175	2,294428505
CASSLAEGPTDTQYF	< 0,0000175	2,24334741
CASSRDRRTEAFF	< 0,0000175	1,998809548
CASSPSGPSTDTQYF	< 0,0000175	1,88820585
CSARDQGYSPLHF	< 0,0000175	1,474771356
CAWRSAGFYNSPLHF	< 0,0000175	1,43887833
CASSERGETQYF	< 0,0000175	1,353732094
CASSTTGRYNEQFF	< 0,0000175	1,05518852
CASSPPGQGLTQYF	< 0,0000175	1,022319813

Clone	% in fresh	% in allo-spec.
CASSQRGGTYEQYF	<0,0000653	6,238132077
CAISEGTDAAYTF	<0,0000653	4,940725361
CASSFRGGNQPQHF	<0,0000653	4,257622226
CSASRAGTGSFSGANVLT	<0,0000653	2,920464657
CASSESTGQPQHF	<0,0000653	2,713760217
CASSLSPGTYEQYF	<0,0000653	2,522956118
CASSVGGNQPQHF	<0,0000653	2,49879983
CASSLYGEETQYF	<0,0000653	2,265492895
CASSPAGRQETQYF	<0,0000653	1,653941297
CASSIGLAGETQYF	<0,0000653	1,606546048
CASSLGLAGALAKNIQYF	<0,0000653	1,600124757
CASSLVPQNYNEQFF	<0,0000653	1,534688736
CASSYGGTGSYEQYF	<0,0000653	1,396172299
CASS*ARGCEKLFF	<0,0000653	1,29710094
CASSLRGQGRETQYF	<0,0000653	1,12678382
CASSYDRVNTEAFF	<0,0000653	1,106296841
CATDRGGGSTDTQYF	<0,0000653	1,013340998

Figure 3: In contrast to polyclonal stimulation, allostimulation by B cell lines leads to clonal expansion of even very low-abundance alloantigen-specific nTreg clones. Genomic DNA was extracted from freshly isolated (fresh) and either polyclonally (polyclonal) or alloantigen-specifically (allogen-specific) expanded nTreg. Next generation sequencing was performed for TCR repertoire analysis. (A and B) Depicted are representative pie charts from freshly isolated (fresh) and either polyclonally (polyclonal) or alloantigen-specifically (allogen-specific) expanded nTreg illustrating schematically the proportional distribution of the 200 most frequent clones in three nTreg products generated from two representative donors. (C, D and E) Depicted are % clonal frequencies of the 200 most frequent clones within the total cell product of fresh (green dots) versus allospecifically expanded (blue dots) nTreg from three representative samples. The tables on the right of each chart show the amino acid sequences of all dominant clones (expanded to >1%) within the allospecific nTreg products and their frequencies within the polyclonal nTreg starting pool before alloantigen-driven expansion. Representative examples are selected from multiple experiments summarized in Table 2. nTreg, natural regulatory T cells.

independent of the degree of clonal expansion. Furthermore, we were able to confirm the purity of our nTreg products by the absence of CD154 up-regulation on both alloantigen-specifically and polyclonally expanded nTreg in response to polyclonal TCR activation (Figure 5C).

Alloantigen-specifically expanded nTreg are superior suppressors of allogeneic skin graft rejection in vivo

Recently, we developed a humanized skin allograft model that is a powerful tool for the *in vivo* testing of the regulatory efficacy of human cellular therapies. Human skin grafts are

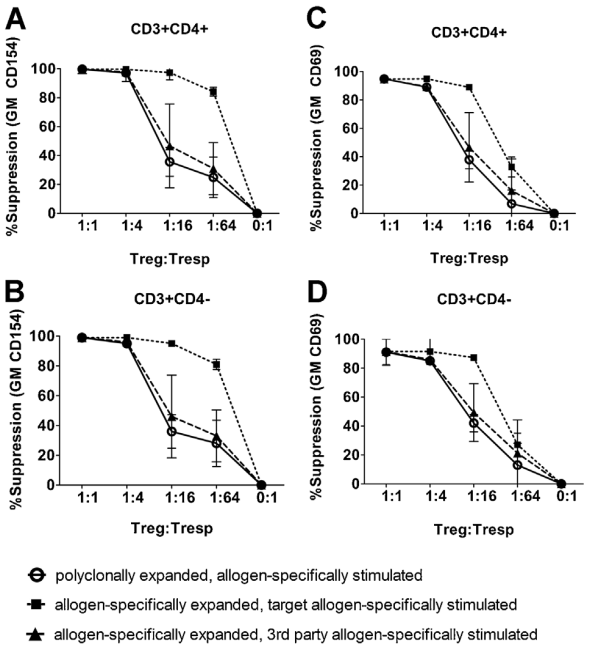


Figure 4: Alloantigen-specific nTreg are superior suppressors of target alloantigen-reactive conventional T cells (Tconv). Suppressive function was assessed by alloantigen-mediated stimulation of nTreg/PBMCs co-cultures. Depicted are mean values ± SD from five individual sample sets (individual cell lines from five different donors) as % suppression based on geometric mean values (MFI) of (A and B) CD154 and (C and D) CD69 expression on (A and C) $CD3^+CD4^+$ and (B and D) $CD3^+CD4^-$ Tconv. MFI, mean fluorescence intensity; nTreg, natural regulatory T cells; PBMCs, peripheral blood mononuclear cells; Tconv, conventional T cells.

permanently accepted if grafted into $Rag2^{-/-}c\gamma^{-/-}$ mice, but rejected within 3.5 weeks after adoptive transfer of 5×10^6 allogeneic Tconv. Moreover, we previously demonstrated that human Tconv reconstitution in mice is not significantly different in the presence of nTreg (37). To mimic the clinical situation we used a donor (skin) and recipient (Tconv/nTreg) pair with five of six mismatches within HLA-A/B/DR. From our ABCB two B cell lines that cover all five mismatches were identified as described in Table 1. These two B cell lines were selected to generate donor-specific nTreg from the recipient. Within 2 weeks, >100-fold expansion was reached starting from approximately 10^6 total freshly isolated nTreg. In parallel, recipient nTreg were polyclonally stimulated to generate a second, polyclonal nTreg product of the same starting population. Purity ($CD4^+CD25^+FoxP3^+$) and impurity (effector cytokine producers and flow cytometric surface marker expression) of both donor-specific and polyclonal nTreg products were comparable and good (Figure 5) as described above (Figures 1 and 2). Both nTreg products were used for functional *in vivo* analyses (Figure 6). Grafts in mice treated with Tconv alone demonstrated a median survival time of 24 days. Adoptive transfer of polyclonally expanded nTreg at a ratio of 1:5 nTreg:Tconv delayed rejection by >1 week. However, at day 37 all grafts in both untreated and polyclonally 1:5 nTreg:Tconv-treated groups were fully rejected. In contrast, all mice treated with either polyclonally or allogen-specifically expanded nTreg survived >100 days if treated at a dose of 1:1 nTreg:Tconv. Most impressively, alloantigen-specifically expanded nTreg given at 1:5 allospecific nTreg:Tconv already induced 100% long-term graft survival.

Discussion

Adoptive transfer of nTreg has become a promising approach to treat both autoimmune and alloimmune triggered immunopathologies. After the recent establishment of GMP-compliant methods for isolation and expansion of polyclonal nTreg, the first promising clinical proof-of-concept trials using adoptive transfer of polyclonally expanded nTreg to treat GvHD have been performed; further studies investigating nTreg treatment approaches for additional indications are ongoing (11,12,16,18,20,43). One major limitation of polyclonal nTreg is the low abundance of the specific clones of interest within the polyclonal repertoire. High cell numbers of polyclonal nTreg are necessary to reach specific suppression but confine its specificity. Therefore, antigen-specific nTreg would have several advantages, but their GMP-compliant isolation and expansion to sufficient yield is challenging.

Here, we describe a novel protocol that is easily transferable to GMP standards of high replicability and enables us to generate sufficient numbers of alloantigen-specific nTreg from 100 mL of peripherally collected blood. With the establishment of an EBV-free ABCB covering >90% of the German HLA-pool, we have overcome significant hurdles: (i) alloantigen-specific nTreg can now robustly be expanded from polyclonal nTreg starting pools without additional antigen-specific isolation steps; (ii) the allogeneic stimulus can specifically be selected on a case-based decision process from an available stock (ABCB) and, in the transplantation setting, allows the generation of an individually tailored nTreg product that covers the respective HLA-donor/recipient mismatch; (iii) the risk of contaminating agents in the adoptively transferred final nTreg product, such as T cell activation beads, is excluded; (iv) the use of conditionally immortalized and lethally irradiated, instead of EBV-infected B cells, ensures self-elimination of stimulator cells and minimizes the risk of B cell survival or EBV-transmission to the patient; (v) allostimulation by B cell lines triggers selective expansion of alloreactive nTreg clones—even if they are of low abundance within the polyclonal nTreg starting population—and reaches up to >10 000-fold expansion within 2–3 weeks as shown by NGS TCR analyses; and (vi) functional *in vitro* and *in vivo* analyses demonstrate that alloantigen-specific nTreg are

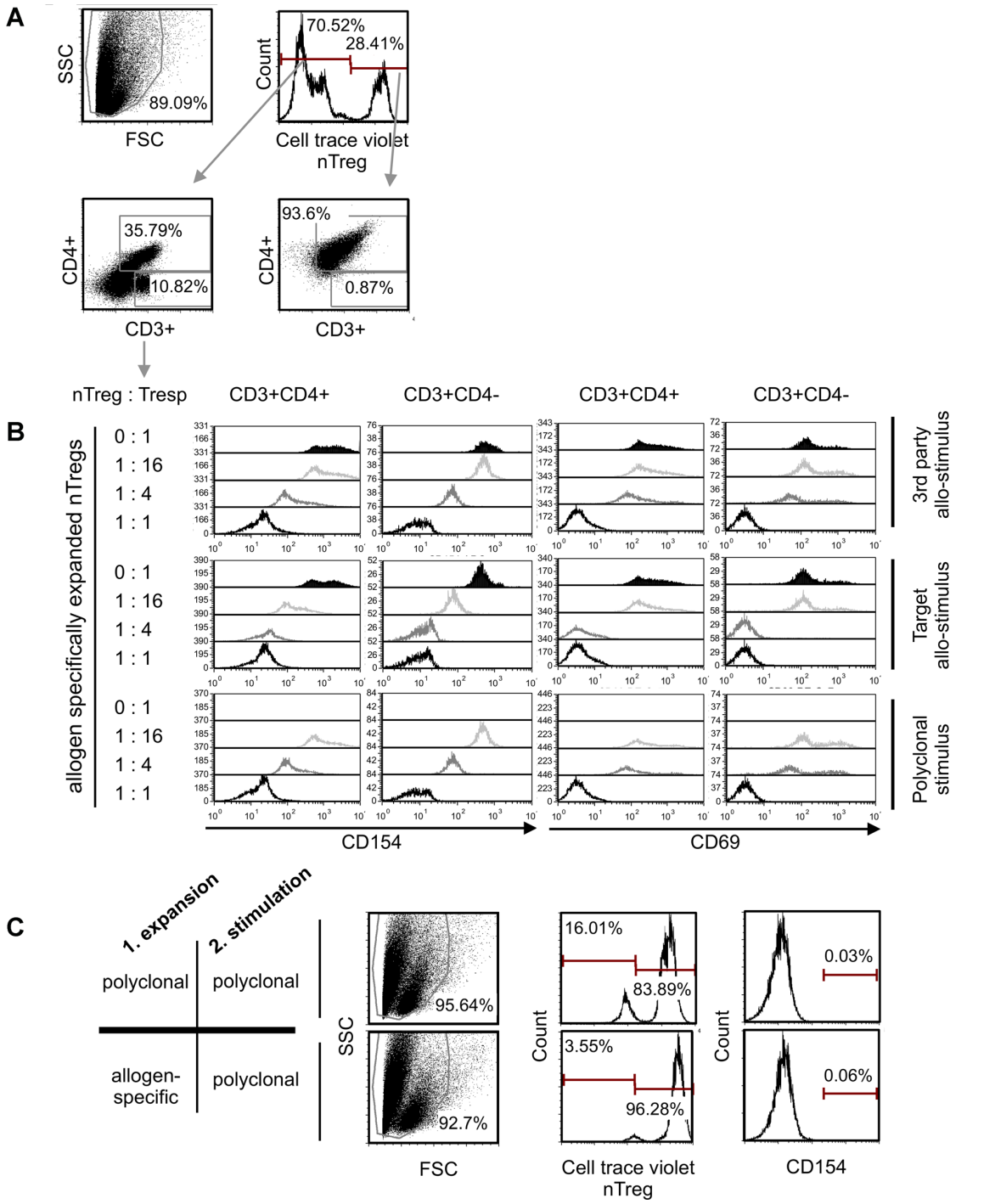
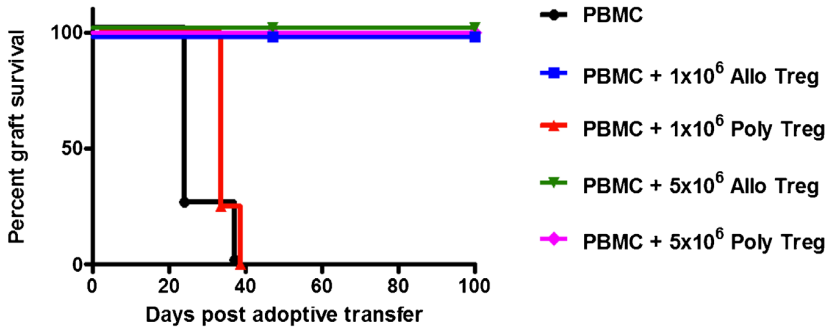


Figure 5: Exemplary FACS data demonstrate the high suppressive capacity and purity of alloantigen-specific nTreg. Depicted is one out of multiple experiments to demonstrate gating strategy and dose dependency. (A) nTreg are fluorescently labeled with CellTrace Violet and rested overnight to allow precise discrimination of nTreg from Tconv in subsequent co-cultures. (B) nTreg and Tresp were co-cultured at increasing ratios, stimulated as indicated and analyzed for specific up-regulation of CD154 and CD69. Left shift of activation marker staining indicates suppression of Tresp activation by nTreg. CD69 and CD154 expression on both $CD3^{+}CD4^{+}$ and $CD3^{+}CD4^{-}$ Tconv are shown. (C) nTreg products are of high purity as indicated by the lack of CD154 up-regulation on nTreg stimulated by CD3/CD28 activation beads. FACS, fluorescence-activated cell sorting; nTreg, natural regulatory T cells; Tconv, conventional T cells; Tresp, responder T cells.



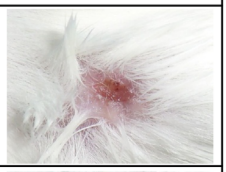
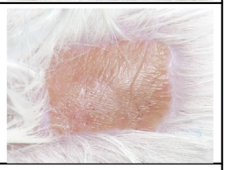
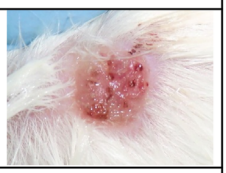
Group	Group size	Graft loss (days)	MST	Skin graft day 24
5x10^6 PBMC	4	22, 22, 24, 37	23	
5x10^6 PBMC + 1x10^6 Allo Treg	4	> 100	>100 p<0.01	
5x10^6 PBMC + 5x10^6 Allo Treg	4	> 100	>100 p<0.01	
5x10^6 PBMC + 1x10^6 Poly Treg	4	32, 32, 32, 37	32	
5x10^6 PBMC + 5x10^6 Poly Treg	4	> 100	>100 p<0.01	

Figure 6: Alloantigen-specific nTreg are superior suppressors of target alloantigen-reactive conventional T cells (Tconv) *in vivo*. Suppressive nTreg function was assessed in a humanized mouse model. To this end, human skin graft rejection was induced by transfer of allogeneic HLA-mismatched Tconv. Additionally, polyclonally versus alloantigen-specifically expanded nTreg were adoptively transferred into these mice at a ratio of 1:1 or 1:5 nTreg:Tconv. The transfer of both polyclonally and alloantigen-specifically expanded nTreg induced long-term survival. In contrast, polyclonal nTreg at a ratio of 1:5 to Tconv significantly delays graft rejection by >1 week while transfer of alloantigen-specific nTreg at the same ratio to Tconv induced long-term skin allograft survival. Depicted are summarized survival data as Kaplan–Meier survival analysis (n = 4 mice in each group) and representative graft pictures on day 24. nTreg, natural regulatory T cells.

functionally superior suppressors of Tconv stimulated by the respective alloantigen compared with polyclonal nTreg pools. These data suggest that the use of specific nTreg will enable us to vastly reduce the amount of adoptively transferred nTreg while improving the clinical efficiency (44). This would further provide the opportunity to fractionate the nTreg product for repetitive treatment cycles without the need of further production rounds.

Putnam et al (45) have very recently demonstrated a similar strategy of alloantigen-specific nTreg expansion. The procedure used for expansion as well as the resulting suppressive capacity and specificity was comparable. Remarkably, our cell product revealed a stronger clonal bias with up to 12 dominant clones (frequency >1%) per cell line. This may be related to the use of more frequent antigen restimulation in our expansion protocol. The clonal dominance is a typical picture of antigen-driven T cell selection processes *in vivo* (46).

Taken together, our results provide strong evidence that alloantigen-specific nTreg therapy is feasible and may be of significant advantage as compared to the adoptive transfer of freshly isolated or polyclonally expanded nTreg pools.

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Authors' contributions

SL-K: designed research, performed the experiments, analyzed the data and wrote the paper. FI: designed, performed and analyzed *in vivo* experiments and wrote the paper. S-HL: established and maintained allogeneic B cell bank. MS, HL and AZ: helped performing experiments. AT: contributed to the establishment of CD154 as activation marker. NB: contributed to the data analysis of next generation sequencing data. KW: designed *in vivo* experiments and contributed to the preparation of the manuscript. H-DV and PR: designed research and contributed to the preparation of the manuscript.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: Activated B cell lines strongly up-regulate the co-stimulatory signaling molecules CD80. Fifty thousand B cells were cultured for 14 days and stimulated weekly with huCD40L-transfected feeder cells in IL-4 supplemented complete medium. Surface molecule expression levels of B cells are compared with corresponding T cell depleted PBMCs (CD3-depl.) and their unstained (FMO) control. Depicted are original data from one representative example and summarized data (MFI ± SD) of six individual experiments from six different, randomly selected B cell lines from the ABCB. FMO, fluorescence minus one control.

Table S1: HLA types covered in the allogeneic 19 donor B cell bank (ABCB). Depicted are all HLA-A, HLA-B and HLA-DR markers represented in the ABCB. The cumulative coverage is calculated with respect to HLA-A, HLA-B and HLA-DR frequencies and based on the two publications (27,29). Full matching and coverage of all mismatched alloantigen specificities is not required because of the well-described phenomenon of linked suppression (47–49): Tregs that recognize several DRB1*04 epitopes suppress Tconv responses to other DRB1*04 epitopes and even nonrelated MHC Class II epitopes as long as these are expressed on the same donor APCs.

Ein genaues Verständnis der phänotypischen und funktionellen Zusammensetzung des Treg-Produktes ist für die Risiko-Nutzen-Abwägung von maßgeblicher Bedeutung. Daher untersuchten wir im Folgenden mittels erweiterten Phänotyp- und Klonotypanalysen Reifungszustände der nach Expansion im Produkt befindlichen Tregs sowie deren genetische Komposition auf Ebene des T-Zell-Rezeptors.

2.2. Humane CD45RA-FoxP3high Gedächtnis-Tregs zeigen ein unterschiedliches TZR-Repertoire im Vergleich zu konventionellen T-Zellen und spielen eine wichtige Rolle in der Kontrolle der frühen Immunaktivierung.

Der folgende Text wurde durch die Autorin übersetzt und entspricht im Wesentlichen dem Abstrakt der Arbeit

Lei H, Kuchenbecker L, Streitz M, Sawitzki B, Vogt K, **Landwehr-Kenzel S**, Millward J, Juelke K, Babel N, Neumann A, Reinke P, Volk HD. Human CD45RA^{-}FoxP3^{hi} memory-type Tregs show distinct TCR repertoires with conventional T cells and play an important role in controlling early immune activation. American Journal of Transplantation, Oktober 2015, Seiten 2625-2635, Band 15. [115]

Adoptive Immuntherapien mit regulatorische T-Zellen (Tregs) stellen eine neue Möglichkeit dar, die immunologische Toleranz nach einer soliden Organtransplantation zu begünstigen. Bei älteren Patienten, die auf eine Transplantation warten, wird das Treg Kompartiment von CD45RA-CD62L+ zentralen Gedächtnis-T-Zellen ($Treg_{CM}$) dominiert und die Isolationsausbeute an gut charakterisierten und stabilen, naiven Tregs ($Treg_N$) ist niedrig. Daher ist es wichtig zu verstehen, ob diese $Treg_{CM}$ im Thymus gereift sind und die gleiche Stabilität und suppressive Kapazität sowie ein ebenso breites Antigenrepertoire aufweisen wie $Treg_N$. Im Rahmen dieser Studie konnten wir mittels Next-Generation-Sequencing aller 24 Vß-Familien mit einer durchschnittlichen Tiefe von 534677 Sequenzen zeigen, dass $Treg_{CM}$ ein anderes T-Zell-Rezeptor-Repertoire (TZR) aufweisen als konventionelle T-Zellen (Tconv). Dabei fanden wir fast keine Kontamination durch induzierte Tregs. $Treg_{CM}$ zeigten gegenüber Tconv auf frühen Ebenen der Immunaktivierung eine verstärkte suppressive Aktivität bezüglich der Expression von Aktivierungsmarkern sowie der Zytokinsekretion, während die

Proliferationshemmung vergleichbar war. Unter Hemmung von mTOR expandierten $Treg_{CM}$ vergleichbar gut wie $Treg_N$ ohne dabei ihre Funktion zu verlieren. Trotz eines relativ begrenzten TZR-Repertoires zeigten $Treg_{CM}$ eine spezifische Alloreaktion, auch wenn diese im Vergleich zu $Treg_N$ leicht reduziert war. Diese Ergebnisse bekräftigen den therapeutischen Nutzen von Treg-Produkten aus einem Treg-angereicherten Ausgangsmaterial mit hohem CD45RA-CD62+ Anteil und deren Einsatz zur adoptiven Treg-Therapie.

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Human CD45RA$^-$ FoxP3^{hi} Memory-Type Regulatory T Cells Show Distinct TCR Repertoires With Conventional T Cells and Play an Important Role in Controlling Early Immune Activation

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Adoptive immunotherapy with regulatory T cells (Treg) is a new option to promote immune tolerance following solid organ transplantation (SOT). However, Treg from elderly patients awaiting transplantation are dominated by the CD45RA$^-$CD62L$^+$ central memory type Treg subset (TregCM), and the yield of well-characterized and stable naïve Treg (TregN) is low. It is, therefore, important to determine whether these TregCM are derived from the thymus and express high stability, suppressive capacity and a broad antigen repertoire like TregN. In this study, we showed that TregCM use a different T cell receptor (TCR) repertoire from conventional T cells (Tconv), using next-generation sequencing of all 24 Vβ families, with an average depth of 534 677 sequences. This showed almost no contamination with induced Treg. Furthermore, TregCM showed enhanced suppressive activity on Tconv at early checkpoints of immune activation controlling activation markers expression and cytokine secretion, but comparable inhibition of proliferation. Following *in vitro* expansion under mTOR inhibition, TregCM expanded equally as well as TregN without losing their function. Despite relatively limited TCR repertoire, TregCM also showed specific alloresponse, although slightly reduced compared to TregN. These results support the therapeutic usefulness of manufacturing Treg products from CD45RA$^-$CD62L$^+$ Treg-enriched starting material to be applied for adoptive Treg therapy.

Abbreviations: CM, central memory; CTLA-4, cytotoxic T-lymphocyte antigen 4; EM, effector memory; iTreg, induced regulatory T cells; MH index, Morisita–Horn similarity; mTOR, mammalian target of rapamycin; NGS, next-generation sequencing; SE, Shannon entropy; SOT, solid organ transplantation; Tconv, conventional T cells; TconvM, memory conventional T cells; TconvN, naïve conventional T cells; TCR, T cell receptor; TregM, memory regulatory T cells; TregN, naïve regulatory T cells; TSDR, Treg-specific demethylation region; tTreg, thymus-derived regulatory T cells

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Introduction

Thymus-derived CD4$^+$CD25^{hi}Foxp3^{hi}CD127^{lo} regulatory T cells (tTreg) are a specialized subpopulation of CD4 T cells that play a central role in the induction and maintenance of allograft tolerance (1–3). For solid organ transplantation (SOT) patients, adoptive tTreg therapy is a promising approach to support graft acceptance while minimizing, or potentially eliminating, immunosuppressive treatments. A Phase I/II international multicenter proof-of-concept clinical trial of Treg therapy for SOT patients is currently in progress in the European Union (The ONE Study) (4). Recent trials in hematopoietic stem cell transplant recipients used Treg generated from cord blood or young stem cell donors, in which the more stable naïve tTreg (TregN) dominate the Treg population (5–7). In contrast, the autologous starting material of the patients awaiting SOT includes a Treg population in which high numbers of memory Treg (TregM) dominate. Consequently, the yield of naïve tTreg is quite

low in these patients, which raises questions regarding the efficacy of a therapeutic Treg cell product manufactured from this TregM dominant population.

In addition to thymus-derived Treg (tTreg), Treg can also be induced from naïve conventional T cells (TconvN) in the presence of antigens and TGF-β (8,9). The induced Treg (iTreg) share the phenotype and function with memory tTreg, and currently there is no specific marker to discriminate between them in human. However, the Treg-specific demethylation region (TSDR) within the FoxP3 promoter of tTreg is fully demethylated while in iTreg it is only partly demethylated (10). This makes iTreg more unstable compared with tTreg as they may lose the Foxp3 expression and become normal Tconv—a putative danger for treatment of SOT patients (11,12). TSDR analysis may provide some information on the relative frequencies of iTreg compared to tTreg, but this method is not sensitive enough to give a definitive answer. The T cell receptor (TCR) provides a unique identity for each cell clone, and is highly diverse with around 2.5×10^7 TCRs for human naïve T cells in each individual (13). Several groups have shown in mouse studies using TCR spectratyping that Treg and Tconv use different TCRs (14–16). Recently, we showed that chronic virus exposure can support the generation of iTreg, which share their TCR repertoire with the corresponding virus-specific Tconv in human healthy donors and patients. This suggests that the virus-specific iTreg and Tconv have shared mother clones (17). However, comprehensive data on human tTreg TCR are lacking, and published studies use either a very limited depth of sequencing, or examine only one of the 24 Vβ families (18,19). In this study, we employ next-generation sequencing (NGS), a new and powerful tool that has been used to analyze the TCR repertoire of different T cell subsets and to track the cells (20,21). Investigation of the TCR repertoires of human Treg subsets with NGS and bioinformatic analysis has the potential to provide a more detailed picture of the Treg population.

It is recognized that pathogens are efficiently controlled by memory conventional T cells (TconvM). $CD45RA^-CD62L^-$ effector memory Tconv (TconvEM) execute immediate intratissue protection, while $CD45RA^-CD62L^+$ central memory Tconv (TconvCM) provide long-lasting protection upon re-exposure to pathogens (22,23). Thus, we hypothesized that a switch of phenotype of Treg to memory Treg (either TregCM or TregEM) might also affect some aspects of functionality, particularly control during early immune activation. Fast activation could be especially important in SOT, as many patients have high levels of donor-specific TconvM that would require fast control to prevent acute rejection (24).

Translation of adoptive Treg therapy from bench to bedside, requires *in vitro* expansion of the autologous Treg cells. Although memory Treg were less stable than naïve ones for expansion without mTOR-inhibitor (7), given the large amount of TregM in the Treg population of SOT patients, it is critical to assess the capacity for expansion, and the stability of the phenotype after expansion, of TregM, under the inhibition of mTOR with rapamycin, which can effectively prevent growth of Tconv (25). It is also important to determine whether the TCR repertoire diversity of TregM allows sufficient alloreactivity to be clinical effective for adoptive Treg therapy.

In this study, we show that about 80% of the Treg population in elderly healthy donors and patients awaiting kidney transplantation were TregCM. Investigation of their TCR repertoire by NGS and bioinformatic analysis showed a different TCR repertoire from Tconv, suggesting a pure tTreg population. The TregCM could effectively control immune activation at early and late timepoints of activation, and could be expanded under mTOR inhibition equally as well as TregN.

Materials and Methods

Subjects

Blood samples for Treg phenotypic analysis were collected from healthy donors and patients awaiting kidney transplantation in Berlin 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.

Flow cytometric analysis

The surface staining was done with the following antibodies: CD3(UCHT1), CD4(RPA-T4), CD8(SK1), CD25(M-A251), CD127(eBioRDR5), CD45RA(5H9) and CD62L(DREG-56). LIVE/DEAD1 Fixable Aqua Dead (Invitrogen, Paisley, UK) was used to exclude dead cells. FoxP3(259D/C7), Helios (22F6) and CTLA-4(BNI3) were stained intracellularly with the FoxP3 staining buffer (eBiosciences, San Diego, CA). Data were acquired on LSRII cytometer (BD Bioscience, Heidelberg, Germany) running FACSDiva software. The analysis was performed with Flowjo Software 9.2 (TreeStar, Ashland, OR).

CD4 enrichment and FACS sorting

Peripheral blood mononuclear cells (PBMCs) were obtained from the buffy coat over Ficoll separation solution and enriched for $CD4^+$ T cells by positive selection by magnetic activated cell separation (MACS) (Miltenyi, Bergisch-Gladbach, Germany). CD4 T cells were further stained with CD4, CD25, CD45RA, CD62L and sorted for TregN ($CD4^+CD25^{hi}CD45RA^+CD62L^+$), TregCM ($CD25^{hi}CD45RA^-CD62L^+$), TregEM ($CD25^{hi}CD45RA^-CD62L^-$) and Tconv ($CD4^+CD25^-$) using a FACSAriaTM (BD) cell sorter. The sorting purity was >95%.

DNA methylation analysis of Treg-specific demethylation region (TSDR)

The DNA methylation analysis of TSDR was performed mainly as described (26). Genomic DNA was extracted and processed for bisulfate conversion according to the manufacturer's instructions (EpiTect Qiagen, Hilden, Germany). Briefly, 60 ng of bisulfate-treated DNA was used for real-time PCR in a final volume of 20 μL containing FastStart Universal Probe Master, Lamda DNA, and methylation or non-methylation-specific probes and primers (Epiontis Company, Berlin, Germany). The proportion of demethylation of the TSDR was calculated by dividing the demethylated copy number by the total genomic FoxP3 copy number.

Next-generation sequencing (NGS) of the TCR repertoire

Total genomic DNA of all the FACS sorted TregN, TregCM, TregEM (cell number between 10^5 and 10^6) and Tconv (2×10^6 cells) from six healthy donors were extracted using QIAamp DNA Blood Mini Kit (QIAGEN). Four hundred nanograms DNA from each sample were amplified and sequenced for the third complementarity-determining region (CDR3) in the TCR-β chain on the ImmunoSEQ platform at Adaptive Biotechnologies (Seattle, WA) using next-generation sequencing technology. The detailed procedure of the deep-sequencing has been described elsewhere (27,28).

Bioinformatic analysis

The Shannon entropy (SE) was calculated as described (29). The SE was normalized by dividing 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. The Morisita–Horn similarity (MH) index was calculated as previously described (30).

Suppression assay of early activation and cytokines production

The FastImmune Regulatory T-Cell Function Kit (BD) was used in combination with CD8 (SK1) and Live/Dead Aqua for the suppression assay, which was performed as described with some modification; the suppression of activation markers expression can also predict the proliferative suppression (31). Autologous PBMCs were used as responder cells; they were cultured either alone or with the Treg subset at a ratio of 1 Treg to 4 responders, in complete medium. Cells were stimulated with anti-CD2/3/28 beads (Miltenyi) at a bead-to-cell ratio of 1:1 for 6 hours. The proportion of $CD69^+$ or $CD154^+$ cells in responder cells alone were used as the control and expression of the activation markers in responder cells co-cultured with Treg were normalized base on the control, i.e. normalized CD69/154 expression=B/A×100%, where A is the frequency of positive cells in the responder cells alone and B is the frequency of positive cells in responder cells co-cultured with Treg subsets. Supernatant of the cells were used to measure cytokines production by cytometric bead array with Th1/Th2/Th17 kit from BD Biosciences accordingly. The cytokine measurements were acquired on a FACS Calibur flow cytometer (BD) and analyzed using the FCAP Array Software V3.0. Normalized cytokine production was calculated as for the activation marker expression described above.

Treg expansion

Treg expansion was done using the human Treg expansion kit (Miltenyi). Briefly, cells were cultured with poly clonal stimulation in X-vivo medium with 10% human AB serum, 500 IU proleukin and 100nm rapamycin. The medium was changed every 3 days. After 3 weeks of expansion, the CD3/CD28 beads were removed and the cells were rested in fresh medium for 4 days.

Allogeniec stimulation of Treg

Freshly FACS sorted naïve and memory Treg were stimulated with an HLA-mismatched human B cell line, obtained from the allogeneic B cell bank of the BCRT. The generation of the B cell lines was described before (32). B Cells in mid-log growth phase were recovered, lethally gamma-irradiated (30 Gy) and used for Treg subsets stimulation with a ratio of 1Treg to 5 B cells in the presence of 500 IU Proleukin for 6 days in 24-well plate. Cells were harvested and stained each day for CD137 expression.

Statistics

Statistical tests were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Significance was defined as $p < 0.05$ ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Comparisons between two paired groups were performed by either paired t-tests or Wilcoxon matched pairs tests. The comparisons among three groups were done by ANOVA followed by Tukey's test.

Results

The dominance of CD45RA$^-$CD62L$^+$ central memory Treg (TregCM) in elderly healthy donors and patients awaiting kidney transplantation

Treg and Treg subsets are gated as shown in Figure 1A. In both healthy donors and patients awaiting kidney transplantation, Treg are dominated by central memory ($CD45RA^-CD62L^+$) rather than effector memory ($CD45RA^-CD62L^-$) and naïve ($CD45RA^+CD62L^+$) population, especially in elderly probands and patients. Additionally, the proportion of TregCM rather than TregEM is positively correlated with age in the healthy donors (Figure 1B and C).

TregCM show more demethylation of the FoxP3 promoter

The phenotype of the *ex vivo*-sorted Treg subsets (N, CM, EM) was analyzed. TregCM showed the highest levels of demethylation of the TSDR compared to TregN, reaching almost 100% (Figure 2A). The proportion of Treg expressing Helios did not differ among Treg subsets, and was approximately 80% (Figure 2B). Moreover, we observed significantly higher expression of CD39 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) in both TregCM and TregEM compared with TregN.

TregCM use different TCR repertoire than Tconv based on next-generation sequencing of all 24 Vβ families

There is a high abundance of TregCM in the total Treg population in both healthy donors and pretransplantation patients. It is, therefore, important to know to what extent these cells are pure tTreg, and how much of the less stable TconvN-derived iTreg are present in the population. In this study, we applied NGS to analyze the TCR repertoire of Treg subsets (from 10^5 to 10^6 cells) and Tconv ($>2 \times 10^6$ cells) from six donors. The average number of total reads was 534 677 and the average number of unique sequences was 8822 in each cell subset. Using this approach, we achieved a depth of the sequencing of 534 677 sequences, which is substantially greater than that of previous human Treg TCR sequencing studies (18,19).

The frequencies of the top 1000 clones from each Treg subset of one representative donor are shown in Figure 3A. Consistent with the TCR repertoire of naïve and memory subsets in Tconv (27), the frequencies of the top ranked clones among TregN rarely reached more than 0.5%, with the majority <0.1%. Among TregM, the frequence of the top expanded clones was close to 1% for TregCM, and exceeded 10% for TregEM. SE is one of the most common and robust tools for measuring diversity and complexity of a data set, and is widely used in analyzing immunoglobulin repertoire diversity (33). We observed the significantly lower SE in TregCM compared with TregN (Figure 3B). To determine whether the source of the expanded clones in

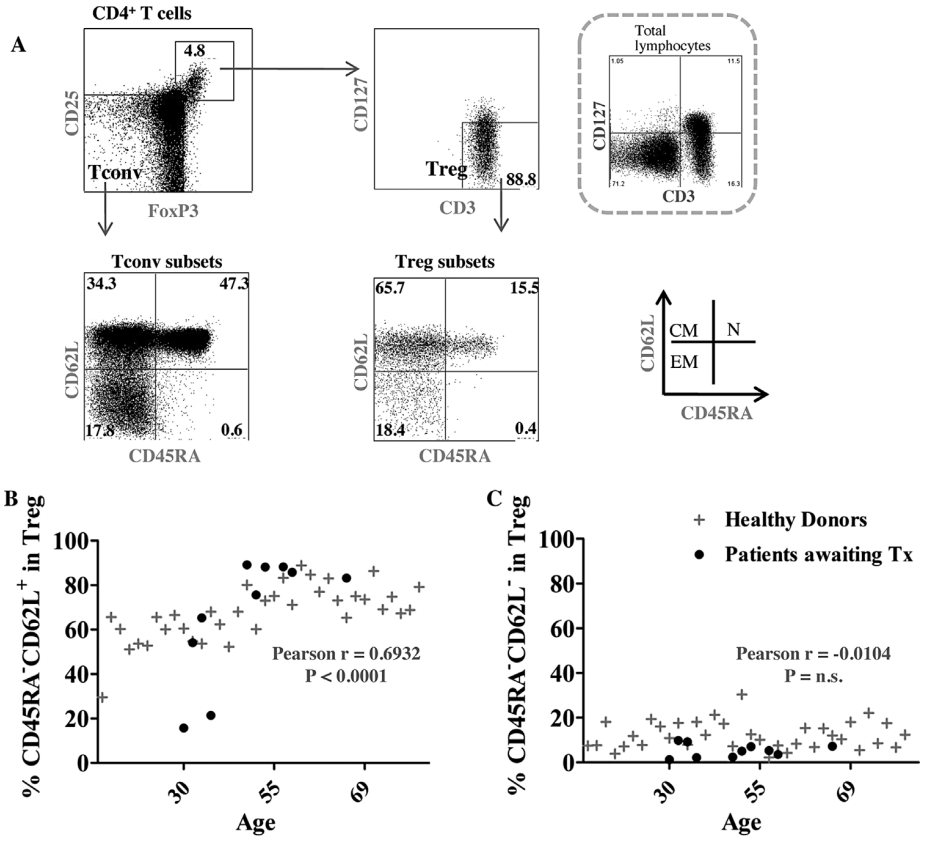

Figure 1: CD45RA$^-$CD62L$^+$ central memory Treg is the dominant Treg phenotype in elderly healthy donors and patients awaiting kidney transplantation. (A) Flow cytometric gating scheme for total Treg and subsets is shown. CD4$^+$CD25^{hi}FoxP3$^+$ cells were further gated on CD127^{lo}-expressing cells as Treg. The gating of CD127 was based on total lymphocytes as shown in the dashed box. (B and C) The proportion of TregCM (CD45RA$^-$CD62L$^+$) and TregEM (CD45RA$^-$CD62L$^-$) in both healthy donors and patients awaiting kidney transplantation is shown individually. The gray crosses represent healthy donors (n = 36, aged 19–87 years) and the dark dots represent patients 1 day before kidney transplantation (n = 10, aged 32–65 years). The correlation of the Treg subsets (CM or EM) with age from healthy donors was calculated by the Pearson correlation method and showed in each graph. CM, central memory; EM, effector memory.

the TCR repertoire of TregCM were from Tconv (especially TconvN-derived iTreg) or from tTregN (as a result of homeostatic expansion or antigen-driven process), we compared all the unique sequences from all Treg subsets. A representative example from one donor was shown in Figure 3C, with frequencies of TCR clones in the TregN and TregCM subpopulations. The shared clone marked with a circle was detected at a frequency of 0.12% in TregN pool and 4.17% in TregCM pool, suggesting an approximately 35-fold expansion of this clone within the TregCM pool.

Additionally, the proportions of the clones overlapping between the TregCM and TregEM, TregCM and TregN and the TregCM and Tconv subpopulations were shown (Figure 3D). The overlap between TregCM and TregEM repertoires was significantly greater than between the other populations examined, up to 25% (Figure 3D). Despite the high diversity of the TCR repertoire of TregN and the consequent rarity of individual clones, even at the high sequencing depth obtainable with NGS, we, nevertheless, observed an overlap of more than 2.5% between the TregN and TregCM repertoire. Consistent with previous studies using less depth of analysis (19), we saw almost no overlapping sequences between the repertoires of TregCM and Tconv subsets (Figure 3D). Furthermore, if we only focused on the top 20 clones in TregCM repertoire, almost 20% of them could be detected in the TregN repertoire, while none of them could be found in Tconv repertoire from our six donors as shown in Figure S1A.

The Morisita–Horn similarity (MH) index is a method used to quantify the similarity of two populations, such as two TCR repertoires (34). Besides the very high similarity of TregCM and TregEM repertoire, we also observed significantly

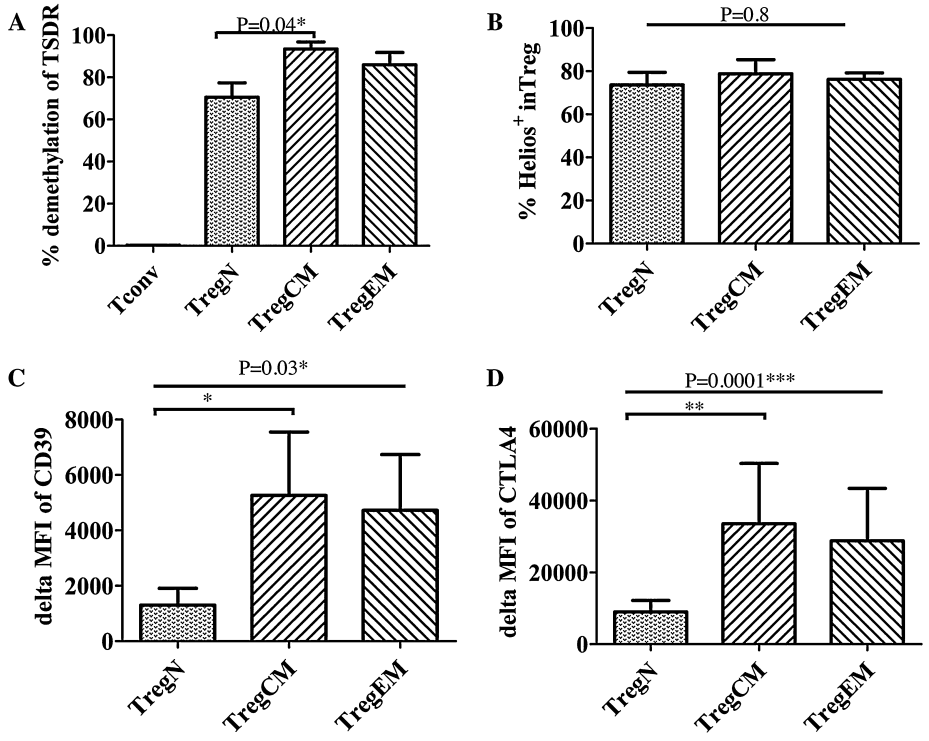

Figure 2: TregCM are fully demethylated in the TSDR and have high expression of CD39 and CTLA-4. (A) The demethylation level of TSDR (Treg-specific demethylation region) in FACS sorted Tconv and Treg subsets were showed individually. (B) Similar expression of Helios was observed in all freshly sorted Treg subsets. (C and D) The delta mean fluorescence intensity (MFI) of both CD39 and CTLA-4 from the unstained control was shown in each Treg subset (n = 6; except TSDR of TregEM were from four of the six donors).

greater repertoire similarity between TregCM and TregN, compared to the similarity between TregCM and Tconv (Figure 3E), although the MH index is quite low due to the super high diversity of the TCR repertoire. This result was consistent if the comparison was between the TregCM and Tconv subset (either TconvN or TconvM) repertoires (Figure S1B).

TregCM show enhanced suppressive capacity at an early timepoint of immune activation

Since the high expression of CD39 and CTLA-4 on TregCM may contribute to the Treg suppression activity (35), we compared the ability of all three Treg subsets to control Tconv at an early time point (6 h) of activation. The gating strategy of this analysis of both CD4 and CD8 Tconv is shown in Figure S2A. A representative example of CD69 and CD154 up-regulation on responder cells upon activation, and a decrease upon exposure to Treg is shown in Figure 4A. Figures 4B–D show normalized expression of CD69 and CD154 on both CD4 and CD8 T cells. CD154 on CD8 T cells were not shown as the expression was very low (<2%) even without exposure to Treg. TregCM show significantly greater suppressive capacity compared with TregN. TregEM showed intermediate suppressive capacity. The suppression by Treg subsets was dose-dependent (Figure S2B–D). Furthermore, cytokines secreted mainly by TconvM rather than TconvN during the first 6 h upon TCR stimulation were also measured. All Treg subsets suppressed the early cytokine responses, particularly TregEM showed the most potent effects for IL-6 production when three groups were compared with ANOVA test; however, when only TregN and TregCM were compared, TregCM showed significantly stronger inhibition for both IL6 and IFNγ production by paired t-test (Figure 4E and F). All Treg subsets showed equivalent inhibition of proliferation of responder cells during 4 days of co-culture (Figure S3).

TregCM and TregN show equivalent expansion capacity and suppressive ability

Expansion of Treg *in vitro* is essential to obtain a sufficient yield for adoptive Treg therapy. We compared the expansion capacity of Treg subsets using anti-CD3/28 plus proleukin-driven stimulation, under the control of partial mTOR inhibition (5). TregCM and TregN showed equivalent expansion rates, while the proliferation of TregEM was very limited (Figure 5A). Therefore, we focused attention on

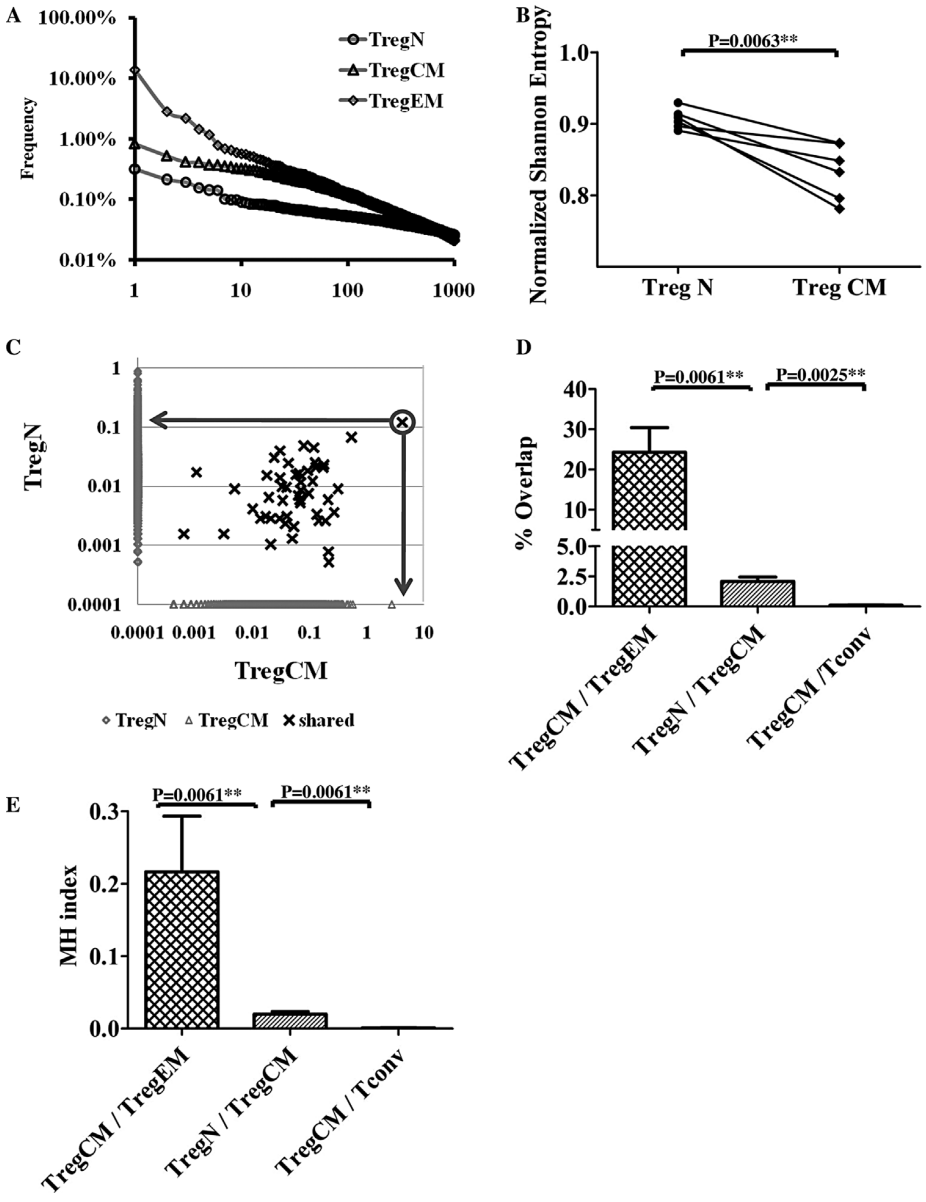

Figure 3: TregCM use different TCR repertoire than Tconv by next-generation sequencing. (A) Frequency of the top 1000 clones of the TCR repertoires in each cell subtype is shown from a representative donor. (B) The normalized Shannon entropy of TregCM repertoire was significantly lower than the value from TregN repertoire. (C) All the detected clones in TregN and TregCM repertoire from a representative donor were shown with their frequency in each population. (Rhombus: Clones detected only in TregN pool; Triangle: Clones detected only in TregCM pool; Cross: Clones shared between the two pools.) (D) The proportion of overlapped clones (% overlap) between the TregCM + EM, TregCM + TregN and TregCM + Tconv populations are shown individually. (E) The Morisita–Horn index of the same three groups are shown (n = 6).

the impact of expansion on the phenotype of TregN and TregCM subsets. After 3 weeks of expansion, there was a dramatic increase in the proportion of TregN that shifted to a $CD45RA^{-}CD62L^{+}$ phenotype, although there was a decrease of the TregCM that kept this phenotype (Figure 5B). Importantly, there was no significant change in expression of FoxP3 following expansion, in both TregN and TregCM (Figure 5C). Considering the $FoxP3^{+}$ cells, the proportion of $Helios^{+}$ cells in the TregN population did not change after expansion, though there was a significant reduction in $Helios^{+}$ TregCM (Figure 5D). Nevertheless, the expanded TregCM maintained a suppressive capacity that did not differ

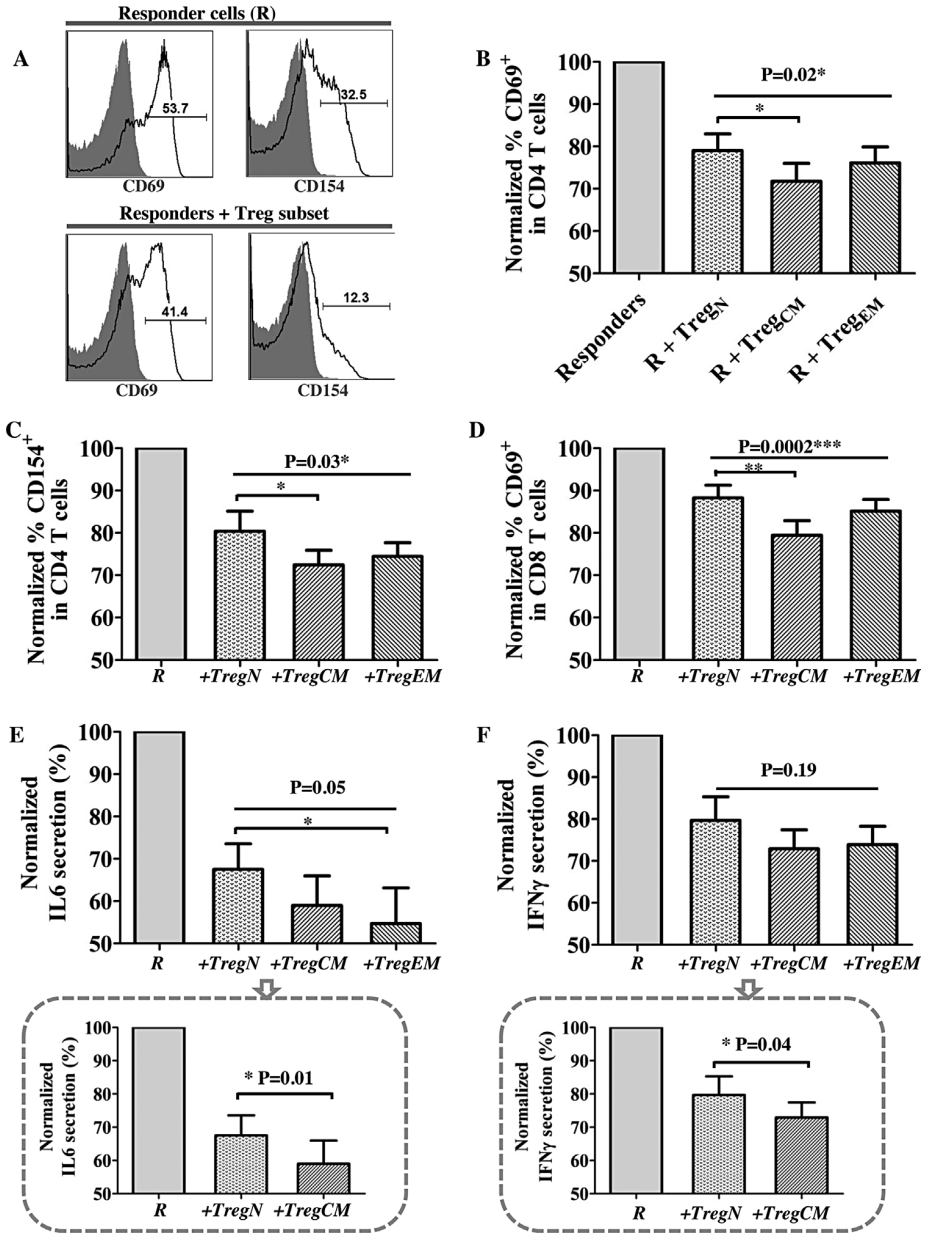

Figure 4: TregCM show increased suppressive capacity at an early timepoint (6 h). (A) CD69 and CD154 expression on CD4 Tconv cultured alone or co-cultured with TregCM is shown from one representative donor; gray area indicated nonstimulated cells. (B–D) CD69 and CD154 expression in both CD4 and CD8 T cells with the presence of each Treg subset were normalized by the expression of responder cells alone upon 6 h TCR stimulation. (E and F) The normalized IL6 and IFNγ production in the supernatant of the same groups are shown separately with ANOVA analysis. The paired test between TregN and TregCM were further shown below in the dashed boxes. The ratio of Treg and responders was 1:4. The comparison among three groups were done with ANOVA followed by Tukey's test (n = 8).

from that of the freshly sorted cells (Figure 5E, F). The TregN showed significantly increased suppressive capacity following expansion. Regarding expansion of TregCM and mixed total $CD4^+CD25^+CD127^{lo}$ cells, both of them show comparable fold expansion and purity (Figure S4).

Memory Treg may have less alloantigen reactivity due to the limited TCR repertoire

Given the reduced TCR repertoire diversity of TregM as shown in Figure 3B, this population might have fewer antigen specificities compared with TregN, which could

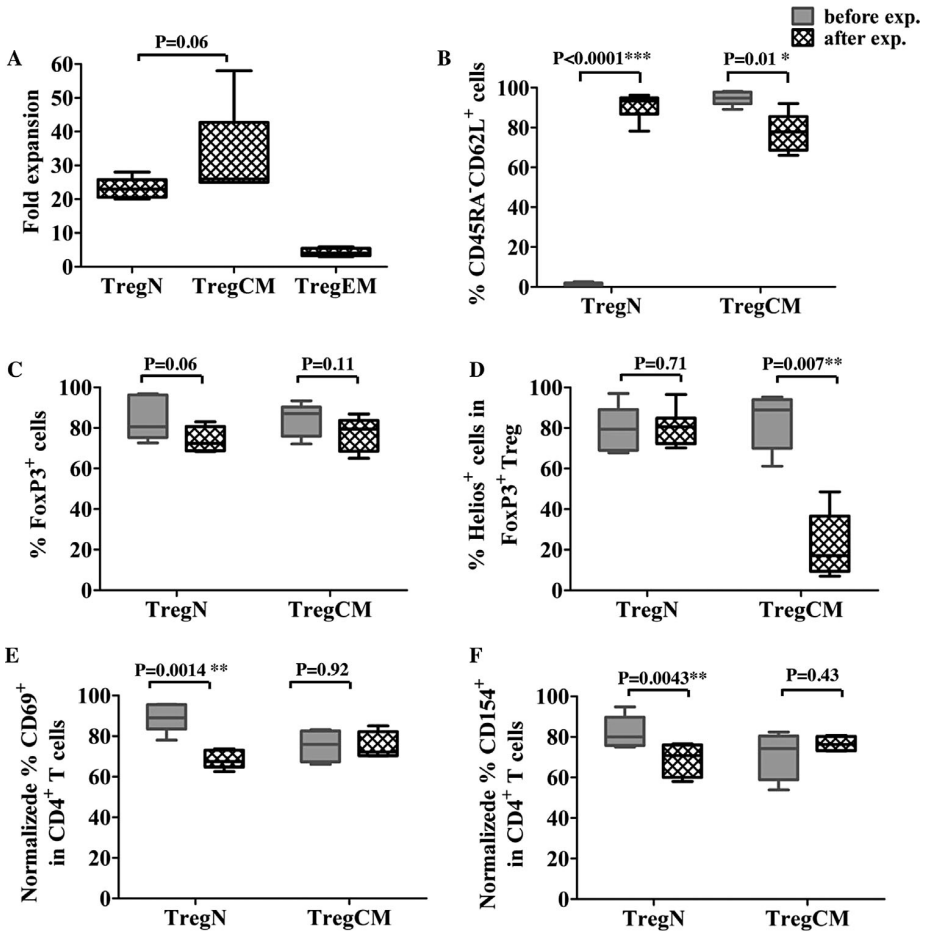

Figure 5: TregCM can be expanded equally as well as TregN under mTOR inhibition without losing phenotype and suppression ability. (A) Similar expansion of TregN and TregCM was observed after 3 weeks of expansion with proleukin and the mTOR inhibitor rapamycin. Proliferation of TregEM was limited. (B and C) The expanded TregN expressed a TregCM phenotype, while majority of TregCM retained their phenotype; there was, nevertheless, a significant decrease after expansion. (D) The proportion of TregCM expressing Helios after expansion reduced significantly, but not in TregN. (E and F) TregN showed significantly enhanced suppression activity after expansion while TregCM kept similar suppression activity after expansion (n = 5, except TregEM were from four of the five donors). The ratio of Treg and responders was 1:4.

result in a loss of alloreactivity. To test this possibility, FACS-sorted TregN and TregM were stimulated with HLA-mismatched allogeneic B cells from an allogeneic B cell bank. CD137 (also known as 4-1BB) was used as a marker for alloantigen activated Treg (36). TregN showed significantly more alloantigen-specific cells compared with TregM (Figure 6).

Discussion

Recent advances in adoptive Treg therapy in hematopoietic stem cell transplantation have yielded promising results (5,6). Nevertheless, in the context of SOT, several important challenges remain as the following: (i) The autologous Treg will be derived largely from elderly patients, in whom the dominant Treg population exhibit a memory phenotype, which might not be as good as naïve Treg; (ii) the presence of donor-specific memory Tconv in many recipients is associated with a need for more immunosuppression and poorer graft function. For these reasons, investigation of the TregM population in terms of their origin, suppressive capacity (especially at early time-points of activation), expansion capacity and numbers of clones with allogeneic specificity is an important issue, which could provide essential insights relevant to adoptive Treg therapy in SOT patients.

In this study, we found that the dominant human Treg subset, $CD45RA^{-}CD62L^{+}$ TregCM, have a TCR repertoire

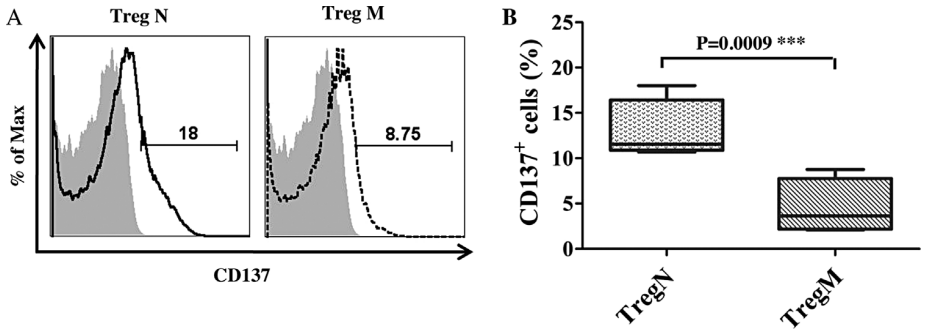

Figure 6: TregM show less alloantigen specificities than TregN. Based on CD137 expression, TregN showed more alloantigen-specific activation than TregM upon allogeneic B cells stimulation for 6 days. The gray area represents unstimulated cells. The ratio of Treg and allogeneic B cells was 1:5 (n = 4).

that is more similar to the TregN rather than the Tconv repertoire. This suggests that the TregCM are not derived from the Tconv repertoire like iTreg, as iTreg share the mother clones with Tconv (17). In light of the distinct TCR repertoires of TregCM and Tconv, we conclude that the TregCM population we examined did not include a substantial contamination with less stable iTreg. The high degree of clonal expansion of TregCM from TregN repertoire suggests some antigen-driven oligoclonal expansion, rather than homeostatic proliferation. However, as some amount of memory like Treg also exist in the thymus at least in mice, it is possible that the Treg phenotype switch from naïve to memory happened directly in the thymus (exposure to many autoantigens), this has to be further investigated in the future by TCR analysis of human thymus/blood pairs. All in all, our results obtained with NGS technology for all 24 Vβ families with an average depth of 534 677 total sequences provide a more accurate picture of Treg TCR usage than has been previously reported (18,19).

We also found that the TregN and TconvN repertoires showed equivalent diversity, as reflected by their Shannon entropy (SE) (Figure S1C). However, the diversity of the TregM repertoire was significantly lower than that of the TconvM repertoire, though still very high (Figure S1C). One concern with using TregM for adoptive therapy is potential limitations in the TCR repertoire, especially from elderly patients, which could reduce the efficacy of polyclonal Treg therapy if desired target allo- or auto-specificities are missing. This was highlighted by the reduction in alloantigen specificity of TregM as compared to TregN when stimulated by HLA-mismatched allogeneic B cells, although lack of Treg proliferation data upon alloantigen re-stimulation might be a shortage in this setting. Nevertheless, TregCM also showed a significant alloresponse from all the tested donors.

Importantly, TregCM showed no loss of suppressive capacity after 3-week expansion. This speaks positively for the impact of TregCM on the potency of tTreg cell products for tTreg therapy. Although TregCM show similar suppression activity regarding the proliferation of responder cells with TregN in the long timepoint, they could faster switch-on the effector function and control the activation of Tconv, particular memory Tconv at the early timepoint, by inhibiting their activation marker expression and early cytokine production. This is especially important in the transplant setting (24).

Consistent with our data, recent reports also show stronger suppression capacity of activated memory type Treg in murine study (37–39). Furthermore, we recently observed that $CD45RA^{-}FoxP3^{hi}$ TregM seem to play a central role in operationally tolerant kidney transplant patients as they have more TregM and their TregM show greater suppressive capacity than TregM from control immunosuppressed patients (40).

We demonstrated that TregCM capable of expanding equally as well as TregN under partial mTOR inhibition. However, a significant proportion of TregCM lost Helios expression after expansion, while TregN did not. The importance of this phenomenon is not yet clear. On one hand, it might suggest a rather intermediate stability of TregCM upon stimulation, since Helios was found to bind to the FoxP3 promoter and up-regulate its expression (41). On the other hand, the use of Helios as a marker for stable tTreg remains controversial. Several studies have shown the presence of both $Helios^{+}$ and $Helios^{-}$ cells coexisting in the human tTreg population (42–44). It is, therefore, difficult to correlate loss of Helios expression with the stability of tTreg. In contrast to TregCM, TregEM could not be expanded. This is consistent with the observation that $CD4^{+}CD25^{hi}CD62L^{-}$ cells show low efficiency in protecting from lethal acute graft-versus-host disease (GVHD) *in vivo* (45). TregEM may represent a terminal differentiation stage for Treg, which can execute rapid intra-tissue regulation by homing to the inflamed tissues, but cannot mediate long-term regulation due to their relative lack of expansion and survival ability.

The results of this study show that TregCM have properties that make the TregCM-enriched products suitable for adoptive Treg therapy, including potent suppressive capacity, which is maintained after expansion. This is particularly important when considering adoptive Treg therapy in the context of SOT, in which TregCM dominate the Treg population of these patients, who generally tend to be elderly. Several issues remain to be resolved, including concerns that limitations in the diversity of the TCR repertoire may compromise the efficacy of adoptive Treg therapy if important target specificities are absent. Nevertheless, this approach holds great promise as an emerging therapeutic option.

Acknowledgments

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Disclosure

The authors have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: (A) Top 20 clones in the TregCM repertoire from each donor were searched in both TregN and Tconv repertoire from the respective donor. Almost 20% of them could be found in TregN repertoire while none of them could be detected in Tconv repertoire. The data were shown with median (n = 6). (B) Total naïve and memory Treg and Tconv subsets were sorted from another four healthy donors (TregN: CD4+CD25hiCD45RA$^+$; TregM: CD4+CD25hiCD45RA−; TconvN: CD4$^+$CD25^{lo}CD45RA$^+$; TconvM: D4$^+$CD25^{lo}CD45RA$^-$). 1200 ng DNA from these samples were further sequenced with an enhanced depth of 1 053 654 total reads by the same procedure on the ImmunoSEQ platform at Adaptive Biotechnologies. The TCR repertoire of memory Treg show no Morisita-Horn (MH) similarity with either naïve or memory Tconv repertoire. (C) The naïve repertoire of both Treg and Tconv show similar diversity while TregM repertoire seems to be less diverse than TconvM repertoire, revealed by their Shannon entropy (n = 4).

Figure S2: (A) Gating strategy of the suppression assay of early activation is shown from one representative donor when the responder cells were cultured alone. Treg are excluded from the analysis by gating on CD25^{lo} cells; responder cells co-cultured with TregCM are used as the reference for this gating as shown in the smaller dot plot. (B and C) Suppression of CD69 and CD154 by TregCM was dose-dependent. (D) Enhanced suppression by TregCM than TregN was also observed with the ratio of 1Treg to 1 responder cell (n = 4).

Figure S3: No significant differences were found between Treg subsets regarding the inhibition of the proliferation of responder cells. Autologous PBMCs were labeled with 2 μM CFSE and co-cultured with or without Treg subsets upon CD2/CD3/CD28 beads stimulation for 3–4 days. Dead cells were excluded by DAPI and CFSE$^+$ cells were gated as responder cells. The proliferation of the responder cells were further analyzed based on the dilution of CFSE. (A) Proliferation of responder cells was showed in responder cells alone, responder cells cultured with Treg N, Treg CM and Treg EM respectively. (B) No significant difference was found between any pair of Treg subsets regarding the inhibition of the proliferation of responder cells. No suppression by Tconv from same donors was also observed. The ratio of Treg and responder cells was 1:1 (n = 6, Treg EM are from five of the total six donors. Paired t-test was used for comparison of any two subsets.)

Figure S4: FACS sorted TregCM and total CD4$^+$CD25$^+$CD127^{lo} cells were expanded *in vitro* under the mTOR inhibitor (rapamycin) in the presence of 500 IU interleukin 2 and CD3/CD28 beads for 21 days. (A and B) Both show similar fold expansion and FoxP3 expression. (n = 5, Mann–Whitney test was used for the comparison.)

T-Zell-Produkte für den klinischen Einsatz können entweder autologer oder allogener Natur sein. Bei der strategischen Entscheidung ob ein autologes oder allogenes Produkt zum Einsatz kommen soll, spielen Faktoren wie Spenderverfügbarkeit, HLA-Merkmale und das bei möglichen Inkompatibilitäten damit verbundene Risiko einer Abstoßungsreaktion und die zu erwartende Produktqualität eine wesentliche Rolle. In der nachfolgend präsentierten Arbeit untersuchten wir daher, ob und inwiefern Patienten, die auf Grund einer länger bestehenden terminalen Niereninsuffizienz Hämodialyse erhalten oder bereits nierentransplantiert sind und daher eine dauerhafte Immunsuppression benötigen, für einen autologen Therapieansatz geeignet sind.

2.3. *Ex vivo* expandierte natürliche regulatorische T-Zellen von Patienten und Patientinnen mit terminaler Niereninsuffizienz oder Nierentransplantation eignen sich für eine autologe Zell-Therapie.

Der folgende Text wurde durch die Autorin übersetzt und entspricht im Wesentlichen dem Abstrakt der Arbeit

Landwehr-Kenzel S, Zobel A, Fuehrer H, Landwehr N, Schmueck-Henneresse M, Schachtner T, Roemhild A, Reinke P. *Ex vivo* expanded natural regulatory T-cells from patients with end stage renal disease or kidney transplantation are useful for autologous cell therapy. Kidney International, Juni 2018, Seiten 1452–1464, Band 93. [116]

> *Neuartige Therapiekonzepte, bei denen autologe, ex vivo expandierte natürliche regulatorische Tregs (nTregs) eingesetzt werden, können die Organabstoßung nach einer Nierentransplantation potentiell verhindern. Die Auswirkungen der Dialyse und der Erhaltungsimmunsuppression auf den Phänotyp und das periphere Überleben von nTregs sind bislang nur unzureichend verstanden, für die Beurteilung bei der Patientenauswahl jedoch essentiell. Die vorliegende Arbeit untersucht daher regulatorische T-Zellen in Dialysepatienten und nierentransplantierten Patienten sowie die Möglichkeit, ein klinisch einsetzbares nTreg-Produkt von diesen Patienten herzustellen. Heparinisiertes Blut wurde von 200 Probanden einschließlich gesunder Kontrollen, Dialysepatienten mit terminaler Niereninsuffizienz und nierentransplantierten Patienten 1, 5, 10, 15 und 20 Jahre nach Nierentransplantation*

analysiert. Die Differenzierung und Reifung von nTregs wurde durchflusszytometrisch untersucht, um Dialysepatienten und nierentransplantierte Patienten unter dauerhafter Immunsuppression mit gesunden Kontrollen zu vergleichen. In niedriger Anzahl waren CD127 exprimierende $CD4^{+}CD25^{high}FoxP3^{+}$ nTregs nachweisbar, die jedoch keinen negativen Einfluss auf die Qualität des nTreg-Endprodukts und die therapeutische Anwendbarkeit von ex vivo expandierten nTregs hatten. Obwohl die Immunsuppression die nTreg-Reifung geringfügig verändert, beeinträchtigten weder die Dialyse noch die pharmakologische Immunsuppression oder vorangegangene akute Abstoßungsreaktionen das Überleben von nTregs in vivo. Entsprechend ist die Herstellung von autologen, hochreinen nTreg-Produkten machbar und ermöglicht Patienten, die auf eine Nierentransplantation warten oder eine allogene Nierentransplantation bereits erhalten haben eine adoptive nTreg-Therapie. Damit kann unser neuartiger Behandlungsansatz das Auftreten von Organabstoßungen und die Notwendigkeit einer langfristigen Immunsuppression reduzieren.

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Ex vivo expanded natural regulatory T cells from patients with end-stage renal disease or kidney transplantation are useful for autologous cell therapy

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Novel concepts employing autologous, *ex vivo* expanded natural regulatory T cells (nTreg) for adoptive transfer has potential to prevent organ rejection after kidney transplantation. However, the impact of dialysis and maintenance immunosuppression on the nTreg phenotype and peripheral survival is not well understood, but essential when assessing patient eligibility. The current study investigates regulatory T-cells in dialysis and kidney transplanted patients and the feasibility of generating a clinically useful nTreg product from these patients. Heparinized blood from 200 individuals including healthy controls, dialysis patients with end stage renal disease and patients 1, 5, 10, 15, 20 years after kidney transplantation were analyzed. Differentiation and maturation of nTregs were studied by flow cytometry in order to compare dialysis patients and kidney transplanted patients under maintenance immunosuppression to healthy controls. CD127 expressing $CD4^+CD25^{high}FoxP3^+$ nTregs were detectable at increased frequencies in dialysis patients with no negative impact on the nTreg end product quality and therapeutic usefulness of the *ex vivo* expanded nTregs. Further, despite that immunosuppression mildly altered nTreg maturation, neither dialysis nor pharmacological immunosuppression or previous acute rejection episodes impeded nTreg survival *in vivo*. Accordingly, the generation of autologous, highly pure nTreg products is feasible and qualifies patients awaiting or having received allogenic kidney transplantation for adoptive nTreg therapy. Thus, our novel treatment approach may enable us to reduce the incidence of organ rejection and reduce the need of long-term immunosuppression.

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Over the past decades, renal allotransplantation has become the treatment of choice in patients with end-stage renal disease (ESRD). With the implementation of lifelong immunosuppression, we have succeeded in preventing early rejection and have significantly improved the quality of life and short-term survival.[1–3] These achievements, however, are a Janus-faced success, and we have traded the short-term benefits for the significant risk of opportunistic or severe courses of viral infections, malignancies, and toxicity-associated graft failure[4–6] but failed to prevent chronic rejection effectively.

With the rapidly growing knowledge on regulatory T cells (Tregs) as mediators of immune homeostasis, there is now increasing confidence that Tregs can serve as a rational target for a new generation of immune-modulatory therapy. Over the past years, Tregs have been characterized as a key mediator of tolerance in the context of autoinflammation,[7,8] infection,[9,10] transplantation,[11,12] and tumor entities.[13] Conversely, absolute or relative superiority of conventional T cells (Tconv) over Tregs is associated with autoinflammation and graft rejection. Despite the progress made so far and although pilot studies are currently ongoing, broad therapeutic implementation of Tregs has not yet been achieved.

The group of Tregs has grown to a large family with multiple subsets.[14,15] Each subset is characterized by a unique, although partially overlapping, composition of phenotypic markers and shows a specific endogenous cytokine profile. The classification as a regulatory cell, however, is based on shared functional characteristics including T-cell receptor (TCR)–dependent suppression of proliferation and cytokine release in Tconv.

Among CD4+ T cells, 4 Treg subsets have been defined: (i) natural Tregs (nTregs) are congenitally present and thymically matured, whereas (ii) induced Tregs, which originate from Tconv, can mature upon specific TCR activation and transiently express both FoxP3 and functional Treg characteristics. (iii) Interleukin (IL)-10–secreting type 1 Tregs[16] and (iv) transforming growth factor-ß–producing T-helper cell type 3 cells[17] are derived from the same precursors as induced Tregs and show comparable suppressive capacities but remain negative for FoxP3.[16–20] In the following discussion, we focus on nTregs as a stable, thymically derived regulatory T-cell population.

A few studies recently addressed nTregs in patients with ESRD and after kidney transplantation (KTx).[21–24] Although there is significant evidence that human nTregs can control alloresponsiveness in KTx,[25–28] it is yet unclear if patients with ESRD are eligible for autologous nTreg therapy; in line, the impact of persistent alloactivation and standard maintenance immunosuppression on phenotypic and functional characteristics of circulating nTregs remains to be elucidated.[24,29–31]

Various protocols for adoptive nTreg therapy exist from stem-cell transplantation settings, in which usually a matched healthy donor is available and can serve as an eligible nTreg source. In contrast, in solid organ transplantation matched healthy Treg donors are usually not available, and several additional hurdles need to be overcome in order to manufacture autologous nTregs from chronically ill or immunosuppressed patients. Most importantly, although a third-party nTreg product is principally possible, the feasibility of finding a human leukocyte antigen–matched nTreg donor is structurally not guaranteed. Accordingly, protocols for nTreg therapy in solid organ transplantation are currently based on an autologous regimen, using *ex vivo* expanded nTregs. Therefore, we aimed to investigate whether patients with chronic ESRD are eligible for autologous nTreg therapy and how nTregs develop under immunosuppressive therapy. The latter is of particular importance in order to evaluate how adoptively transferred nTregs behave in immunosuppressed hosts and whether autologous nTreg therapy is also feasible at later stages post-transplantation. To this end, we first investigated circulating nTregs in patients with ESRD and further addressed the course of peripheral nTreg subsets in patients on immunosuppression. Prospectively, the implementation of adoptive nTreg therapy may enable us to significantly reduce or completely withdraw maintenance immunosuppressive therapy and minimize the risk of graft rejection, drug toxicity, and susceptibility to infections.

RESULTS

Despite CD4+ lymphocytosis in ESRD and posttransplantation, $CD4^+CD25^{high}FoxP3^+$ nTreg frequencies remain stable during disease and under maintenance immunosuppression

Lymphocyte analysis was performed in freshly collected blood from healthy controls (HCs), patients with ESRD, and KTx patients between 18 and 87 years of age (Figures 1 and 2a–l). Patient characteristics are listed in Table 1. All KTx patients received combination therapy for long-term maintenance immunosuppression. Two representative patients were chosen as examples to show the gating strategy (Figure 1) used for the results depicted in Figures 2–4. Total CD3+ lymphocyte frequencies were comparable between HCs and all patients groups (Figure 2a), whereas both ESRD and KTx patients showed mild CD4+ lymphopenia (Figure 2b). nTreg frequencies appeared to be slightly elevated in ESRD and KTx patients, although this effect was not statistically significant (Figure 2c). Because mTor inhibitors were previously postulated to increase the amount of circulating nTregs, we were further interested in the drug-specific effects of the immunosuppressive agents used for maintenance therapy. Interestingly, no drug-specific differences were observed. In particular, neither mTOR inhibitors nor calcineurin inhibitors, mycophenolate mofetil, steroids, nor azathioprine altered the frequencies of CD3+ (Figure 2e), CD4+ (Fig. 2f), or regulatory (Figure 2g) T cells *in vivo*. We then went on to assess nTreg maturation using CD45RA as a marker expressed by naïve, unresponsive T cells that have not yet encountered their TCR-specific antigen.[32] Here, KTx patients with maintenance immunosuppression tend to have increased naïve $CD45RA^+$ nTregs, indicating partially impaired nTreg maturation (Figure 2d). This phenomenon was again independent of the immunosuppressive agent (Figure 2h) and the number of previous acute rejection episodes (Figure 2i–l).

KTx patients show increased frequencies of naïve and effector memory nTregs, whereas central memory nTregs are reduced

To further investigate nTreg maturation, we assessed additional naïve and memory maturation states (Figure 3a–j). CD62L is expressed on naïve T cells; upon activation, CD62L mediates T-cell recruitment to peripheral lymphoid organs through high endothelial venules.[33] Subsequently, CD62L expression fades and CD62L- cells are considered as "antigen-experienced." Accordingly, whereas naïve, unresponsive cells are $CD45RA^+CD62L^+$ (T_{NAIVE}), central memory T cells have lost CD45RA expression and shifted to a $CD45RA^-CD62L^+$ (T_{CM}) phenotype. These cells can be recruited to lymph nodes where they mature to effector memory T cells, defined as $CD45RA^-CD62L^-$ (T_{EM}).[34,35] In our KTx patient cohorts, $nTreg_{NAIVE}$ (Figure 3a) and $nTreg_{EM}$ (Figure 3c) were elevated compared with HC and ESRD patients, whereas $nTreg_{CM}$ were substantially reduced (Figure 3b). These findings were independent of the maintenance immunosuppressive regimen (Figure 3d–f) and previous rejection episodes (Figure 3g–i). Drug-specific alterations were not observed, and although it seemed that the amount of $nTreg_{EM}$ correlates with the frequency of rejection episodes, this effect was not statistically significant (Figure 3i). Figure 3j summarizes T-cell subset markers and specific distributions of $nTreg_{NAIVE}$, $nTreg_{CM}$, and $nTreg_{EM}$ in our cohorts. In order to exclude that the observed maturation shifts of nTreg subsets in KTx patients are secondary, transplantation-associated effects due to

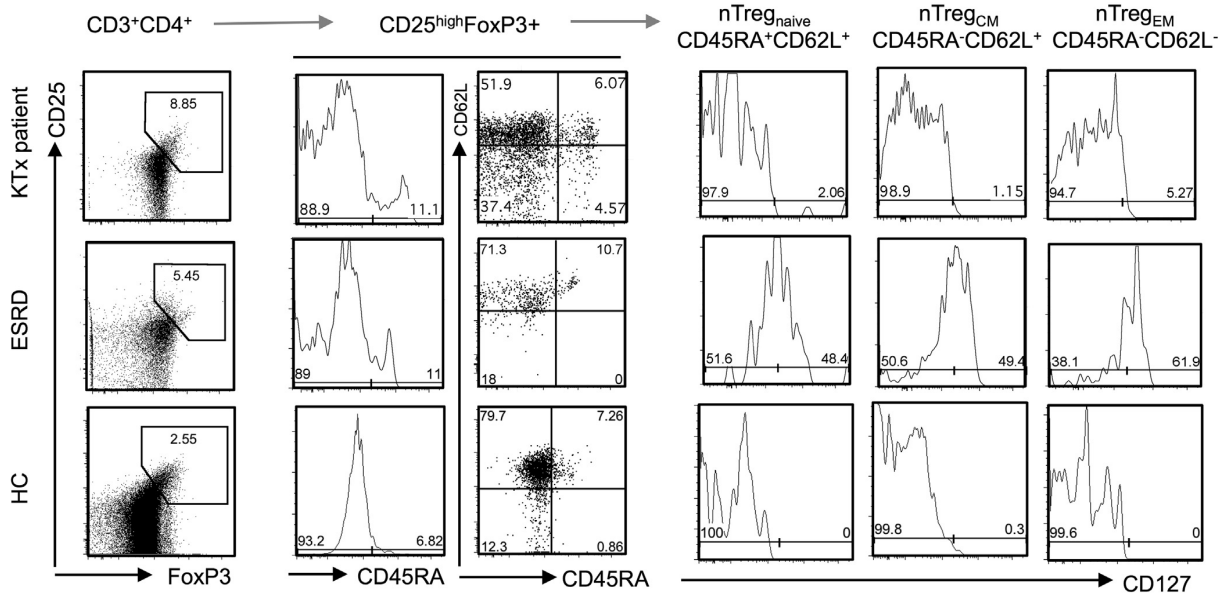

Figure 1 | Gating strategy of 10-color flow cytometry for detection and analysis of CD4$^+$CD25^{high}FoxP3$^+$ natural regulatory T cells (nTreg cells). Gating strategy as outlined in the Materials and Methods section on representative data from 1 kidney transplantation (KTx), 1 end-stage renal disease (ESRD) patient, and 1 healthy control (HC). Cells were hierarchically gated as leukocytes, singlets, CD3+ lymphocytes, and CD4+ T cells. nTregs were defined as CD4$^+$CD25^{high}FoxP3$^+$ cells and further analyzed as naïve (CD45$^+$CD62L$^+$), central memory (CD45RA$^-$CD62L$^+$), effector memory (CD45RA$^-$CD62L$^-$), and CD45RA$^+$ effector memory Tregs (CD45RA$^+$CD62L$^-$) for the expression of CD127.

persistent allostimulation and consecutive peripheral FoxP3 upregulation in Tconv, we went on and assessed CD127 expression in all nTreg subsets.

Patients with ESRD express CD127+ nTregs that lack effector cell characteristics

In 2006, low expression of the IL-7 receptor CD127 was introduced as specific characteristic of nTregs and correlated with FoxP3 expression in these cells.[36,37] Conversely, high expression of CD127 in CD3+ was later associated with activated T cells and rejection in KTx patients, and activation-induced coexpression of CD127 and FoxP3 can occur in Tconv.[38] Accordingly, we analyzed naïve and memory nTreg subsets with respect to their CD127 expression. Interestingly, CD127 expression was not elevated in KTx patients compared with HCs (Figure 4a–c). This strongly indicates that increased nTreg$_{EM}$ are not due to increased activation-dependent FoxP3 expression in peripheral Tconv, but due to increased maturation of nTregs from a central memory to an effector memory phenotype. We confirmed these observations by data analysis in light of previous rejection episodes (Figure 4d–f). Although it seems that increased frequencies of CD127 expression nTregs circulate in KTx patients and correlate with the number of previous rejection episodes, this effect is not statistically significant. In contrast to our expectations, however, we observed significantly increased CD127 expression on all nTreg subsets (Figures 1 and 4a–c) of patients with ESRD. Hence, because depletion of CD127+ cells has been suggested to improve nTreg products when aiming for adoptive nTreg therapy, we went on to further characterize CD127$^+$ cells in ESRD.

CD154, also known as the CD40 ligand, is a member of the tumor necrosis factor superfamily expressed on T and B cells. It can bind its ligand CD40 on antigen-presenting cells and act as costimulatory molecule in TCR-dependent signaling and T- and B-cell maturation (summarized in Grewal *et al.*[39]). In previous studies, it was shown that CD154 is robustly upregulated on Tconv upon TCR stimulation and is indispensable for the development of antigen-specific CD4+ effector T cells.[40,41] Furthermore, it has been demonstrated that nTregs can express CD154, but this expression is hardly inducible compared with Tconv.[42] Therefore, we assessed the expression of CD154 in resting and TCR-specifically activated cells. Raw data are depicted in Figure 4g for 2 representative donors of each cohort summarized in Figure 4h: Tconv of both HC and ESRD patients strongly upregulated CD154 on stimulation, although CD154 upregulation was markedly stronger in HCs than in patients with ESRD. In contrast, upregulation of CD154 was barely detectable on CD4$^+$CD25^{high}FoxP3$^+$ nTregs (Figure 4h) of ESRD and HCs. This confirmed the phenotypic and functional stability of CD127-expressing nTregs (Figure 4a–c). Interestingly, activation-induced CD154 expression on Tconv was detectable but markedly reduced in ESRD patients compared with HCs (Figure 4h).

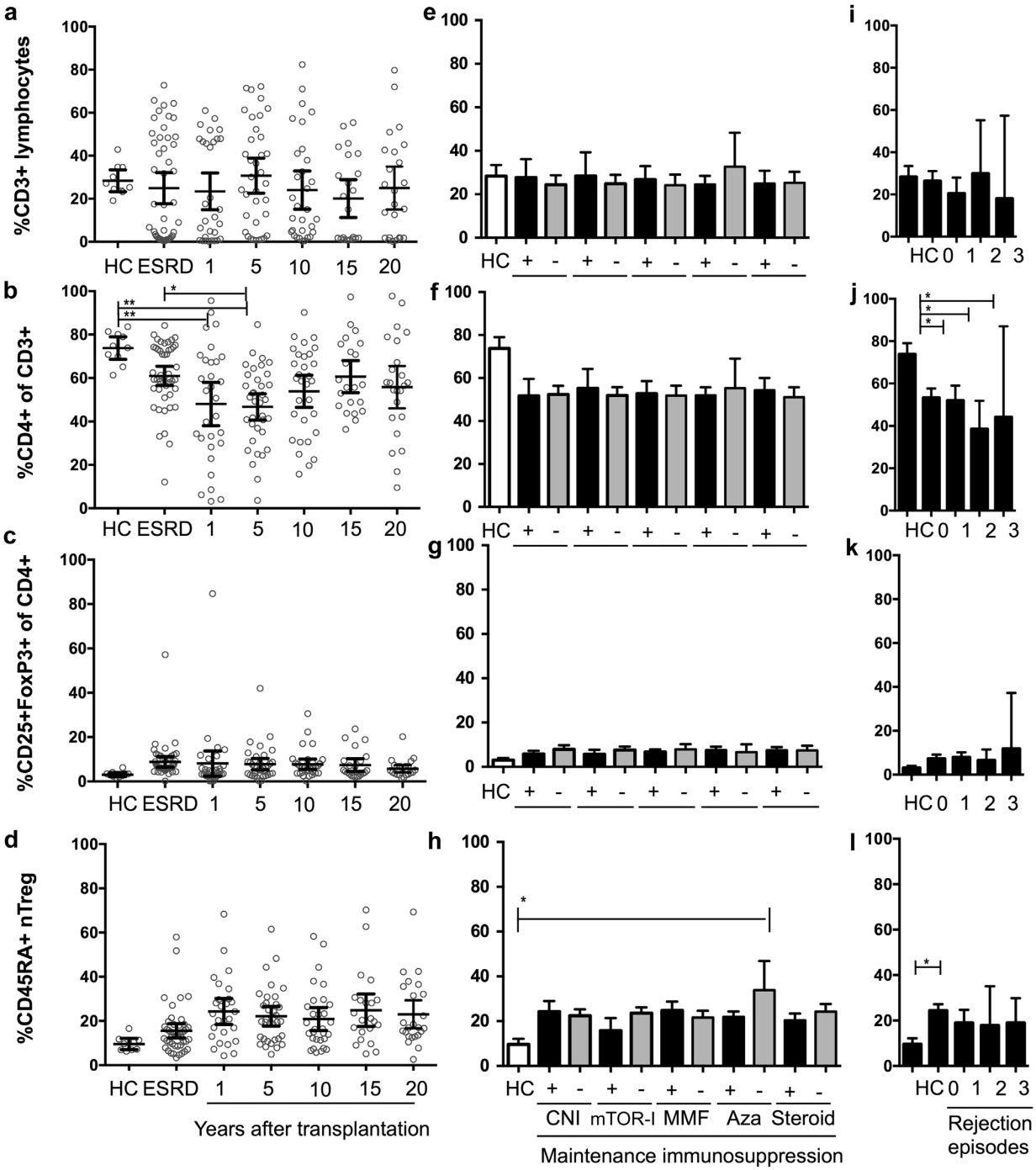

Figure 2 | T-cell subset analysis. Whole blood from healthy controls (HC) and patients with end-stage renal disease (ESRD) or 1, 5, 10, 15, and 20 years after kidney transplantation was analyzed by flow cytometry and gated as outlined in Figure 1. Further analysis was performed according to time after transplantation (**a–d**), immunosuppressive maintenance therapy (**e–h**), and previous rejection episodes (**i–l**). Depicted are cell frequencies for total CD3+ lymphocytes (**a**, **e**, **i**), $CD4^+$ T cells (**b**, **f**, **j**), and $CD4^+CD25^{high}FoxP3^+$nTreg (**c**, **g**, **k**) cells. Natural regulatory T cells (nTregs) were further analyzed for the expression of CD45RA as a marker for naïve ($CD45RA^+$) cells (**d**, **h**, **l**). Shown are individual data and mean values ± 95% confidence intervals. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Number of individuals analyzed: HC, $N = 10$; ESRD, $N = 46$; time after transplantation: 1 year, $N = 30$; 5 years, $N = 36$; 10 years, $N = 31$; 15 years, $N = 22$; 20 years, $N = 24$; immunosuppression calcineurin inhibitor (CNI)+, $N = 33$; CNI−, $N = 110$; mTOR inhibitor (mTor-I)+, $N = 12$; mTOR-I−, $N = 131$; mycophenolate mofetil (MMF)+, $N = 57$; MMF−, $N = 86$; azathioprine (Aza)+, $N = 132$; Aza−, $N = 11$; steroid (Ste)+, $N = 50$; Ste−, $N = 93$; 0 rejection episodes, $N = 105$; 1 rejection episode, $N = 29$; 2 rejection episodes, $N = 6$; 3 rejection episodes, $N = 3$.

Table 1 | Patient characteristics and comedications of kidney transplant patient cohort

No. of years after KTx	No. of patients	Age, yr	Age, N	Sex, N		Average time on dialysis, mo	Kidney source, N		Immunosuppression, N					Patients with a history of transplant rejection, N
				M	F		Deceased	Living	CNI	mTOR-I	MMF	Aza	Ste	
ESRD	46	18–39	7	3	4	58.7								
		40–59	17	11	6	74.3								
		≥60	22	9	13	59.8								
		Primary renal disease				Diabetes, 21.7% ($N = 10$); hypertensive arteriopathy, 10.9% ($N = 5$); glomerulonephritis, 39.1% ($N = 18$); familial hereditary, 15.2% ($N = 7$); infective and obstructive, 2.2% ($N = 1$); congenital, 2.2% ($N = 1$); toxic nephropathies, 2.2% ($N = 1$); systemic disease, 4.3% ($N = 2$); cause uncertain or missing, 2.2% ($N = 1$).								
1 y	30	18–39	8	6	2		4	4	5	0	5	0	5	3
		40–59	13	6	7		7	6	10	0	10	0	6	4
		≥60	9	5	4		6	3	6	0	4	0	6	2
5 y	36	18–39	11	9	2		7	4	6	3	8	1	7	4
		40–59	14	11	3		10	4	11	3	12	0	8	7
		≥60	11	6	5		10	1	9	0	7	0	4	5
10 y	31	18–39	7	3	4		2	5	6	0	4	0	6	1
		40–59	14	6	8		11	3	13	3	8	0	9	2
		≥60	10	4	6		9	1	7	3	7	0	10	1
15 y	22	18–39	4	3	1		3	1	3	0	2	1	3	1
		40–59	7	2	5		7	0	5	0	1	0	3	2
		≥60	11	5	6		11	0	11	0	6	2	8	3
20 y	24	18–39	0	0	0		0	0	0	0	0	0	0	0
		40–59	16	7	9		15	1	12	0	9	5	12	5
		≥60	8	3	5		7	1	6	0	3	1	6	0

Aza, azathioprine; CNI, calcineurin inhibitor; ESRD, end-stage renal disease; F, female; KTx, kidney transplantation; M, male; MMF, mycophenolate mofetil; mTOR-I, mTOR inhibitor; Ste, steroids.

The generation of autologous nTreg products from patients with ESRD is feasible

Last, to demonstrate the eligibility of ESRD patients for autologous nTreg therapy, 50 ml of blood was peripherally collected from patients with ESRD. Healthy volunteers served as independent controls (Figure 5a–e). nTregs were isolated by CliniMACS and polyclonally expanded under Good Manufacture Practice clean room conditions from patients with ESRD and from HCs. In our hands, nTregs from patients with ESRD expanded rapidly and reached cell numbers partially exceeding the yield of nTreg cultures from healthy volunteers (Figure 5a). Expansion rates, yield, and purity were comparable between all patients with ESRD independent of the primary renal disease. Further, the end-product purity, defined as $CD25^{high}FoxP3^{+}$ cells within the end product was comparable to nTreg cultures from HCs and previously published data (Figure 5b and c).[43] To exclude the presence of Tconv or induced Tregs, which may transiently express CD25 and FoxP3 but can be distinguished by the characteristic formation of effector cytokines upon activation, cells were stimulated with phorbol 12-myristate 13-acetate/ionomycin and analyzed for IL-2 and interferon-γ. We found that the amount of IL-2– and interferon-γ–producing cells in ESRD patients was slightly higher compared with our HCs but remained very low (Figure 5c). Further, expression of CD127 in *ex vivo* expanded nTregs from ESRD patients was barely detectable (Figure 5d). Finally, the capacity of the nTreg cell product to suppress effector T-cell activation on a functional level was determined in mixed lymphocyte reactions. Effector T cells upregulate CD154 and CD69 upon activation, which can be used as a surrogate marker. We found that nTregs expanded from patients with ESRD effectively suppressed both CD154 and CD69 upregulation on effector T cells in a dose-dependent fashion. The suppressive capacity was consistent with our data from healthy donors reported previously (Figure 5e).[43] In summary, these data demonstrate that the generation of autologous nTregs from dialysis-dependent ESRD patients is feasible and safe. Increased levels of $CD127^{+}$ nTregs are detectable under hemodialysis before transplantation but do not constitute a safety issue during the expansion process.

DISCUSSION

The objective of this study was to investigate phenotypic and functional characteristics of $CD4^{+}CD25^{high}FoxP3^{+}$ nTregs in patients receiving dialysis before or maintenance immunosuppression after renal transplantation. In particular, we were interested in whether renal replacement therapy or maintenance immunosuppression affects circulating nTregs, which might be a limiting factor when planning cell therapeutic approaches with autologous *ex vivo* expanded nTregs. These data are of crucial importance because according to the European Medicines Agency nTreg cell products, manufactured for clinical applications, are defined as advanced therapy medicinal products, and licensing is tightly regulated.[44] Accordingly, a detailed description of the patients' immunologic phenotype at the time of cell harvesting and following adoptive transfer, the manufacturing process, and the product specifications is required when applying for approval of clinical trials.

To our best knowledge, this is the largest cross-sectional analysis of ESRD patients and long-term analysis of KTx patients. We found that continuous alloactivation in KTx

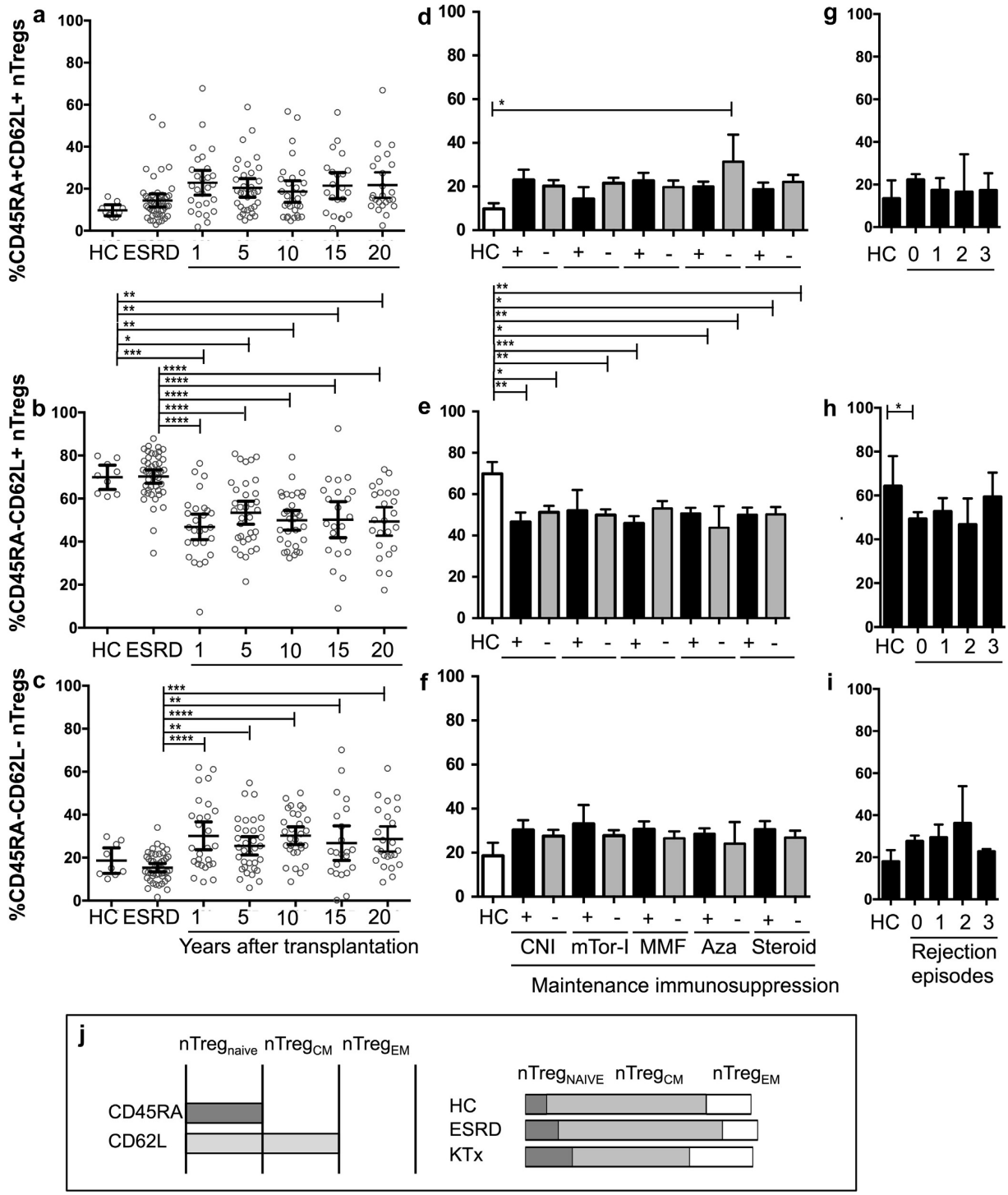

Figure 3 | Regulatory T cell (Treg) subset analysis. Frequencies of naïve CD45RA$^+$CD62L$^+$ (natural [n]Treg$_{NAIVE}$) (**a,d,g**), central memory CD45RA$^-$CD62L$^+$ (nTreg$_{CM}$) (**b,e,h**) and effector memory CD45RA$^-$CD62L$^-$ (nTreg$_{EM}$) (**c,f,i**) were determined in healthy controls (HC) and patients with end-stage renal disease (ESRD) or 1, 5, 10, 15, and 20 years after kidney transplantation (KTx). (**j**) Further summarizing of patterns of subset-defining marker expression and subset distribution in HC, ESRD patients, and as the average of all KTx patients. Depicted are data from the individual patients and mean values ± 95% confidence interval. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. (Continued)

patients seems to decelerate maturation of naïve nTregs but promotes further maturation from central memory to effector memory phenotypes. Neither pharmacologic immunosuppression nor the incidence of previous rejection episodes specifically altered the frequency, phenotype, or maturation of nTregs. In ESRD patients, expression of CD127 is increased on nTregs. However, we were able to demonstrate that these $CD4^+CD25^{high}FoxP3^+CD127^+$ cells are functionally stable nTregs. Because CD127 expression was not further detectable on expanded nTregs, we consider depletion of $CD127^+$ cells dispensable when isolating cells for the generation of clinical grade nTreg products. Finally, we were able to show that the generation of high-quality polyclonally expanded nTreg products from ESRD patients is feasible.

Operational tolerance, describing a spontaneously stable long-term graft function in the absence of maintenance immunosuppression, has been observed in few KTx patients.[45–48] The underlying mechanisms that mediate tolerance against the transplanted organ in these patients have not yet been elucidated. Yet, it seems that regulatory B cells[49] and Tregs[50–52] significantly contribute to the immunologic shift.[53] Vice versa, poor graft survival has been correlated to low nTreg numbers.[48,52,54,55] Therapeutic benefits of adoptively transferred $CD4^+CD25^{high}FoxP3^+$ nTregs were recently acknowledged in the context of acute and chronic graft versus host disease following hematopoietic stem cell transplantation.[56–59] Our experience with nTregs in the context of solid organ transplantation is, however, limited and experimental at this stage.[43,60–62] The ONE Study, a large-scale collaborative clinical research trial of which we are part, has been initiated to evaluate safety and collect first indicators on the efficiency of different cell therapeutic approaches (e.g., nTregs, among others) in KTx.

Factors that determine the fate of *in vivo* maturation and behavior of circulating nTregs under chronic hemodialysis and, even more importantly, under chronic allostimulation but continuous maintenance immunosuppression is only scarcely understood. The interpretation of recent publications is further hampered by differing definitions of nTregs.[53,63–65] Additionally, published data describing nTregs under renal replacement therapy are partially contradictive.[66–68] In our cohort, nTreg frequencies in dialysis and KTx patients appear slightly increased, although this increase was not statistically significant, likely due to high patient-to-patient variance. Long-term maintenance immunosuppression appears associated with increased frequencies of naïve and effector memory nTregs, whereas central memory nTregs appear decreased; again not all of these results are significant due to population variance. At the same time, the high patient-to-patient variance, which leads to a large overlap between groups, also means that not every significant difference between groups will directly translate into clinical relevance. It is, however, tempting to speculate that increased frequencies of naïve and effector memory nTregs are due to decelerated maturation at early stages while maturation to effector memory differentiation states is accelerated by continuous exposure to non–self-HLA antigens present on the transplanted organ. A correlation of nTreg maturation with the number of previous rejection episodes was not detectable. Data on the influence of pharmacologic immunosuppression on nTreg biology are largely contradictive. Inconsistent definition of phenotypic nTreg markers and limited patient numbers might substantially contribute to contradictive or at least heterogeneous results, but differences between human *in vivo* and *in vitro* biology and animal models have also to be taken into account. Additionally, the investigation of drug-specific effects in humans is difficult because solid organ– as well as hematopoietic stem cell–transplanted patients are routinely treated with a combination of at least 2 different immunosuppressive drugs. In summary, published data suggest that not only inhibition of mTOR (e.g., by rapamycin, or everolimus)[29,38,64,66,69–75] but also administration of antithymocyte globulin[76,77] or IL-2[78–85] promotes the frequency and survival of circulating nTregs. In contrast, the use of calcineurin inhibitors[60,86–95] was suggested to reduce nTreg frequencies. Data on the effect of mycophenolate mofetil[72,88] and steroids[96–101] are even less conclusive. In our large patient cohort, we found no drug-specific effects with respect to $CD4^+CD25^{high}FoxP3^+$nTreg frequency and nTreg maturation. This underlines the substantial differences between *in vivo* and *in vitro* nTreg biology as, in our hands, the addition of rapamycin to *ex vivo* nTreg cultures is crucial in order to generate a high-quality, clinical grade nTreg product.

Currently licensed technologies (e.g., from CliniMACS, Miltenyi Biotec, Bergisch Gladbach, Germany) to isolate nTregs for clinical applications are based on magnetic bead–based depletion of CD8 T cells followed by the enrichment of CD25-expressing subsets.[102–108] On a scientific level, additional markers have further been associated with the functionally suppressive capacity of nTregs. Most importantly, FoxP3, an X-chromosome expressed forkhead protein, has long been recognized as a Treg-defining protein,[109–111] but FoxP3 is constitutively expressed by nTregs, and its nature as an intracellular protein precludes it from being an isolation marker. Further, FoxP3 can be induced in non-nTreg cells and is therefore a necessary but insufficient criterion for the definition of nTregs (for review, see Sakaguchi[112]). CD127 expression has additionally been described at a characteristically low level on nTregs.[36] Accordingly, some authors suggest

Figure 3 | (Continued) Number of individuals analyzed: HC, $N = 10$; ESRD, $N = 46$; time after transplantation, 1 year, $N = 30$; 5 years, $N = 36$; 10 years ($N = 31$), 15 years ($N = 22$), 20 years, ($N = 24$); immunosuppression: calcineurin (CNI)+ ($N = 33$), CNI− ($N = 110$), mTOR-inhibitor (mTOR-I)+ ($N = 12$), mTOR-I− ($N = 131$), mycophenolate mofetil (MMF)+ ($N = 57$), MMF− ($N = 86$), azathioprine (Aza)+ ($N = 132$), Aza− ($N = 11$), Steroid+ ($N = 50$), Steroid− ($N = 93$); rejection episodes: 0 ($N = 105$), 1 ($N = 29$), 2 ($N = 6$), and 3 ($N = 3$).

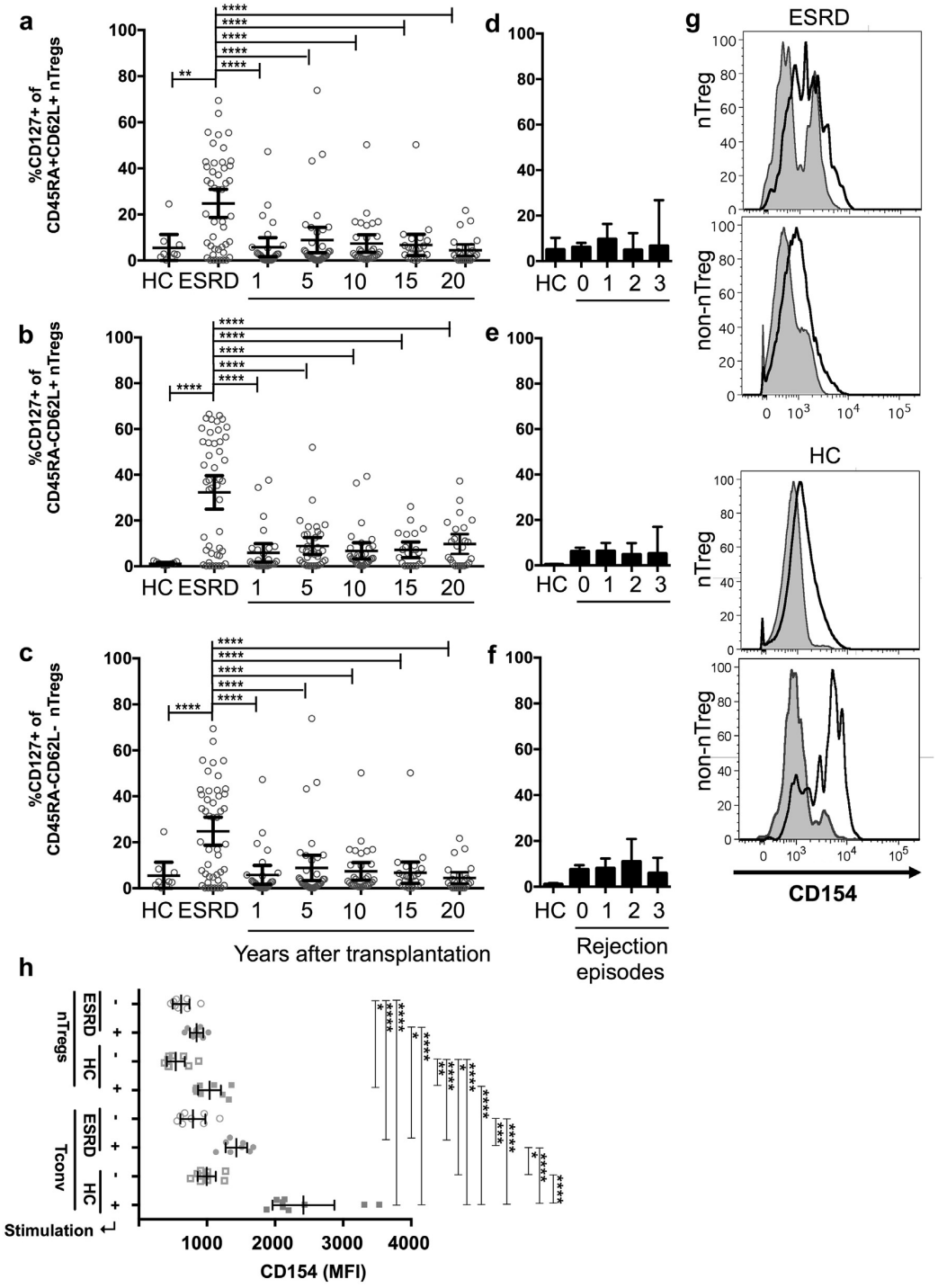

Figure 4 | Natural regulatory T cells (nTregs) of end-stage renal disease (ESRD) express higher levels of CD127 but lack CD154 expression upon activation. nTregs, defined as $CD4^{+}CD25^{high}FoxP3^{+}$, from whole-blood staining of healthy controls (HC), ESRD, and kidney transplantation (KTx) patients 1, 5, 10, 15, and 20 years after transplantation were analyzed for the expression of CD127 on $nTreg_{NAIVE}$ (**a,d**), $nTreg_{CM}$ (**b,e**), and $nTreg_{EM}$ (**c,f**) as described in Figure 1. Depicted are %CD127 nTreg frequency mean values ± 95% confidence interval. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (**g,h**) Peripheral mononuclear blood cells were isolated and stimulated with phorbol 12-myristate 13-acetate/ionomycin. nTregs were identified by FACS as $CD4^{+}CD25^{high}FoxP3^{+}$ cells and analyzed for the expression of CD154. Non-Tregs were identified as $CD4^{+}CD25^{low/int}FoxP3^{-}$ by Boolean gating. Depicted is CD154 expression of 1 representative HC and 1 (continued)

depletion of CD127$^+$ cells when isolating nTregs for clinical applications to increase product purity,[54,67,113–117] and most studies investigating nTregs did not address or excluded CD127$^+$ cells from Treg analysis.[21,54,67,68,91,117–122] This is of particular importance because different isolation techniques result in protocol-specific cell products, most of which are not licensed or impractical for clinical applications. Further, it must be considered that Tconv downregulate CD127 upon stimulation, which indicates that this marker should, if at all, only be used for Treg definition in resting T-cell populations and in combination with high CD25 coexpression (Figure 4).[36,37] In 2010, Simonetta *et al.*[123] suggested that CD127 can be upregulated on activated Tregs. Rosenblum and colleges further demonstrated that *in vivo* functionally active Tregs can express CD127 and that the expression of the IL-7 receptor is associated with strongly suppressive Treg characteristics and coexpression of high levels of CTLA-4 and KLRG-1.[124] Accordingly, it seems that the paradigm of nTregs being CD127^{low} needs to be reconsidered. In line with these 2 publications, we identified a Treg population with increased CD127 expression in dialysis patients, which was detectable in neither healthy controls nor in KTx patients. Functional analysis showed a clear and stable nTreg phenotype lacking activation-induced CD154 upregulation, a hallmark of non-nTreg cells. *Ex vivo* expansion of these cells resulted in a highly pure and functionally stable nTreg product. Accordingly, we consider depletion of CD127$^+$ cells dispensable for the nTreg selection process.

In summary, we were able to demonstrate that the generation of high-quality polyclonally expanded nTreg product from ESRD patients is feasible without depletion of CD127$^+$ cells. Maintenance immunosuppression after kidney transplantation slightly alters nTreg maturation, but overall survival seems unaffected. Accordingly, adoptive transfer of nTregs constitutes a promising therapeutic approach. It may enable us to shift the immunologic response toward a more tolerant state and might allow tapering of a standard immunosuppressive regimen after kidney transplantation.

METHODS

Study population

Ten healthy probands, 46 patients requiring chronic hemodialysis due to ESRD, and 143 patients 1 year (±2 months), 5, 10, 15, and 20 years (±1 year) after KTx were enrolled in this study. Blood collection was performed in the ESRD cohort after a long interdialytic interval directly before initiation of routinely scheduled dialysis (3 times weekly regimen) and in KTx patients during a regular follow-up visit. To control for age, patients were recruited to the age groups younger than 40 years of age, 40 to 59 years of age, and 60 years of age or older. Detailed patient characteristics are summarized in Table 1. This study was approved by the Charité University Medicine ethics committee (Institutional Review Board). Informed consent was documented.

Peripheral mononuclear blood cell preparation

Peripheral mononuclear blood cells (PBMCs) were separated from heparinized venous blood by density centrifugation using Ficoll-Paque (Amersham Bioscience, Freiburg, Germany). Isolated cells were counted using the Casy Cell Counter System (Omni Life Sciences, Bremen, Germany). The viability was routinely >95%. PBMCs were stimulated overnight with anti-CD3/anti-CD2/anti-CD28–coated T-cell activating beads as previously described by Meier *et al.*[125] To enable intracellular staining of activation-induced CD154 expression, 2 μg/ml Brefeldin A (Sigma-Aldrich, Taufkirchen, Germany) were added 2 hours after of activation onset. Cells were incubated overnight at 37 °C in a 5% humidified CO_2 environment.

nTreg isolation and expansion

nTreg isolation and polyclonal expansion was performed as previously described.[43] Briefly, nTregs were isolated from PBMCs of freshly collected blood by CliniMACS isolation (Miltenyi Biotec) performing CD8 depletion followed by CD25+ enrichment. For expansion, nTregs were suspended in X-VIVO15 medium (Lonza, Cologne, Germany) containing 10% serum, 500 U/ml human recombinant IL-2 (Chiron Behring, Marburg, Germany), and 100 nM rapamycin (Sigma-Aldrich); rested overnight; and stimulated with (CD3/CD28) Treg expansion beads (Miltenyi Biotec). For nTreg product analysis, cells were directly stained or stimulated with phorbol 12-myristate 13-acetate/ionomycin for 16 hours in the presence of 2 μg/ml Brefeldin A (Sigma-Aldrich) to allow intracellular cytokine staining.

Flow cytometry

Whole blood, PBMC, and nTreg staining was performed using the FoxP3 Staining Buffer Set purchased from eBioscience (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's recommendations. The following antibodies were used for whole-blood staining: CD14-PacificOrange (Invitrogen, Darmstadt, Germany); CD3-PacificBlue, CD4-AlexaFluor700, CD25-PE (BD Biosciences, Heidelberg, Germany); CD127-APC-AlexaFluor780, CD62L-PerCP-Cy5.5 (BioLegend, San Diego, CA); CD45RA-ECD (Beckman Coulter, Krefeld, Germany); and FoxP3-AlexaFluor488 (BD Pharmingen, Heidelberg, Germany). The following antibodies were used for PBMC stainings: CD3-PE-Cy7, CD4-AlexaFluor700, CD25-PE (BD Biosciences), CD14-PacificOrange (Invitrogen), CD127-APC-AlexaFluor780, CD45RA-ECD (Beckman Coulter); FoxP3-AlexaFluor488, CD137-PE-Cy5 (BD Biosciences), and CD154-PacificBlue (BioLegend). Dead cells were routinely detected by staining with aqua fluorescent reactive dye (Invitrogen). Data acquisition was performed on a FACS LSRII (BD Biosciences) or Navios flow cytometer (Beckman Coulter). FlowJo software V8 (Treestar) was used for further analysis.

Figure 4 | (continued) ESRD patient (**g**) and summarized data showing individual expression levels of CD154 as mean fluorescence intensity (MFI) (**h**). Data are given as the percentage of frequencies with mean values ± 95% confidence interval. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Number of individuals analyzed: HC, $N = 10$; ESRD, $N = 46$; time after transplantation: 1 year ($N = 30$), 5 years ($N = 36$), 10 years ($N = 31$), 15 years ($N = 22$), 20 years ($N = 24$); 0 rejection episodes ($N = 105$), 1 rejection episode ($N = 29$), 2 rejection episodes ($N = 6$), 3 rejection episodes ($N = 3$).

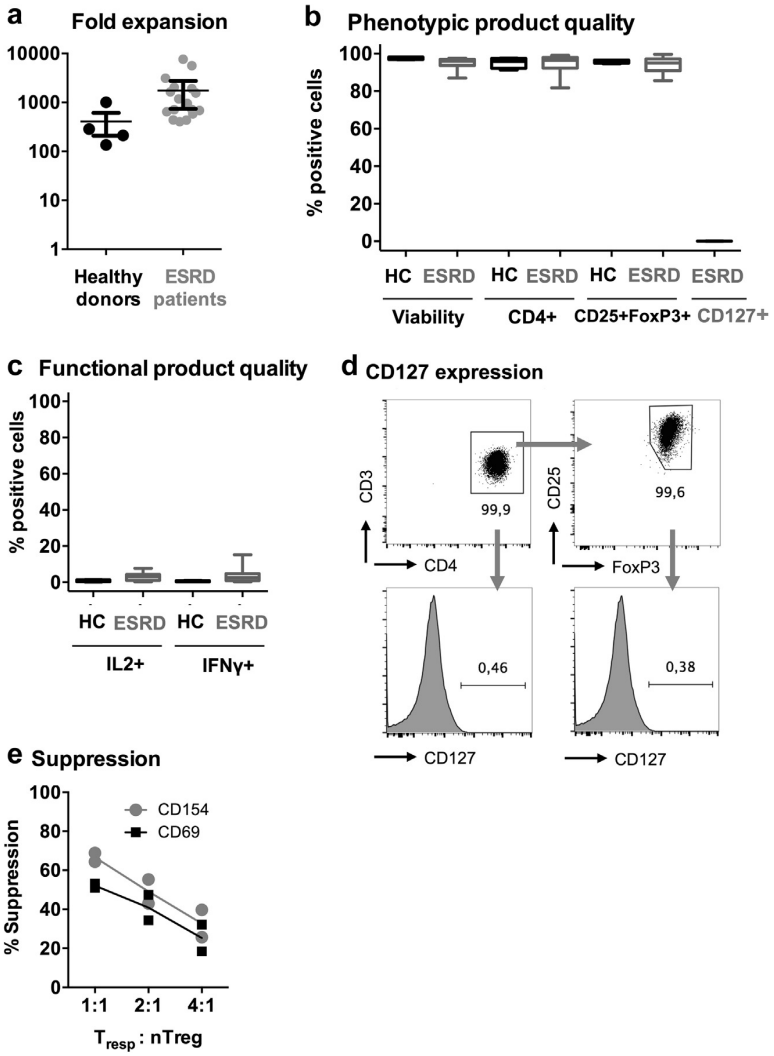

Figure 5 | CD127 depletion is dispensable in the production process of *ex vivo* expanded natural regulatory T cells (nTregs) from end-stage renal disease (ESRD) patients. nTregs were isolated and expanded from health controls (HC) ($N = 4$) and ESRD patients ($N = 17$) under Good Manufacture Practice conditions. The cell products were analyzed for (**a**) x-fold expansion rates after 3 weeks and both (**b**) phenotypic and (**c**–**e**) functional end-product quality. (**c**) Cells were stimulated with phorbol 12-myristate 13-acetate/ionomycin and assessed for the expression of proinflammatory cytokines including interleukin-2 (IL-2) and interferon-γ (IFN-γ). Representative data of CD127 expression in clinical grade Treg products as summarized in (**b**) are depicted in (**d**) from 1 representative ESRD patient. (**e**) Suppressive capacity of *ex vivo* expanded nTregs was functionally assessed in 2 nTreg products by mixed lymphocyte reactions and subsequent quantification of CD154 and CD69 upregulation on non-nTregs (responder T cells, T_{resp}) upon T-cell receptor activation.

nTregs were defined as $CD4^{+}CD25^{high}FoxP3^{+}$ cells. By Boolean gating, non-Treg cells were identified and considered Tconv.

Suppression analysis

Expanded nTregs were rested overnight and cocultured with freshly isolated or cryopreserved autologous PBMCs. Cells were stimulated with T-cell activation beads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 7 hours and subsequently stained using the FastImmune Human Regulatory T-cell Function Kit (Becton Dickinson) according to the manufacturer's recommendations. FACS analysis was performed on a Navios flow cytometer (Beckman Coulter). Further analysis and graph design were performed using Microsoft Excel 2010 and GraphPadPrism5.

Statistical analysis

Data analysis was conducted using a MATLAB-Simulink environment and GraphPadPrism5 by 1-way analysis of variance followed by Tukey's test. P values <0.05 were considered significant. Data are depicted as scatter or bar plots plus the mean and 95% confidence interval.

DISCLOSURE

All the authors declared no competing interests.

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Im nächsten Schritt untersuchten wir den Einfluss von konventionellen Immunsuppressiva auf intrinsische und adoptiv transferierte Tregs, um im Rahmen der adoptiven Treg-Therapie pharmakologische Synergien zu nutzen und antagonistische Effekte zu vermeiden. Hierfür nutzen wir ein humanisiertes Maus-Modell, das es uns ermöglichte, funktionelle Phänomene in vivo zu betrachten.

2.4. Im Gegensatz zu Glukokortikoiden unterstützt Cyclosporin A die Wirksamkeit von *ex vivo* expandierten, adoptiv transferierten humanen Tregs bei GvHD.

Der folgende Text wurde durch die Autorin übersetzt und entspricht im Wesentlichen dem Abstrakt der Arbeit

Landwehr-Kenzel S, Zobel A, Schmitt-Knosalla I, Forke A, Hoffmann H, Schmueck-Henneresse M, Klopfleisch R, Volk HD, Reinke P. Cyclosporine A but Not Corticosteroids Support Efficacy of *Ex Vivo* Expanded, Adoptively Transferred Human Tregs in GvHD Frontiers Immunology, Oktober 2021, Band 12, Artikel 716629. [117]

Die Modulation des Immungleichgewichts durch adoptiven Transfer von regulatorischen T-Zellen (Tregs) entwickelte sich zu einer vielversprechenden Strategie zur Behandlung unerwünschter Immunreaktionen, einschließlich der Graft-versus-Host-Erkrankung, welche jenseits von Rezidiven die am häufigsten zum Tode führenden Komplikation nach allogener Stammzelltransplantation darstellt. Derzeit ist jedoch wenig über die potenziell inhibitorischen in-vivo-Effekte von konventionellen immunsuppressiven Medikamenten, die routinemäßig zur Behandlung der GvHD eingesetzt werden, auf adoptiv transferierte Tregs bekannt. Im Rahmen dieser Arbeit untersuchten wir in einem humanisierten NOD/SCID/IL2Rgamma -/- GvHD Mausmodell arzneimittelspezifische Wirkungen der konventionellen Immunsuppressiva Cyclosporin A, Mycophenolatmofetil und Methylprednisolon auf adoptiv übertragene Tregs. Sowohl der klinische Verlauf der GvHD als auch die post mortem durchgeführte Organhistologie einschließlich der Analyse zellulärer Organinfiltrationen zeigten, dass eine kombinierte Therapie mit Cyclosporin A und Tregs äußerst vorteilhaft ist, da sie die Akkumulation von Tregs in entzündetem Gewebe von Leber und Lunge fördert. In ähnlicher Weise verbesserte die Kombination

von Mycophenolatmofetil mit Tregs die klinischen Zeichen der GvHD. Im Gegensatz dazu führte die gleichzeitige Verabreichung von Methylprednisolon und Tregs zu einer verringerten Treg-Rekrutierung in entzündetes Gewebe und einer schnellen Verschlechterung des klinischen Zustandes einiger Tiere. Daher empfehlen wir bei der Planung klinischer Studien, die die Sicherheit und Wirksamkeit einer adjuvanten Treg-Therapie untersuchen, die Treg-Therapie mit Cyclosporin A zu kombinieren, während hohe Dosierungen von Glukokortikoiden vermieden werden sollten.

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Cyclosporine A but Not Corticosteroids Support Efficacy of *Ex Vivo* Expanded, Adoptively Transferred Human Tregs in GvHD

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Reshaping the immune balance by adoptive transfer of regulatory T-cells (Tregs) has emerged as a promising strategy to combat undesired immune reactions, including in Graft-versus-Host Disease (GvHD), which is the most lethal non-relapse complication of allogeneic hematopoietic stem cell transplantation. Currently however, little is known about the potentially inhibitory *in vivo* effects of conventional immunosuppressive drugs, which are routinely used to treat GvHD, on adoptively transferred Tregs. Here we demonstrate drug-specific effects of the conventional immunosuppressive drugs Cyclosporine A, Mycophenolate mofetil and methylprednisolone on adoptively transferred Tregs in a humanized NOD/SCID/IL2Rgamma-/- GvHD mouse model. The clinical course of GvHD and *postmortem* organ histology, including cellular organ infiltration, showed that co-administration of Cyclosporine A and Tregs is highly beneficial as it enhanced Treg accumulation at inflammatory sites like lung and liver. Similarly, co-administration of Mycophenolate mofetil and Tregs improved clinical signs of GvHD. In contrast, co-administration of methylprednisolone and Tregs resulted in reduced Treg recruitment to inflammatory sites and the fast deterioration of some animals. Consequently, when clinical trials investigating safety and efficacy of adjunctive Treg therapy in GvHD are designed, we suggest co-administering Cyclosporine A, whereas high doses of glucocorticosteroids should be avoided.

Keywords: transplantation, regulatory T-cells, adoptive cell therapy, tolerance, drug interaction, Graft-versus-Host Disease, immunosuppressive drugs

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative treatment option for many patients with malignant and non-malignant diseases of the hematopoietic system. Graft-versus Host-Disease (GvHD) however, still constitutes one of the most lethal post-transplant complications of HSCT (1). While pharmacologic immunosuppression has dramatically improved short-term outcomes, long-term morbidity and mortality is still poor in patients suffering from GvHD. Despite the beneficial factor of graft-versus leukemia effects in patients with malignant underlying disease, transplantation related mortality rises from 5-13% in patients without GvHD to 51-75% in patients suffering from any form of GvHD (2). The current paradigm holds that GvHD is primarily a T-cell mediated disease caused by donor effector-T-lymphocytes recognizing allogenic structures as non-self in the host, and thereby eliciting their proinflammatory and cytolytic program. Our understanding of immunoregulatory mechanisms profoundly advanced with the identification of thymic derived regulatory T-cells (tTregs) as physiologic counterplayers of effector T-cells and critical mediators of immune homeostasis after T-cell activation by self- and non-self structures (3). tTregs are characterized by high expression of CD25 and the transcription factor FoxP3, and characteristically low expression of CD127. All three proteins are established as markers for Treg diagnostics. A numeric loss of tTregs is associated not only with a higher incidence and severity of GvHD (4–8) but also with autoimmune diseases (9–11). In contrast, therapeutic benefits can be achieved by enrichment of circulating tTregs after HSCT (12–19) and in the context of solid organ transplantation (20–22) and autoimmune diseases such as hepatitis C vasculitis (23), systemic lupus erythematosus or Type 1 diabetes (24–26).

Recent technological developments have enabled the purification and *ex vivo* expansion of Tregs on a large scale and under clinical grade conditions. This has allowed the translation of adoptive tTreg transfer from animal models to clinical protocols. In order to investigate safety and efficacy, Tregs will have to be administered as adjunctive therapy in addition to current state of the art protocols in early clinical trial phases. Therefore, in the context of HSCT, Treg transfer will have to be combined with the conventional immunosuppressive drugs (cISD) Cyclosporine A (CSA), mycophenolate mofetil (MMF) and prednisolone or methylprednisolone (MP) as components of prophylactic as well as first-, second- and third-line treatment regimens.

The question of "if" and "how" cISD influence the function and frequency of tTregs *in vivo* has previously been addressed, but some of the data are contradictory. Furthermore, there is little data available describing the effects of these drugs on the *in vivo* fate of *ex vivo* manipulated, adoptively transferred tTregs (27). Direct comparison of individual cISD in a clinical setting is difficult, since standard immunosuppressive regimens in HSCT but also for solid organ transplantation are comprised of a combination of at least two agents. Therefore, most data investigating individual drug-specific effects were generated by *in vitro* or animal experiments. The prevailing opinion assigns a beneficial effect of mTOR (Rapamycin, Everolimus) (28) and histone deacetylase inhibition (29, 30) but also low-dose Interleukin-2 (18, 23, 31–36) and anti-thymocyte globulin (37, 38) to tTreg maturation, function and survival. In contrast, the calcineurin inhibitors (CNI) CSA and Tacrolimus (20, 27, 28, 39–48), and blockage of CTLA-4 (49) and Interleukin-2 (50) seems to hamper both tTreg development and function. Even less consistent data exists on the effect of complement inhibitors, steroids (51–56) and MMF (43, 57). The effect of cISD on *ex vivo* expanded and adoptively transferred tTregs are just now starting to receive attention, however, study designs and results are diverse.

Therefore, the aim of this study was to determine the drug-specific influence of the standard prophylactic and therapeutic first-, second- and third-line drugs, CSA, MMF and MP used in GvHD therapy, on adoptively transferred Tregs. In order to investigate the pharmacological effects of cISD at the cellular level *in vivo*, as well as to obtain a deeper understanding of beneficial combinations of cISD with adjunctive Treg therapy in the clinical setting of GvHD, a humanized GvHD mouse model was employed. Insights obtained from this work will pave the way for rational design of prospective Phase I and II clinical trials investigating safety and efficacy of adjunctive Treg therapy in patients with acute and chronic GvHD.

MATERIALS AND METHODS

Cell Isolation

Peripheral blood mononuclear cells (PBMC) were isolated from freshly collected blood of healthy volunteers or from buffy coats by Ficoll density-gradient centrifugation (PAA Laboratories, Pasching, Austria). Treg isolation was performed using the CD4+CD25+ Treg isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's recommendations. This study was approved by the Charité-Universitätsmedizin Berlin ethics committee (Institutional Review Board) and all blood donors provided written informed consent.

Treg Expansion

Freshly isolated Tregs were polyclonally expanded as described previously (13). Briefly, Tregs were cultured in X-VIVO15 medium (Lonza, Basel, Switzerland) containing 10% serum, 500 U/mL human recombinant IL-2 (Chiron Behring, Marburg, Germany) and 100 nM rapamycin (Sigma–Aldrich, St. Louis, MO) and stimulated with CD3/CD28 Treg expansion beads (Miltenyi Biotech). Cells were restimulated every 3-4 days. After 3-weeks expansion time tTreg product purity was assessed by specific staining of CD3-APC750 (Clone UCHT1), CD4-APC (Clone 13B8.2), CD8-A700 (Clone B9.11), CD25-PeCy7 (Clone B1.49.9) from Beckman Coulter, Krefeld Germany) and FoxP3-A488 (Clone 259D/C7, Beckton Dickinson, Heidelberg,

Germany). Mean Treg purity after isolation was 80% for $CD3^+CD4^+$ and 60% for $CD3^+CD4^+CD25^{high}$ cells. After isolation Tregs were expanded in cell culture medium supplemented with IL-2 and rapamycin in order to promote Treg expansion and suppress expansion of non-Treg cells. The final Treg products consisted of ~90% $CD3^+CD4^+$ T-cells with a frequency of 94% $CD25^{high}FoxP3^+$ Tregs.

Humanized Animal Experiments for Xenogenic GVHD

The adult offspring of NOD/SCID/IL-2Rgamma^{-}/$^{-}$ mice, originally purchased from Charles River Laboratories (Research Models and Services, Sulzfeld, Germany), were used according to the approval by the Landesamt für Gesundheit und Soziales Berlin (G0483/09). Mice were maintained under specific pathogen-free conditions. Xenogenic GvHD was induced, after sublethal radiation (300 cGy), by single intravenous injection of $3x10^6$ human PBMCs/mouse. *Ex vivo* expanded human tTregs, that originate from the same donor as the PBMCs used to induce GvHD, were administered i.v. at a single dose of $1.5x10^6$, $3x10^6$, or $6x10^6$, equivalent to a PBMC:tTreg ratio of 2:1, 1:1 and 1:2. Additionally $1.5x10^6$ tTregs were combined with each of the cISD. Conventional immunosuppressive therapy was administered daily from day+1 until mice were sacrificed on day+35 or sacrificed due to progressive disease: Cyclosporine A 4 mg/kg s.c. (Novartis Pharma GmbH, Nürnberg, Germany), Mycophenolate Mofetil 0.5 mg p.o. (MMF, CellCept® 1g/5ml Roche Pharma, Grenzach-Wyhlen Germany), Methylprednisolone 20 mg/kg/d i.p. daily (MP, Urbason ® solubile 32 mg Sanofi Aventis Deutschland GmbH, Frankfurt am Main, Germany). cISD dose finding was based on our own experience (20) and previously published data (58–60) in order to mimic the clinical situation with serum levels similar to target levels used in human patients. During the experimental setup we aimed for equal distribution of male and female animals in all groups (**Supplementary Figure S1**). The first group included mice that received no form of therapy after the PBMC administration. To further control for the effect of tTregs alone, tTregs were administered to a second control group without prior administration of PBMCs. Animals were monitored daily for body weight and clinical signs of GvHD (behavior, fur alterations, skin inflammation, hunched back, **Supplementary Table ST1**) and scored as either no GvHD (0); mild GvHD (1 – 1.5), moderate GvHD (2 – 2.5) or severe GvHD (3). Livers, lungs, spleens and intestines were collected on day+35 or when clinical signs required euthanasia. Euthanasia due to a poor clinical condition was performed when body weight decreased by >20% of initial body weight or at a clinical score of 3. Details of experimental setup are depicted in **Figure 1** and days of analysis further listed in **Table 1**.

Organ Flow-Cytometry

After animals were sacrificed their organs were collected, divided and either fixed in 5% formalin for histologic analysis or resuspended in saline for direct organ flow cytometry. To isolate cells for FACS analysis, organs were mechanically disrupted and further digested by DNAse and collagenase (Sigma Aldrich, Germany). Subsequently, erythrocytes were lysed and remaining cells were washed in PBS and culture medium. Lung sample cells were flushed through 100 µm and 70 µm pore sized filters before staining. Hepatic samples were centrifuged at 50 xg for 3 minutes after cellular disruption and

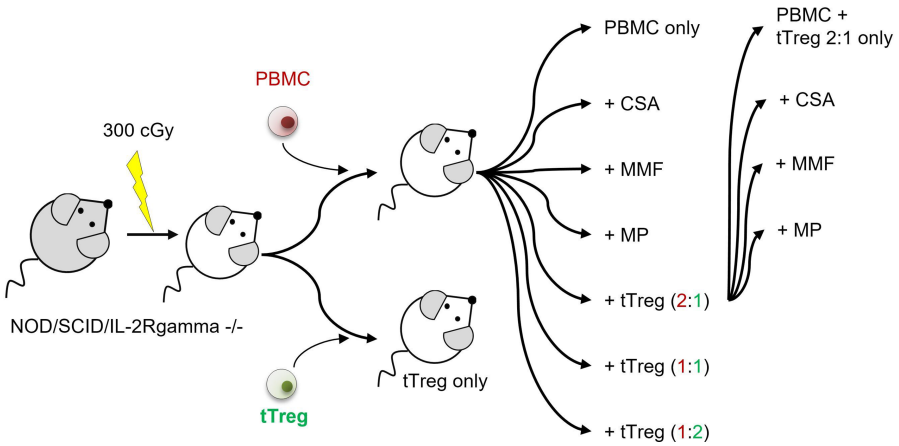

FIGURE 1 | Experimental setup. NOD/SCID/IL-2Rgamma^{-}/$^{-}$ mice were sub-lethally radiated with 300 cGy (day -1). Xenogenic GvHD was induced by intravenous application of $3x10^6$ human PBMCs. GvHD control mice did not receive further therapy. tTreg control mice received $3x10^6$ *ex vivo* expanded tTregs. If indicated tTregs were simultaneously administered with PBMCs at a dosage of $1.5x10^6$ (PBMC:tTreg ration of 2:1), $3x10^6$ (PBMC:tTreg ratio of 1:1) or $6x10^6$ (PBMC:tTreg ratio of 1:2). Mice that received either PBMCs alone or in combination with tTregs were further treated daily with the cISD CSA (4 mg/kg/d s.c.), MMF (0.5 mg/d p.o) or MP (20 mg/kg/d i.p.) starting day +1 after cell administration. Animals were monitored daily for body weight and clinical signs of GvHD (behavior, fur alterations, skin inflammation, hunched back). Liver, spleen, and lungs were collected on day +35 or when clinical signs required euthanasia.

TABLE 1 | Summary of experimental setup.

Group	Total number of animals	Number of independent experiments	Survival in days (=Days of organ analysis)
PBMC alone	6	3	18, 18, 24, 27, 29, 29
tTreg alone	3	2	35, 35, 35
PBMC:tTreg 2:1	6	3	15, 20, 26, 33, 34, 35
PBMC:tTreg 1:1	7	3	27, 29,31, 32, 35, 35, 35
PBMC:tTreg 1:2	5	2	22, 30, 25, 35, 35
PBMC + CSA	5	3	35, 35, 35, 35, 35
PBMC + MMF	5	3	10, 30, 35, 35, 35
PBMC + MP	5	4	35, 35, 35, 35, 35
PBMC + CSA + tTreg	5	2	35, 35, 35, 35, 35
PBMC + MMF + tTreg	6	3	31, 32, 35, 35, 35, 25
PBMC + MP + tTreg	6	3	24, 25, 31, 35, 35, 35

Animals were assigned to 10 treatment groups, including mice who received PBMCs alone, PBMCs plus Treg monotherapy at a PBMC:tTreg ratio of 1:2, 1:1 and 1:2, PBMCs plus cISD CSA (4 mg/kg/d s.c.), MMF (0.5 mg/d p.o) or MP (20 mg/kg/d i.p.) or PBMCs plus co-administration of tTregs and cISD as listed in column 1.
The total numbers of animals per group and the numbers of individual experiments per group are listed in the second and third column, respectively. Days of survival are listed for every mouse of each group in column four. All organs were processed for analysis directly after harvesting.

collagen digestion. Most hepatocytes sedimented whilst lymphocytes remained in the supernatant. The supernatant was collected and spun at 200xg for 2 minutes. The lymphocytes sedimented and were subsequently collected. Lymphocytes were additionally enriched by density centrifugation using Lymphocyte Separation Medium (LSM1077, PAA Laboratories, Pasching, Austria) before erythrocytes were lysed and cells subjected to the routine staining procedure. Antibodies were titrated to establish optimal staining conditions before use: CD3-APC-A750 (Clone UCHT1), CD4-APC (Clone 13B8.2), CD8-APC-A700 (Clone B9.11), CD19-ECD (Clone J3-119), CD25-PeCy7 (Clone B1.49.9), CD45-PacificBlue (Clone J33), CD56-PE (Clone N901), CD127-APC-A700 (Clone R34.34) from Beckman Coulter, Krefeld Germany; CD45 mouse-PerCP-Cy5.5 (Clone 30-F11, Biolegend, Germany); L/D PacificOrange (Life Technologies GmbH, Darmstadt, Germany); FoxP3-A488 (Clone 259D/c7), Ki67-PerCP-Cy5.5 (Clone B56) from Beckton Dickinson, Heidelberg, Germany. Flow cytometry was performed using Beckman Coulter NAVIOS cytometer. Data were analyzed using Kaluza® software (Beckman Coulter). For staining, up to $2x10^6$ cells were suspended in 100 µl FACS buffer and unspecific Fc receptors were blocked using FcR blocking reagent (Miltenyi Biotech GmbH, Germany). Extracellular staining was performed as indicated. Cells were fixed and permeabilized using FoxP3 staining buffer set purchased from eBioscience, San Diego. Subsequently, cells were intracellularly stained, washed and analyzed by flow cytometry.

Histology

Tissue samples of liver, lung, spleen, and kidney were fixed in 5% formalin, embedded in paraffin, and cut into 5 µm-thick slices. Slices were dewaxed and stained with hematoxylin/eosin. For microscopy an Olympus BX41 (Olympus, Hamburg, Germany) microscope with a 20-fold magnification lens was used. The grade of infiltration of human cells was calculated based on a semi-quantitative scoring system. The scoring system ranged from 0 (no); 1 (mild); 2 (moderate) up to 3 (massive) infiltration by inflammatory cells.

Statistics

Unless stated otherwise, data are presented as scatter plots showing individual values and means ± SEM. Statistical significance was determined by Kaplan-Meier analysis and Log-rank test (Mantel-Cox-Test) or One-Way-ANOVA with Tukey correction. Significance levels are shown as indicated in the legends.

Data Sharing

Most of the data are presented in the manuscript text, figures, and legends. Any additional data will be made available upon request by the corresponding author (sybille.landwehr-kenzel (at)charite.de).

RESULTS

Co-Administration of CSA or MMF With Adoptive Treg Transfer Improves the Clinical Course of GvHD

To investigate the clinical effect of conventional immunosuppressive drugs on adjunctive tTreg therapy, GvHD was induced in NOD/SCID/IL-2Rgamma-/- mice by infusion of $3x10^6$ human peripheral blood mononuclear cells (PBMCs) (**Figure 1**, **Table 1** and **Figures 2A–M**). Severe GvHD, substantial weight loss and a higher GvHD score were observed in all mice (**Figures 2A, B, J**). The timing of clinical GvHD manifestation resembled the clinical course known from human HSCT, with disease onset usually occurring during the first 15 days after transplantation. Mice showed signs of GvHD such as hunched posture, scurfy hair and overall reduced general condition. One control group received only tTregs in the absence of PBMCs (**Figures 2A, B**). These mice did not show clinical signs of GvHD (**Figures 2A, B**). Following PBMC-mediated GvHD induction mice were treated once with escalating doses of *ex vivo* expanded tTregs in the absence of immunosuppressive drugs. Remarkably, monotherapy with tTregs led to a statistically significant dose-dependent reduction in weight loss (**Figure 2A**) and reduced GvHD activity (**Figure 2J**). In line, overall survival was significantly improved by higher doses of tTregs (**Figures 2B, I, J, K–M** and **Table 1**). Alternatively, mice were treated daily with the cISD CSA (4 mg/kg s.c.), MMF (0.5 mg p.o.) or Methylprednisolone (MP, 20 mg/kg i.p.) (**Figures 2C, D**). cISD dosages were based on previously published data in order to reach target serum levels similar to target trough levels in the clinical setting (20, 58–60). To investigate the effects of co-administered cISD on adoptively transferred tTregs, the lowest tTreg dose (PBMC: tTreg = 2:1) was combined with CSA, MMF or MP (**Figures 2A–J**). Mice that received any of the cISD alone suffered from substantial weight loss and in case of MMF monotherapy, showed partially reduced survival (**Figures 2C, D, I, J** and **Supplementary Figure S2**). Despite their poor clinical condition, some mice receiving CSA or MMF alone did not fulfill the endpoint

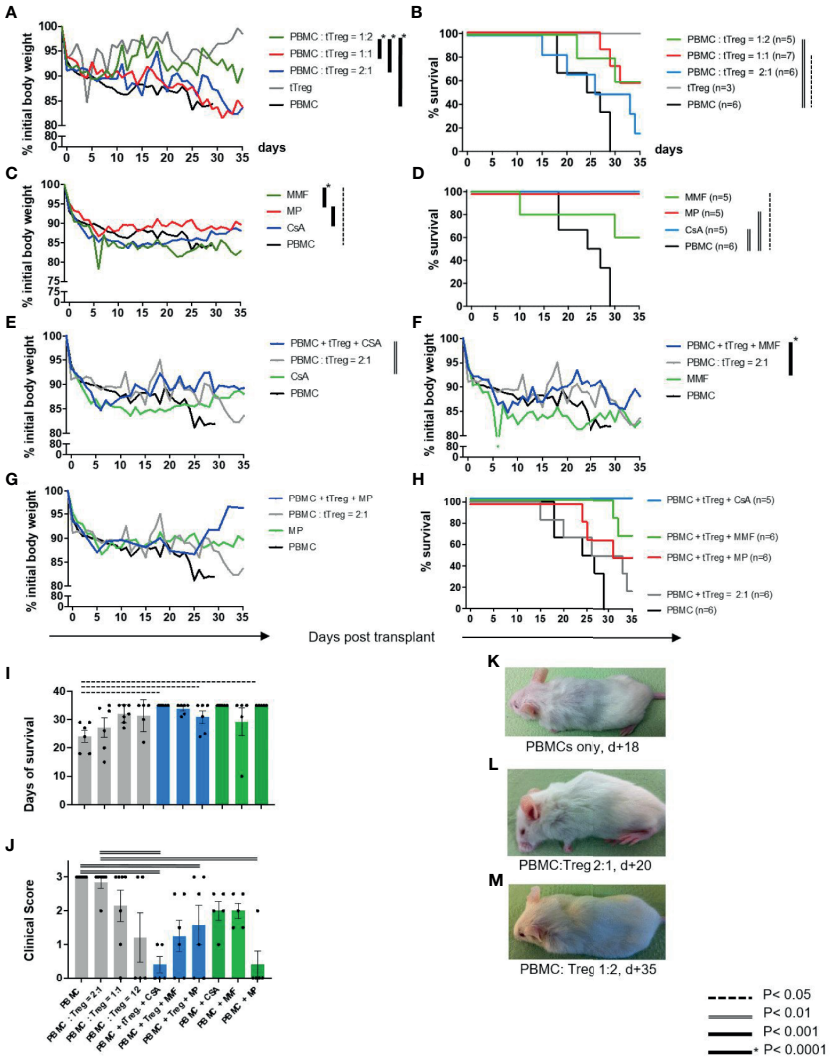

FIGURE 2 | Clinical course and survival rates. As described in **Figure 1** GvHD was induced in NOD/SCID/IL-2Rgamma -/- mice by infusion of $3x10^6$ human PBMC [GvHD control, **(A–M)**]. tTreg control mice received *ex vivo* expanded human tTreg alone ($3x10^6$) but no PBMCs [tTreg, **(A, B)**]. **(A, B)** Mice were treated with escalating doses of *ex vivo* expanded tTregs at a ratio of 2:1 (n=6), 1:1 (n=7) or 1:2 (n=5). **(C, D)** Alternatively, mice were treated with the clSD CSA (4 mg/kg s.c.), MMF (0.5 mg p. o.) or MP (20 mg/kg i.p.) (n=5) alone. **(E–H)** After GvHD induction by PBMCs mice were treated with tTregs in combination with CSA **(E, H)**, MMF **(F, H)** or MP **(G, H)**. **(J)** Clinical assessment further included clinical signs of GvHD (behavior, fur alterations, skin inflammation, hunched back, **Supplementary Table ST1**) and was quantified as either no GvHD (0); mild GvHD (1 – 1.5), moderate GvHD (2 – 2.5) or severe GvHD (3). **(K–M)** Representative images demonstrating the clinical condition of mice treated with no [**(K)**, d+18], low-dose [**(L)**, d+20] or high dose [**(M)**, d+35] tTregs are shown. Depicted are mean weight curves as percentage of initial body weight **(A, C, E–G)** and Kaplan-Meier-Survival curves **(B, D, H)**. Individual body weight values are depicted in the **Supplementary Figure S2**. To support survival curves, days of survival are further listed in **Table 1**. Statistical analysis of weight curves was performed as One-Way-ANOVA with Tukey correction for multiple comparison. Survival data were statistically analyzed using Log-Rank (Mantel-Cox) test. CSA, Cyclosporine A; MMF, Mycophenolate Mofetil; MP, Methylprednisolone.

criteria for euthanasia (**Figures 2D, I, J**). Their prolonged survival was associated with a lower clinical GvHD score (**Figures 2I, J**). CSA or MMF administration in addition to tTreg therapy, substantially improved the clinical course of GvHD (**Figures 2E, F, H–J** and **Table 1**). In contrast, co-administration of tTreg and MP was associated with fast deterioration of all animals and a poor clinical outcome (**Figures 2G, H-J**). Most importantly, combined therapy with tTreg/CSA was clinically more effective than any other treatment approaches investigated (**Figures 2A–J** and **Supplementary Figure S2**), resulting in minimal GvHD activity

(**Figure 2H**) and 100% survival (**Figures 2H, I**). In summary, tTreg monotherapy ameliorated the clinical signs of GvHD and improved overall survival, whilst co-administration of CSA or MMF in addition to tTregs significantly improved the clinical outcome, even when combined with the lowest investigated tTregs dose.

Combined Treatment of tTreg Therapy With CSA Reduces Lymphocytic Infiltration and Tissue Inflammation

Histopathologic organ morphology was assessed at the time of euthanasia in liver, lungs (**Figure 3**) and the intestine by

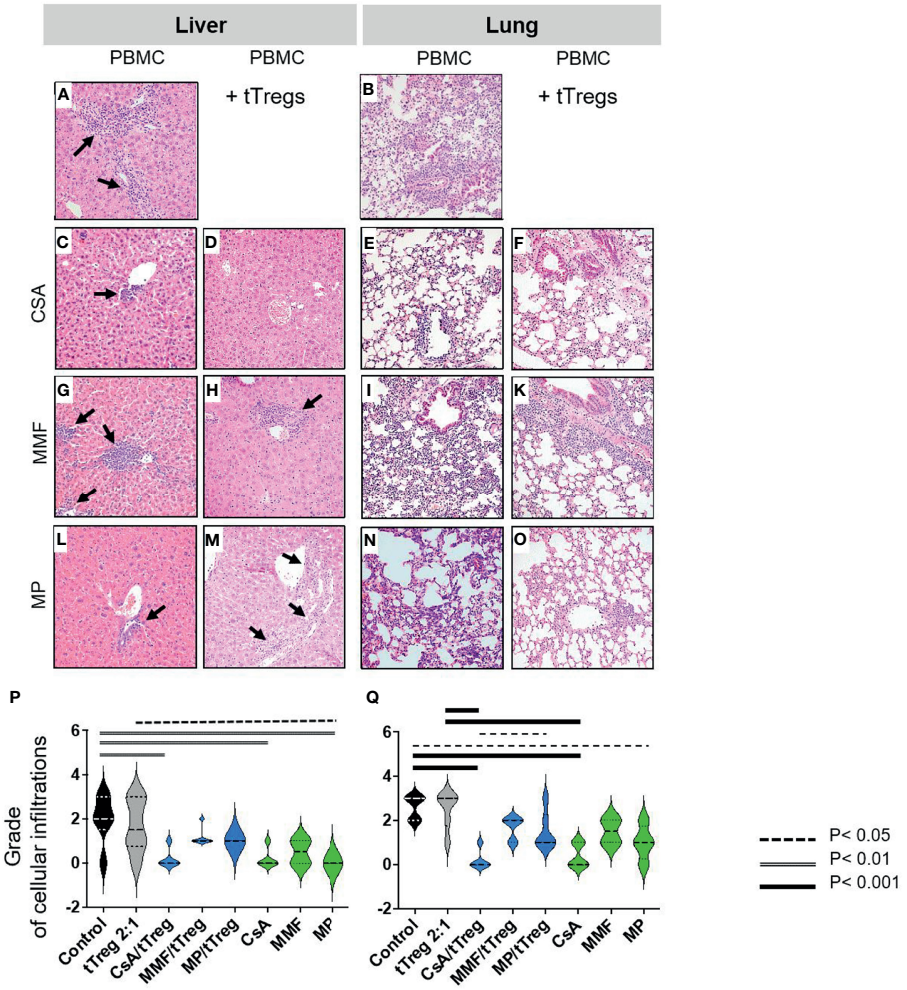

FIGURE 3 | Histopathologic changes of liver and lung. Liver and lungs of mice described in **Figure 1** were stained with Hematoxylin/Eosin (HE). Depicted are **(A–O)** representative images from liver and lung samples at 20-fold magnification and **(A, B)** moderate to high-grade cellular infiltration and inflammation in liver and lungs of animals that received PBMCs but no GvHD therapy. **(A)** Liver shows significant bile duct damage, grade 3 lobular inflammation with dense lymphocyte infiltration and lymphocyte ballooning (arrows). **(B)** Lung tissue shows massive intra-alveolar and intraepithelial lymphocyte infiltration with inflammation of the pulmonary epithelium and apoptotic bodies as well as loss of alveolar tissue architecture. **(C–F)** Only mild lymphocyte infiltration and almost complete resolution of inflammation with preserved tissue architecture in both liver **(C, D)** and lung **(E, F)** were observed in mice treated with CSA **(C, E)** or CSA + Tregs **(D, F)**. **(G, H)** Significant reduction of hepatic lymphocyte infiltration and inflammation after MMF monotherapy; moderate infiltration of lymphocytes and signs of inflammation in mice which received combined MMF/tTreg therapy with preserved hepatic tissue architecture. **(I–K)** Only partial reduction of lymphocyte infiltration and inflammation in lung of mice which received MMF alone or in combination with tTregs. **(I)** Dense lymphocyte infiltration leading to diffuse alveolar damage and septal edema. **(K)** Alveolar architecture partially preserved after MMF/tTreg co-administration. **(L–O)** Significant reduction of cellular infiltration and preserved tissue architecture in both liver and lung upon treatment by MP alone or in combination with tTregs. **(P, Q)** shows quantification of lymphocyte infiltration of liver **(P)** and lung **(Q)**. Statistical analysis was performed as One-Way-ANOVA with Tukey correction for multiple comparisons. Statistical significance levels are indicated as $p < 0.05$ (dotted line), $p < 0.01$ (dashed line), $p < 0.001$ (bold line), $p < 0.0001$ (solid line with asterix). CSA, Cyclosporine A; MMF, Mycophenolate Mofetil; MP, Methylprednisolone.

microscopic analysis of tissue architecture, lymphocyte infiltration and signs of acute inflammation (**Figures 3A–Q**). To histologically semi-quantify these results, human $CD3^+$ cells were stained and quantified in all samples of **(P)** livers and **(Q)** lungs. In line with previously published data (61–63) indicating that the lack of chemotherapeutic conditioning protects from intestinal GvHD, we did not find intestinal infiltrates or alternative signs of GvHD (**Supplementary Figure S3**). Accordingly, intestinal samples were not further analyzed here. In contrast, both the liver and lungs of animals that received PBMCs but not GvHD therapy showed moderate to high grade signs of GvHD (**Figures 3A, B**). In particular, we found hepatic bile duct damage with dense lymphocyte infiltration, inflammation and lymphocyte ballooning (**Figure 3A**). Lung pathology showed a significant loss of alveolar tissue architecture and was dominated by massive intra-alveolar and intraepithelial lymphocyte infiltration with inflammation of the pulmonary epithelium and apoptotic bodies (**Figure 3B**). Co-administration of CSA and tTregs (**Figures 3D, F**), and to a slightly lesser extent CSA monotherapy (**Figures 3C, E**), significantly reduced the histopathological grade of GvHD (**Figures 3P, Q**). This effect was characterized by preserved tissue architecture, mild lymphocyte infiltration and almost complete resolution of inflammation in both the liver and lung. Both MMF and MP administered as monotherapy or combined with tTregs only partially reduced lymphocyte infiltration but ameliorated signs of inflammation and largely preserved organ architecture (**Figures 3G–K**). In summary, therapy with both MMF and MP alone or in combination with tTregs was inferior to CSA and CSA/tTreg therapy. Accordingly, the beneficial effect of tTreg therapy on clinical parameters as described in **Figure 2** correlated with reduced lymphocytic organ infiltration.

The superior efficacy of CSA and CSA/Treg co-administration as compared to the alternative treatment regimen tested became clearer when cellular infiltrates were analyzed in more detail (**Figures 4**, **5**), yielding statistically significant differences.

To more accurately quantify cellular infiltration and further characterize the phenotype of lymphocytic infiltrates, liver (**Figure 4A**), lung (**Figure 4B**) and spleen (**Figure 4C**) tissues were analyzed by flow cytometry. In line with clinical experience, which classifies the liver as primary target organ of GvHD, we found massive infiltration by human CD45+ leukocytes in the liver (**Figure 4A**, black dots), but also in lungs (**Figure 4B**, black dots) and the spleen (**Figure 4C**, black dots). The mildly increased frequency of human leukocytes in case of low-dose Treg monotherapy, reflected the increased amount of circulating human cells but failed to reduce the frequency of infiltrating cells (**Figures 4A–C**, grey dots). Addition of CSA to Treg therapy significantly reduced organ infiltration in the liver, lung and spleen (**Figures 4A–C**, blue triangles). In contrast, neither tTreg/MMF (**Figures 4A–C** blue squares) nor tTreg/MP (**Figures 4A–C**, blue dots) co-administration was sufficient to reduce the hepatic, pulmonary or splenic infiltration of human leukocytes as compared to the untreated PBMC control (**Figures 4A–C**, black dots). Reassuringly, if only tTregs but no

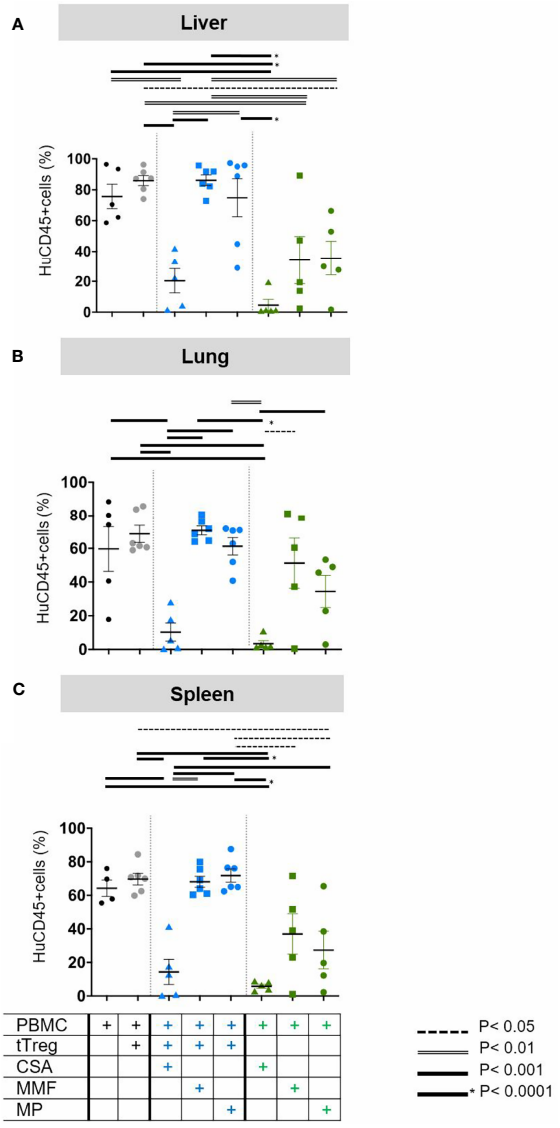

FIGURE 4 | Quantification of human leukocyte infiltration. As described in **Figure 1** GvHD was induced by $3x10^6$ human PBMCs in NOD/SCID/IL2Rgamma -/- mice. Mice were subsequently treated with (i) the individual clSD (green symbols), CSA (4 mg/kg s.c., green triangles), MMF (0.5 mg p.o., green squares) or MP (20 mg/kg i.p., green dots) (n=5) alone (n=5), (ii) with *ex vivo* expanded tTregs at a PBMC:tTreg ratio of 2:1 (n=6, grey dots), or (iii) with a combination of clSD and low dose tTregs (PBMC:tTreg ratio 2:1; CSA/tTreg n=5, blue triangles; MMF/tTreg, blue squares; MP/tTreg n=6, blue dots). Postmortem liver **(A)**, lung **(B)** and spleen **(C)** were analyzed by flow cytometry to quantify the amount of infiltrating CD45+ cells. Depicted are individual values and means ± SEM. Statistical analysis was performed as One-Way-ANOVA with Tukey correction for multiple comparison. Statistical significance levels are indicated as $p < 0.05$ (dotted line), $p < 0.01$ (dashed line), $p < 0.001$ (bold line), $p < 0.0001$ (solid line with asterix). CSA, Cyclosporine A; MMF, Mycophenolate Mofetil; MP, Methylprednisolone.

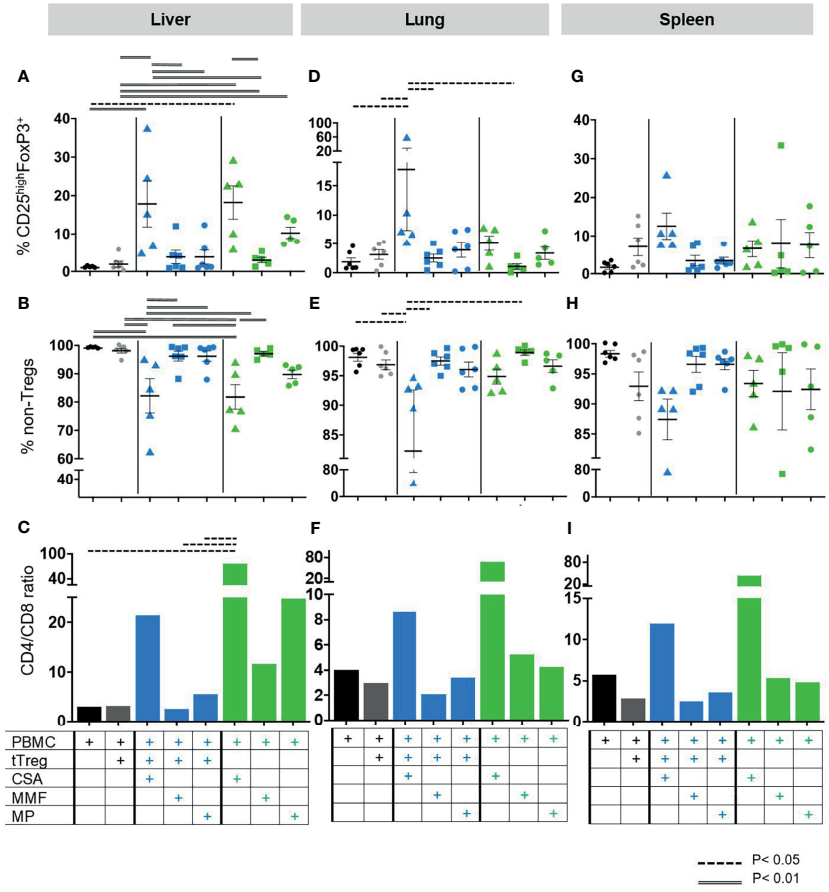

FIGURE 5 | Composition of lymphocytic organ infiltrates. As described in **Figure 1** GvHD was induced by 3x10^6 human PBMCs in NOD/SCID/IL2Rgamma-/- mice. Mice were subsequently treated with (i) the cISD (green symbols) CSA (4 mg/kg s.c., triangles), MMF (0.5 mg p.o, squares) or MP (20 mg/kg i.p., dots) (n=5) alone (n=5), (ii) with *ex vivo* expanded tTregs at a PBMC:tTreg ratio of 2:1 (n=6, grey dots), or (iii) with a combination of cISD and low dose tTregs (PBMC:tTreg ratio 2:1; CSA/tTreg n=5; MMF/tTreg, n=6; MP/tTreg n=6, blue symbols). Organ samples from liver, lung and spleen were further analyzed by flow cytometry to assess lymphocytic infiltration and proliferation. All samples were analyzed directly after harvesting as outlined in **Table 1**. To this end human live lymphocytes were identified as human CD45+ cells and subsequently gated as CD3+ cells. CD3+ T-lymphocytes were subdivided into CD4+ and CD4- cells. Treg cells were identified as CD25highFoxP3+ cells within the CD4+ compartment **(A, D, G)**, while the remaining CD4+ cells were considered as non-Treg CD4+ T-cells **(B, E, H),** CD8$^+$ T-cells were identified as CD3$^+$CD4$^-$. Subsequently CD4/CD8 ratios were analyzed for each group. **(C, F, I)**. Depicted are individual values and mean values ± SEM. Statistical analysis was performed as One-Way-ANOVA with Tukey correction for multiple comparisons. Statistical significance levels are indicated as $p < 0.05$ (dashed line), $p < 0.01$ (doubled line), $p < 0.001$ (solid line). CSA, Cyclosporine A; MMF, Mycophenolate Mofetil; MP, Methylprednisolone.

PBMCs were administered we did not observe infiltration of human cells into any organ (data not shown).

tTreg Recruitment to GvHD Target Tissues Is Promoted by CSA Co-Administration

As PBMC induced GvHD resulted in high frequencies of human leukocytes in the liver, lungs, and spleen, we further aimed to differentiate the composition of leukocytic organ infiltrates and assess the effect of cISD on tTregs in samples from livers (**Figures 5A–C**), lungs (**Figures 5D–F**) and spleens (**Figures 5G–I**). To this end these organ samples were analyzed for the specific frequency of CD4$^+$CD25^{high}FoxP3$^+$ tTreg (**Figures 5A, D, G**) and CD4+ non-Tregs (**Figures 5B, E, H**). Additionally, the CD4/CD8 ratio was assessed in all samples (**Figures 5C, F, I**). Cellular infiltrates in all organs consisted of mainly CD3$^+$CD4$^+$ non-Treg lymphocytes (**Figures 5B, E, H**). In line with our clinical observations, co-administration of CSA and Tregs led to strong recruitment of circulating CD4$^+$CD25^{high}FoxP3$^+$ Tregs to liver, lungs and spleens (**Figures 5A, D, G**) and substantially reduced the frequency of CD3$^+$CD4$^+$ non-Treg lymphocytes in these organs (**Figures 5B, E, H**). This effect was superior to any other therapeutic approach in lungs and spleens **Figures 5D, E, G, H**) and, in hepatic tissues, co-administration of CSA and

Tregs was comparable to CSA monotherapy (**Figures 5A, B**). In line with the survival data presented in **Figure 2**, co-administration of tTregs and MP (**Figures 5A, G**) substantially reduced the hepatic and splenic proportion of tTregs as compared to MP monotherapy (**Figures 5A, G**). Similarly, combined administration of Treg and CSA reduced the frequency of infiltrating CD8 T-cells as expressed by increased CD4/CD8 ratios in these organs and was with this respect superior to both Treg/MMF and Treg/MP therapy (**Figures 5C, F, I**).

DISCUSSION

The main focus of this study was to assess if any of the cISD used in first and second line GvHD prophylaxis or therapy either hamper or support the immunomodulatory functions of adoptively transferred tTregs *in vivo.* To this end we employed a humanized GvHD mouse model and found that *in vivo* survival and function of adoptively transferred tTregs are supported by combining them with CSA therapy, leading to significant reduction of harmful lymphocytic organ infiltration and limited clinical progression of GvHD. Similarly, the clinical course of mice treated with MMF and low-dose tTregs significantly improved. Conversely, we found that co-administration of steroids and tTregs negatively impacts the histological state in the studied tissues and the clinical outcome of GvHD.

During acute GvHD, glucocorticoids are usually administered at high doses to control allo-specific immune responses and tapered thereafter to the minimal dose required to prevent GvHD recurrence. The mechanism of action is mediated by DNA-binding steroid-receptor complexes that form in the cytosol and translocate to the nucleus where they directly interfere with the transcription factors AP-1 and NFkB (64, 65). In-depth data on the direct effects of corticosteroids on tTregs are lacking. Previous studies have shown that corticosteroids increase the frequency of circulating Tregs (51–53), and may amplify expansion of Tregs in an IL-2 (54, 55) or dendritic cell (66–70) dependent manner. These data however were not consistently confirmed (56) and we can only speculate on whether the *ex vivo* manipulation and expansion protocol of tTregs or the setting of GvHD might be responsible for the observed differences. Another reason for the aforementioned discrepancies might be the imprecise definition of Tregs, with some groups including induced Tregs in their analysis, while others used additional markers such as CD127, CD45RA and HLA-DR. However, steroid-induced FoxP3 expression (71) seems to not correlate with suppressive activity of these cells (72), and after recruitment to an inflamed tissue environment, these cells might exert effector T-cell function and promote a corticosteroid-dependent condition in patients suffering from GvHD. Last but not least, glucocorticoid-induced T-cell apoptosis is well described on a functional level, but is not well understood on a molecular level (73, 74). In light of the detrimental effect of MP on the clinical outcome we would strongly suggest minimizing the use of glucocorticoids in the case of tTreg therapy.

MMF is typically used in combination with prednisone and CSA. MMF is converted to its pharmacologically active form, Mycophenolic acid (MPA), by enteric and hepatic bioactivation. MPA inhibits proliferation of B- and T-lymphocytes primarily *via* the enzyme inosine monophosphate dehydrogenase, which is crucial for *de novo* purine synthesis. Substantial data on the specific effects of MPA on tTreg function and survival are insufficient as, in most studies, MMF was combined with CSA or steroids, but according to prevailing opinion MMF is compatible with adoptive tTreg transfer (43, 57, 75). A beneficial effect of MMF was postulated by the observation that the amount of circulating tTregs is significantly higher in kidney transplant patients treated with a combined therapy consisting of CSA, steroids and MMF as compared to patients who received CSA, steroids and Everolimus; however, it should be to taken into account that in this study CSA trough levels were significantly higher in the MMF treated group (46). In conclusion, we present, for the first time, data that indicates MMF combined with adoptively transferred tTregs may work synergistically to ameliorate the clinical signs of GvHD, which was reflected in histologically reduced cellular infiltration in the lung and liver. We were, however, unable to quantitatively confirm the additive effect of MMF and tTregs on a cellular level by organ flow cytometry, most likely due to the technical limitations of small organ anatomy and the overall limited cell number yield during organ preparation.

Calcineurin inhibitors, in particular CSA, are one of the most commonly used immunosuppressive compounds for maintenance immunosuppression in transplantation medicine, irrespective of the transplanted organ. By inhibition of the intracellular phosphatase calcineurin, cytosolic activation and nuclear translocation of the nuclear factor of activated T-cells is reduced, thereby hampering *de novo* synthesis of proinflammatory cytokines such as IL-2. Accordingly, the current paradigm holds that under most circumstances CSA leads to reduced *de novo* conversion of allospecific Tregs (45), reduced frequency of circulating tTregs (46), and reduced suppressive capacities (47). However, these results were generated in the clinical setting of solid organ or hematopoietic transplantation where cISD are administered at high doses. Therefore, conceptually different approaches were necessary to recognize that low-dose CSA can increase the frequency of circulating tTregs and promote tTreg function and survival (47, 76–78). In line with these findings, we previously reported that tTreg therapy can replace permanent pharmacologic immunosuppression only if tTreg transfer was combined with early-phase, short-term and low-dose CNI treatment (20). Within a Phase I/IIa clinical trial, we were able to further demonstrate that autologous Treg therapy allows tapering of standard triple immunosuppression in kidney transplanted patients to CNI (Tacrolimus) monotherapy if Tregs are administered early after kidney transplantation (79). The mechanism behind this was previously elucidated by Ruppert and colleagues, who showed that Tregs resist CSA-induced cell

death *via* CD44-mediated signaling pathways in a dose-dependent fashion (39). In contrast to our data presented here, Zeiser et al., suggested that coadministration of CSA and Tregs hampers the suppressive capacity of tTregs (57). The authors further describe that the combination of CSA and tTregs strongly reduced survival in a GvHD mouse model, whereas this was not the case when tTregs were combined with MMF. However, the model employed by Zeiser et al., does not recapitulate the clinical state where we face either ongoing GvHD or the post-transplant phase with a particularly high risk of clinically relevant allo-responses (57). Additionally, Zeiser et al., administered 10 mg/kg CSA while in the model presented here, we use only 4 mg/kg body weight, which more closely corresponds to the clinical dosage in human patients (20, 58, 59). Therefore, it is perhaps unsurprising that in our present study we were able to confirm the beneficial effect of CSA and tTreg co-administration.

In summary, we have demonstrated that the efficacy of *ex vivo* pre-activated tTregs to control GvHD reactions by alloreactive T-cells is increased when combined with the calcineurin inhibitor CSA and, to a lesser extent, when combined with MMF. In contrast, the combination of tTregs with steroids in this context reduced tTreg treatment efficacy. In our mouse model, GvHD is due to clonal expansion of naïve xenoreactive T-cells and their differentiation to pathogenic effector T-cells. The data support synergistic inhibitory effects of CSA and tTreg on this process, whilst the functionality of pre-activated tTregs used for adoptive transfer appears to be resistant to CSA. The model presented here has one limitation: *in vivo* expansion of human tTreg in the humanized mouse graft is inherently poor, therefore, a relatively high tTreg/Teff ratio (*e.g.* >1:2) is necessary to see beneficial effects, confirming findings from numerous other groups (80–83). Thus, the use of CSA supports tTreg activity, but in a xenogenic mouse model high cell doses are necessary to compensate for poor tTreg clonal expansion. To date, the numbers of adoptively transferred tTregs required to obtain beneficial effects in a human context remains unclear. However, we conclude that CSA should be the agent of choice when adoptive transfer of a sufficient number of tTregs for GvHD is scheduled. MMF may be a further beneficial combination partner if dual or alternative therapy is required. Steroids should be avoided or, if necessary, be restricted to minimal doses.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Charité-Universitätsmedizin Berlin, Ethics Committee (Institutional Review Board), Charitéplatz 1, 10117 Berlin. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Landesamt für Gesundheit und Soziales Berlin (G0483/09), Berlin, Germany.

AUTHOR CONTRIBUTIONS

SL-K supervised the project, designed the experiments, performed some experiments, analyzed the data, and wrote the manuscript. AZ, AF, HH, and IS-K performed and analyzed experiments. MS-H contributed to the data analysis and assisted in writing the manuscript. RK performed the histological analysis of solid organs. H-DV and PR led the project, designed the research, assisted in data interpretation, and contributed the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.716629/full#supplementary-material

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Der erste klinische Einsatz unseres Treg-Produktes erfolge im Rahmen einer Phase I/IIa Studie. Hierbei wurden Patienten, die Lebendspende-Nierentransplantation erhielten, mit autologen, *ex vivo* expandierten Tregs behandelt und über bis zu drei Jahren nachbeobachtet.

2.5. Regulatorische T-Zell-Therapie zur Minimierung der Immunsuppression bei Nieren-Transplantationen: eine klinische Phase I/IIa-Studie.

Der folgende Text wurde durch die Autorin übersetzt und entspricht im Wesentlichen dem Abstrakt der Arbeit

Roemhild A, Otto NM, Moll G, Abou-El-Enein M, Kaiser D, Bold G, Schachtner T, Choi M, Oellinger R, **Landwehr-Kenzel S,** Juerchott K, Sawitzki B, Giesler C, Sefrin A, Beier C, Wagner DL, Schlickeiser S, Streitz M, Schmueck-Henneresse M, Amini L, Stervbo U, Babel N, Volk HD, Reinke P. Regulatory T cells for minimizing immune suppression in kidney transplantation: phase I/IIa clinical trial. British Medical Journal, Oktober 2020, Seiten m3734, Band 371. [118]

Zielsetzung: *Ziel dieser Arbeit war zu untersuchen, ob eine Modulation des immunologischen Gleichgewichts durch eine Infusion von autologen natürlichen regulatorischen T-Zellen (nTregs) bei Patienten nach einer Nierentransplantation sicher und machbar ist und ein Ausschleichen der lebenslang hoch dosierten Immunsuppression, mit ihrer begrenzten Effektivität, ihren Nebenwirkungen und den hohen direkten sowie indirekten Kosten ermöglicht. Gleichzeitig sollten im Rahmen eines sinnvollen Proof-of-Concept Krankheitsmodells einige zentrale Herausforderungen der nTreg-Therapie wie beispielsweise ein einfacher und stabiler Herstellungsprozess, die Gefahr der zu starken Immunsuppression, die Interaktion mit Medikamenten der Standardtherapie und die funktionelle Stabilität in einem inflammatorischen Milieu adressiert werden.*

Design: *Investigator-initiated, monozentrisch, nTreg Dosiseskalation, klinische Phase I/IIa-Studie (ONEnTreg13).*

Setting: *Charité-Universitätsklinikum, Berlin, Deutschland, innerhalb des ONE-Study Konsortiums (gefördert durch die Europäische Union).*

Teilnehmer: *Empfänger einer Lebendspende-Nierentransplantation (ONEnTreg13, n=11) und entsprechende Teilnehmer der Referenzgruppe (ONErgt11-CHA, n=9).*

Interventionen: *$CD4^+CD25^+FoxP3^+$ nTreg-Produkte wurden sieben Tage nach einer Nierentransplantation als intravenöse Dosis von 0,5, 1 oder 2,5-3 $x10^6$ Zellen pro Kilogramm Körpergewicht verabreicht. Nachfolgend wurde die Dreifach-Immunsuppression über 48 Wochen bis zu einer niedrigen Tacrolimus-Monotherapie ausgeschlichen.*

Wichtigste Beobachtungsparameter: *Die primären klinischen und sicherheitsrelevanten Endpunkte setzten sich aus einem Endpunkt nach 60 Wochen sowie einer weiteren 3-jährigen Nachbeobachtungszeit zusammen. Die Bewertung schloss folgende Punkte ein: eine durch eine Biopsie bestätigte akute Abstoßungsreaktion, die Beurteilung von unerwünschten Ereignissen, die im Zusammenhang mit der nTreg-Infusion stehen und Zeichen einer zu starken Immunsuppression. Die wissenschaftliche Begleitforschung beinhaltete ein umfassendes, exploratives Biomarker-Portfolio.*

Ergebnisse: *Für alle Patienten konnten nTreg Produkte mit ausreichender Ausbeute, Reinheit und Funktionalität aus 40-50 ml peripherem Blut, das zwei Wochen vor Transplantation entnommen wurde, hergestellt werden. Keine der drei Dosis-Eskalationsgruppen zeigte eine dosislimitierende Toxizität. Sowohl die nTreg als auch die Referenzgruppen zeigten zu 100% ein Transplantatüberleben und vergleichbare Profile hinsichtlich Sicherheit sowie des klinischen Verlaufes. In der Patientengruppe, die nTregs erhielten, konnte eine immunsuppressive Monotherapie in acht von elf Patienten (73%) erreicht werden, während die Referenzgruppe weiterhin eine immunsuppressive Doppel- oder Tripel-Therapie erhielt (p=0.002). Auf mechanistischer Ebene war die Aktivierung von konventionellen T-Zellen reduziert und der das T-Zell-Rezeptor-Repertoire von nTregs verschob sich in vivo von einem polyklonalen zu einem oligoklonalen Profil.*

Zusammenfassung: *Die Anwendung von autologen nTregs erwies sich selbst bei Patienten, die eine Nierentransplantation erhalten hatten und immunsupprimiert waren als sicher und machbar. Diese Ergebnisse sollten Anlass zur weiteren Untersuchung der Wirksamkeit von nTregs sein und als eine Grundlage für die weitere Entwicklung neuerer nTreg-Generationen im Kontext der Transplantation und jeder anderen Immunpathologie dienen.*

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Regulatory T cells for minimising immune suppression in kidney transplantation: phase I/IIa clinical trial

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ABSTRACT

OBJECTIVE

To assess whether reshaping of the immune balance by infusion of autologous natural regulatory T cells (nTregs) in patients after kidney transplantation is safe, feasible, and enables the tapering of lifelong high dose immunosuppression, with its limited efficacy, adverse effects, and high direct and indirect costs, along with addressing several key challenges of nTreg treatment, such as easy and robust manufacturing, danger of over immunosuppression, interaction with standard care drugs, and functional stability in an inflammatory environment in a useful proof-of-concept disease model.

DESIGN

Investigator initiated, monocentre, nTreg dose escalation, phase I/IIa clinical trial (ONEnTreg13).

SETTING

Charité-University Hospital, Berlin, Germany, within the ONE study consortium (funded by the European Union).

PARTICIPANTS

Recipients of living donor kidney transplant (ONEnTreg13, n=11) and corresponding reference group trial (ONErgt11-CHA, n=9).

INTERVENTIONS

CD4+ CD25+ FoxP3+ nTreg products were given seven days after kidney transplantation as one intravenous dose of 0.5, 1.0, or 2.5-3.0×10^6 cells/kg body weight, with subsequent stepwise tapering of triple immunosuppression to low dose tacrolimus monotherapy until week 48.

MAIN OUTCOME MEASURES

The primary clinical and safety endpoints were assessed by a composite endpoint at week 60 with further three year follow-up. The assessment included incidence of biopsy confirmed acute rejection, assessment of nTreg infusion related adverse effects, and signs of over immunosuppression. Secondary endpoints addressed allograft functions. Accompanying research included a comprehensive exploratory biomarker portfolio.

RESULTS

For all patients, nTreg products with sufficient yield, purity, and functionality could be generated from 40-50 mL of peripheral blood taken two weeks before kidney transplantation. None of the three nTreg dose escalation groups had dose limiting toxicity. The nTreg and reference groups had 100% three year allograft survival and similar clinical and safety profiles. Stable monotherapy immunosuppression was achieved in eight of 11 (73%) patients receiving nTregs, while the reference group remained on standard dual or triple drug immunosuppression (P=0.002). Mechanistically, the activation of conventional T cells was reduced and nTregs shifted in vivo from a polyclonal to an oligoclonal T cell receptor repertoire.

CONCLUSIONS

The application of autologous nTregs was safe and feasible even in patients who had a kidney transplant and were immunosuppressed. These results warrant further evaluation of Treg efficacy and serve as the basis for the development of next generation nTreg approaches in transplantation and any immunopathologies.

TRIAL REGISTRATION

NCT02371434 (ONEnTreg13) and EudraCT:2011-004301-24 (ONErgt11).

WHAT IS ALREADY KNOWN ON THIS TOPIC

No consensus protocol exists for tapering immunosuppression in patients after solid organ transplantation and pharmacological induction treatment; preclinical data have indicated adoptive transfer of regulatory T cells (Tregs) as a promising option

Although currently published protocols on good manufacturing practice are heterogeneous and inconsistent, several groups have been able to generate Treg products from peripheral blood, cord blood, or thymus with sufficient yield and purity if enough starting material is available; however, manufacturing protocols that are easy to implement and clear defined criteria of product characteristics are lacking

Data from 12 patients who received Treg treatment at later time points after kidney transplantation have been published with limited information on mechanistic side studies to better understand the mode of action and reasons for patients responding or not responding to treatment

WHAT THIS STUDY ADDS

Our natural Treg (nTreg) manufacturing process uses small amounts of blood and is easy to use, especially for patients with comorbidities and for those receiving solid organ transplantation from deceased donors, with a short time window for cell collection

These data show stable minimisation of immunosuppression in most patients receiving nTreg treatment after kidney transplantation

Our analyses indicate that inhibition of conventional T cell activation and natural killer cell maturation without signs of over immunosuppression and (allo)antigen driven clonal expansion in the nTreg T cell receptor repertoire might be important mechanisms of nTreg treatment

Introduction

Adoptive cellular therapies based on thymus derived natural regulatory T cells (nTregs) are promising candidates for sustainable reshaping of undesired immune reactions in various medical indications.[1 2] Conventional immunosuppression targets undesired effector mechanisms, but unfortunately also protective pathways like nTreg control. Consequently, in patients undergoing immunosuppression the immune balance remains disturbed and chronic immunosuppression is often necessary. Reshaping the immune balance by nTreg infusion in immunopathology related diseases might overcome the need for lifelong high dose immunosuppression with its limited efficacy, adverse effects, and high direct and indirect costs. Preclinical studies have already shown the ability of nTregs to delay or prevent graft rejection after solid organ transplantation or graft versus host disease, and to control autoimmunity and undesired immunogenicity of biological drugs and advanced treatment products after adoptive transfer in vivo. However, several open questions and obstacles remain before adoptive cellular treatment with nTregs can be widely implemented.

Transplantation with long term immunosuppression is the current standard of care in solid organ transplantation.[3 4] Despite progress in one year patient and allograft survival, long term results have not improved during the past two decades. Chronic rejection and long term complications of chronic immunosuppression such as infections, malignancies, cardiovascular diseases, and pharmacological toxicity associated graft failure (eg, chronic allograft nephropathy) continue to be issues, which have spurred the search for novel treatment strategies.[5-10] Therefore, minimising or weaning long term immunosuppression after solid organ transplantation remains a major goal.[4] Several promising approaches have failed mainly because of the high clonal size of alloreactive naïve and memory or effector T cells and B cells.[4] Macrochimerism inducing protocols have shown promising results of tolerance induction, but their complexity limits broad implementation.[11]

CD4+ CD25+ FoxP3+ nTregs have been identified as key players of immune homoeostasis and are now a major research focus in transplantation.[12-16] In a preclinical kidney transplantation model, which mimics the cellular presensitisation frequently found in patients undergoing transplantation, regulatory T cells (Tregs) combined with calcineurin inhibitor and T cell depletion controlled donor reactive memory T cells without the need for permanent calcineurin inhibitor treatment.[12] Although clinical experience with nTregs is limited, during the past decade a few phase I clinical trials of Treg treatments have been carried out for different clinical indications. Two small studies have included patients undergoing kidney transplantation and more recently patients with coronavirus disease 2019.[1 2 17 18] However, crucial knowledge gaps still exist about the optimal use of Treg treatment, reflected among the large heterogeneity in good manufacturing practice (GMP) protocols, dosing, and timing of Treg applications.

We used a single dose of nTreg treatment after living donor kidney transplantation in an investigator initiated phase I/IIa trial, accompanied by an extensive biomarker programme, as a proof-of-principle study. We addressed several issues relating to the broad implementation of nTreg treatment for various indications, including easy and robust manufacturing, risk of over immunosuppression, interaction with standard care drugs, and biomarkers for monitoring safety and efficacy.

Methods

Trial objectives, study design, safety, and efficacy monitoring

Our trials were independent studies within the European Union funded ONE study consortium, applying different site specific adoptive cellular therapies in patients undergoing living donor kidney transplantation in conjunction with using a consensus immunosuppression protocol and biomarker portfolio in comparison to the reference patients.[19 20]

The primary objective of the ONEnTreg13 phase I/IIa trial was to assess safety and feasibility of our in-house developed autologous CD4+ CD25+ FoxP3+ nTreg product in patients undergoing living donor kidney transplantation (n=11). The results were compared with the ONErgt11-CHA reference trial (n=9), which was conducted at our centre before the ONEnTreg13 trial, to establish safety margins and biomarker panels (fig S1A). We used a composite primary safety endpoint, consisting of adverse infusion related effects, infections, acute rejection, and graft function or failure (table S1). The secondary objective of the trial was to evaluate whether nTregs allow safe tapering of conventional maintenance immunosuppression from triple drug treatment to monotherapy within 48 weeks of transplantation (fig 1). Figure 1, figure S1, and tables S1 and S2 summarise the primary and secondary trial objectives and endpoints, the assessment of adverse events and efficacy, and patient eligibility criteria. Baseline parameters were similar for the reference group and the nTreg group (table 1 and table S5). Additionally, both groups followed the same approach (fig 1 and table 2) for visits and initial immunosuppression (except the nTreg group did not receive induction treatment with the anti-interleukin 2 receptor basiliximab), and the adjunct biomarker programme.

In total, 17 patients were assessed two weeks before kidney transplantation for eligibility to be enrolled in the ONEnTreg13 trial. However, six patients did not receive the nTreg product because of patient related issues occurring before cell infusion which was scheduled seven days after surgery (such as kidney transplantation not performed, surgical complications, withdrawal of consent, early biopsy proven acute rejection; fig S1). The remaining 11 patients were split into cohorts of three or four patients and received nTregs in escalating doses of 0.5, 1.0, or 2.5-3.0×10^6 fresh cells/kg of body weight seven days after kidney transplantation. Because the targeted dose of 3.0×10^6

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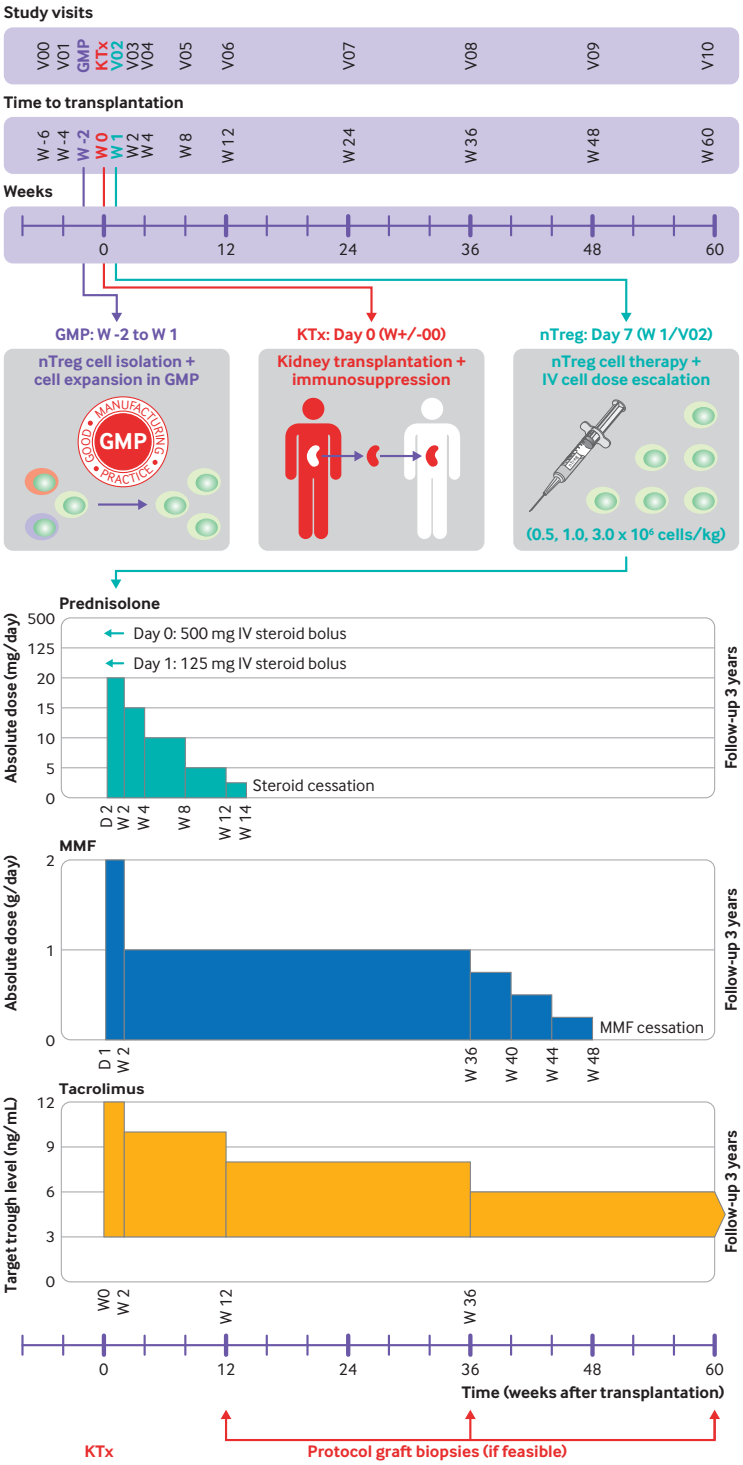

Fig 1 | ONE study ONEnTreg13 clinical trial design. Upper panel: time schedule of ONEnTreg13 clinical trial: patient enrolment, cell collection, living donor kidney transplantation, nTreg adoptive cellular therapy (dose escalation of 0.5, 1.0, or 2.5-3.0×10^6 fresh cells/kg of body weight), and primary 60 week study follow-up. Lower panel: overview of protocol immunosuppressive regimen of ONEnTreg13 clinical trial with doses adjusted to specified levels at indicated time points: first steroid reduction until week 14, followed by MMF reduction at week 36-48, with continuation of tacrolimus monotherapy in nTreg group until study end at week 60 and three year follow-up. GMP=good manufacturing practice; IV=intravenous; KTx=kidney transplantation; MMF=mycophenolate mofetil; nTreg=natural regulatory T cell

Table 1 | Baseline characteristics of patients before transplantation. Data are numbers unless indicated otherwise

Characteristics	Charité nTreg group ONEnTreg13 (n=11)	Charité reference group ONErgt11-CHA (n=9)
Median (range) recipient age (years)	36 (33-58)	43 (30-63)
Recipient female/male	5/6	4/5
Median (range) donor age (years)	56 (33-63)	53 (30-67)
Donor related/unrelated	7/4	7/2
Cause of end stage renal disease		
Glomerulonephritis or vasculitis	4	5
Diabetic nephropathy	2	2
Polycystic kidney disease	2	—
Uropathy	1	—
Other or undetermined	2	2
Median (range) time receiving dialysis (months)	11 (0-110)	14 (4-118)
Human leukocyte antigen mismatch		
0	2	1
1-3	7	6
4-6	2	2
Panel reactive antibody test		
<10%	10	8
>10%	1	1
Cytomegalovirus serology donor/recipient		
D−/R−	3	3
D-/R+	2	—
D+/R−	2	1
D+/R+	4	5

nTreg=natural regulatory T cell.

cells/kg could not be obtained in two patients owing to cell loss at bead depletion, two patients in the high dose group received only 2.5×10^6 cells/kg body weight. The cells were administered through slow intravenous infusion over 30 minutes by Perfusor Space pump (fig 1 upper panel). Paracetamol (acetaminophen) and antihistamine were given prophylactically before cell infusion. With the exception of basiliximab (to prevent targeting of Tregs), nTreg treatment was used in conjunction with the standard immunosuppression regimen (steroids, mycophenolate mofetil, and tacrolimus) as adjunct treatment with the intention to reduce maintenance immunosuppression (fig 1 lower panel).

GMP manufacturing and characterisation of nTregs

The clinical grade nTregs were manufactured at our in-house GMP facility (Berlin Institute of Health Center for Regenerative Therapies, and Berlin Center for Advanced Therapies).[21] Table S3 gives an overview of the process and materials. We were able to establish a robust GMP process for isolation and expansion of autologous polyclonal nTregs with good purity and sufficient yield (table 3) from 40-50 mL peripheral blood samples. We studied whether our nTreg manufacturing process is feasible for patients before kidney transplantation in terms of robustness, yield, and product composition. In total, we generated 17 nTreg products from patients before kidney transplantation and 11 were administered to patients in the ONEnTreg13 trial (table S4).

For validation purposes and to better understand the manufacturing process and to study potential differences in product characteristics, we also generated nTreg products from healthy donors. A sufficient yield of nTregs (>1×10^9 cells) could be consistently generated from 40-50 mL of heparinised whole blood obtained by venipuncture from the patient's forearm two weeks before kidney transplantation. All of the donors in the ONE study cohort had end stage renal disease and half were receiving dialysis. The Tregs were enriched by using the CliniMACS Plus system (CD8+ T cell depletion and CD25+ T cell enrichment) with subsequent stimulation and expansion for 23

Table 2 | Clinical outcomes after 60 week study period and three year follow-up. Data are numbers unless indicated otherwise

	60 weeks			3 years		
Clinical outcome	nTreg (n=11)	Reference (n=9)	P value	nTreg (n=11)	Reference (n=9)	P value
Immunosuppression						
Monotherapy (tacrolimus only)	8	0	0.002	8	—	0.001
Dual therapy (tacrolimus, MMF)	—	5		—	4	
Triple therapy (tacrolimus, MMF, steroid)	3	4		3	5	
Alloreactivity						
T cell mediated acute rejection			0.56			1.00
Borderline	—	1		—	—	
Ia/Ib	1	1		—	—	
IIa/IIb/III	1	—		—	—	
Mixed T cell and antibody mediated acute rejection	1	—		—	—	
De novo donor specific antibodies	2	—	0.18	2	1	0.66
Infections and malignancies						
Cytomegalovirus viraemia	1	1	0.51	—	—	1.00
Cytomegalovirus disease	—	—	1.00	—	1	0.26
Polyomavirus viraemia	—	1	0.26	—	—	1.00
Other infections*	1	1	0.88	—	—	1.00
Cancer	—	—	1.00	—	1	0.26
Graft function						
Delayed graft function	—	2	0.10	—	—	1.00
Median (range) creatinine (mg/dL)	1.6 (1.3-1.8)	1.1 (1.0-2.0)	0.65	1.5 (1.2-1.6)	1.2 (0.9-2.0)	0.80
Median (range) estimated glomerular filtration rate (mL/min)	51 (46-58)	60 (37-64)	0.45	53 (47-49)	59 (38-66)	0.79
Median (range) proteinuria (mg/g Krea)	192 (92-336)	130 (93-430)	0.89	120 (89-221)	151 (62-278)	0.89

MMF=mycophenolate mofetil; nTreg=natural regulatory T cell.
nTreg and reference groups were compared after 60 weeks (study endpoint) and at three year follow-up.
*Urinary tract infection and pneumonia.

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Table 3 | Robust GMP manufacturing of nTregs from small volumes of blood from patients with ESRD and healthy donors. Data are medians (minimum-maximum)

Quality control analyses	Release criteria	Patients with ESRD (n=17)*	Healthy donors (n=6)
Viability (%)	≥70	96 (87-97.5)	96.9 (95.27-98.2)
Purity			
CD4+ CD25+ FoxP3+ (% of CD4+)	≥70	95.44 (90.4-99.6)	96.48 (94.55-98.82)
CD4+ CD25+ FoxP3+ (% of total)	FIO	91.9 (80.8-99.6)	94.0 (90.4-98.8)
Impurity			
Interleukin 2 production (% of total)	≤10	2.9 (0.15-6.05)	0.82 (0-1.5)
Interferon γ production (% of total)	≤10	2.2 (0.2-7)	0.35 (0-1.14)
Starting material and cell yield			
Blood collected (mL)	FIO	45 (38-50)	50 (50-50)
Treg cell No start (E+06)	FIO	3.9 (2.3-8.02)	5.5 (2.2-7)
Treg cell No end (E+09)	FIO	4.7 (0.96-37.7)	3.78 (0.73-13.4)

ESRD=end stage renal disease; FIO=for information only (no release criteria); GMP=good manufacturing practice; nTreg=natural regulatory T cell.
Quality control analyses of 23 GMP nTreg products from validation and clinical trial runs. Figure S4 shows further details on the process.
*Eleven products were infused seven days after kidney transplantation to Treg group.

days under nTreg promoting conditions, followed by magnetic expansion bead depletion (>99.997%) to obtain the final nTreg product (fig S4). We performed several analytical procedures before product release with validated methods (table 3), as described in detail in the supplementary methods (fig S4, tables S3 and S7). The nTreg products from all patients showed high expansion rates from the starting material (mean >2000-fold), high viability (mean 96%) and purity (mean 96% and 91% of CD4+ T cells and total cells, respectively), and low effector cytokine production (<10% interleukin 2 and interferon γ) upon phorbol myristate acetate or ionomycin activation. These data were comparable to those of healthy donors. T cell receptor repertoire analysis of our nTreg GMP products by next generation sequencing confirmed their polyclonal pattern, as described previously.[22]

Clinical monitoring and exploratory biomarker analysis

More than 100 parameters for determining safety, efficacy, and mechanism of action were monitored in the nTreg and reference groups over the study period of 60 weeks, with follow-up of up to three years, consisting of the consensus biomarker portfolio within the ONE study consortium[19] and additional site specific markers. Monitoring included assessing biopsy confirmed acute rejection, clinical analysis and biochemical indices for renal function, and immune monitoring for measures of safety, immune activation or tolerance, and pharmacokinetics or dynamics, as outlined in supplementary table S7. Specifically, patient assessment included monitoring of viral load, monocytic human leukocyte antigen (HLA)-DR expression,[23] standardised multiparameter flow cytometry analysis of 60 immune cell subsets according to the ONE study protocol[20 24]; monitoring of humoral and cellular allosensitisation by screening of antidonor HLA panel reactive antibodies with Luminex and donor reactive T cell frequencies with interferon γ ELISPOT[25]; cytokine measurements in plasma and urine; T cell receptor repertoire analysis of nTreg GMP products and circulating nTregs in patient blood[26-29]; and gene expression of selected tolerance or rejection pattern.[30]

Statistical analysis

A statistical analysis plan defined the conventions and analysis, and emphasised the exploratory nature of the study; accordingly the proposed statistical examination of clinical and immunological data was in principle descriptive.[19] Table S6 summarises all statistical subset definitions, testing methods, and P values. We assessed clinical parameters and biomarkers over the whole time course by using non-parametric analysis of the variance type statistic with F approximation developed by Brunner and colleagues[31] in a two way factorial repeated measures design (fig 2, fig 3, fig 4, and fig S2). Testing was conducted for significant interaction; that is, non-parallel response profiles of the two treatment groups and within group changes over time using the nparLD R package.[32] Post hoc multiple comparisons between individual time points were performed with P value adjustment by using Holm's method. A two tailed Wilcoxon matched pairs signed rank test was used when comparing two time points within the nTreg group. A P value less than 0.05 was considered statistically significant for all tests. All analyses were performed with R (version 3.5.1). GraphPad Prism software (version 8, GraphPad, La Jolla, CA) was used to generate graphs. Longitudinal data are presented as medians and interquartile ranges in all line graphs or individual comparisons as boxplots with whiskers indicating the data ranges.

Patient and public involvement

No patients were involved in setting the research question, nor were they involved in the design and implementation of the study.

Results

Patients and study design

Two weeks before kidney transplantation, patients were assessed for eligibility and blood samples were collected to manufacture the nTreg products. The definitive enrolment of patients for cellular treatment and for the reference group took place seven days after kidney transplantation according to the inclusion and exclusion criteria (fig S1A and table S2). The clinical results of patients treated with Tregs (n=11) were compared with the ONE study reference group patients (n=9), who were enrolled at our centre by using the same criteria and who received the standard of care. Patient characteristics and initial immunosuppression were similar for the two groups (table 1), but the nTreg group did not receive anti-interleukin 2 receptor induction treatment to prevent interaction with the nTreg product. No apparent cell dose-response association was found for any of the parameters investigated.

Safety of nTreg treatment

The primary objective was to assess the safety of cellular treatment. At the time of this analysis after three years

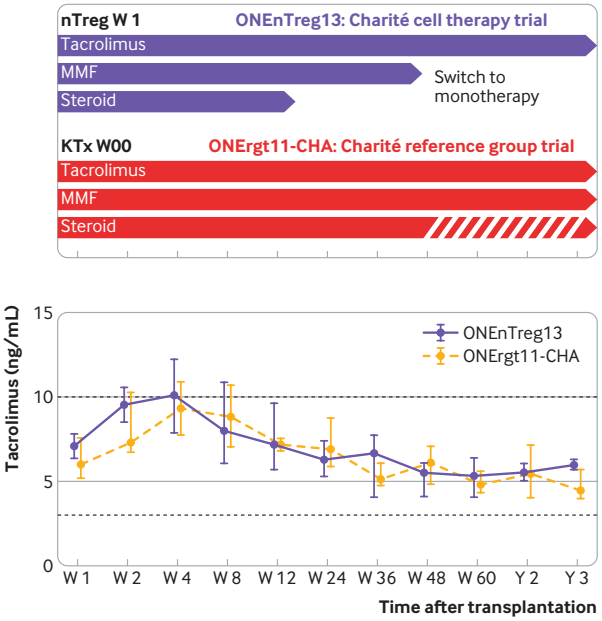

Fig 2 | Autologous nTreg infusion might enable minimisation of immunosuppression in patients with kidney transplant. Tapering of immunosuppression in the nTreg trial group (switch to monotherapy) compared with reference group (continuous dual or triple drug regimen) with monitoring of patients' tacrolimus levels (median±interquartile range, shown on lower panel). KTx=kidney transplantation; MMF=mycophenolate mofetil; nTreg=natural regulatory T cell

of follow-up, all patients in both groups are alive with good graft function. No serious adverse events have been reported; that is, immediate or long term adverse effects arising directly from nTreg cell infusion (fig S1B and table 2). Importantly, no differences were found in the following parameters: tacrolimus blood levels during the three year follow-up (fig 2); renal functional parameters (including estimated glomerular filtration rate, serum creatinine, proteinuria, serum albumin, and serum urea; fig 3 and fig S2A); liver functional parameters (including bilirubin, aspartate aminotransferase, alanine transaminase, and alkaline phosphatase; fig S2B); or systemic inflammation and other safety parameters (such as C reactive protein, urinary interferon induced protein 10 inflammation marker, fibrinogen, and free haemoglobin; fig 4 and fig S2C). In contrast to anticancer T cell treatment, we did not observe any cellular infusion related systemic increase of proinflammatory or anti-inflammatory plasma cytokines, such as interferon γ, tumour necrosis factor α, interleukin 1, interleukin 6, interleukin 8, interleukin 10, and urinary interferon induced protein 10 (fig 4). A trend was observed towards decreased intrarenal inflammation (P=0.1, fig 4). Furthermore, we did not observe any signs of immediate and long term over immunosuppression by nTreg treatment, as shown by monitoring monocytic HLA-DR expression (an immunocompetence marker; fig 4 lower panel), and the rate of infectious complications (eg, cytomegalovirus, Epstein-Barr virus, polyomavirus, urinary tract infections, septic events) and cancer until the three year follow-up (table 2).

First hints for efficacy of nTreg treatment

The secondary objective of the ONEnTreg13 trial was to evaluate whether nTregs allow safe tapering of conventional maintenance immunosuppression from triple drug treatment to monotherapy (fig 1 and

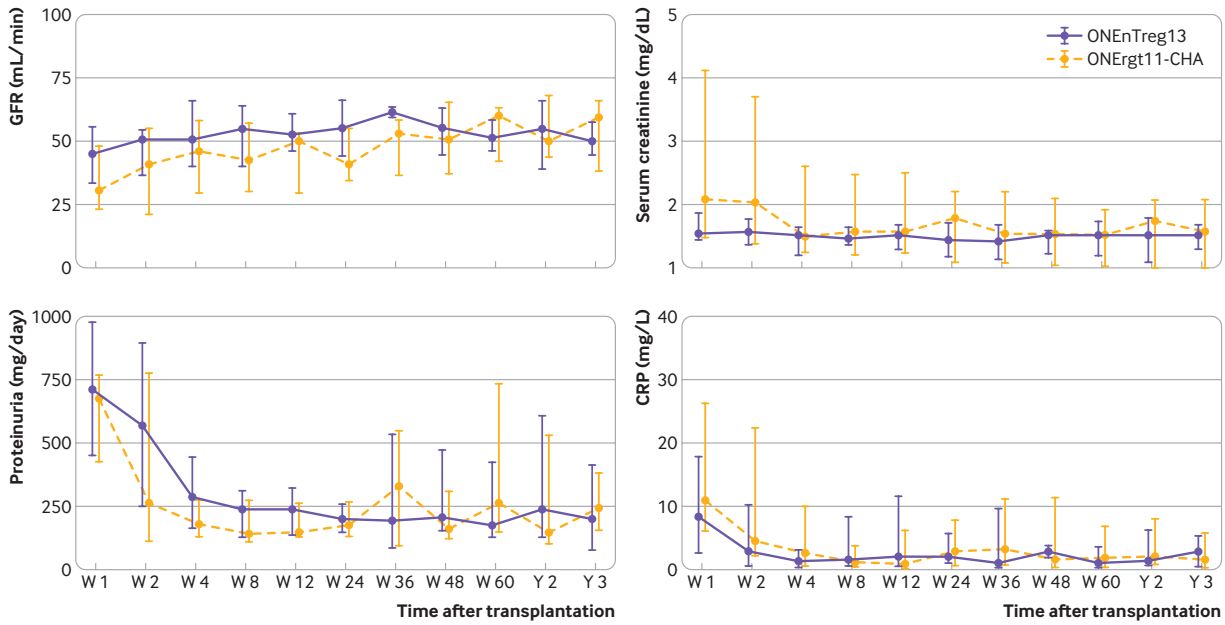

Fig 3 | Long term follow-up of renal allograft function (median±interquartile range): glomerular filtration rate (GFR, mL/min), serum creatinine (mg/dL), proteinuria (mg/day), and C reactive protein (CRP, mg/dL)

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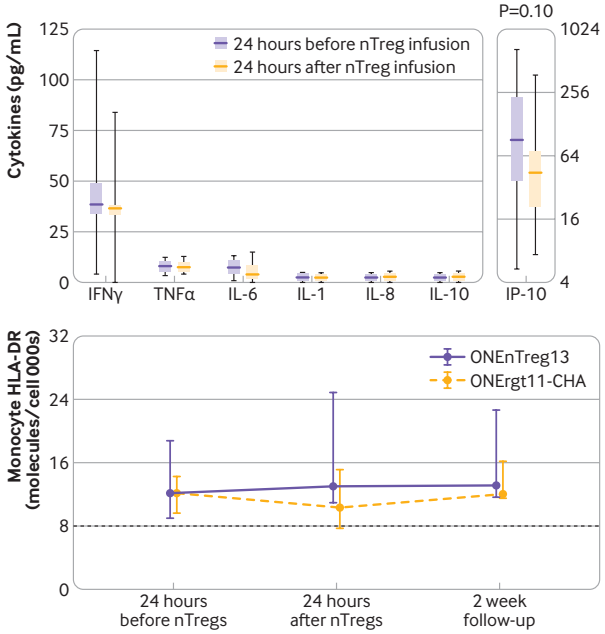


Fig 4 | No immune complications after infusion: monitoring of cytokine release after infusion by measuring inflammatory cytokine serum or urinary levels (box plot, minimum-maximum range) with respective typical normal values (serum: TNFα<15.0 pg/mL, IFNγ=10-30 pg/mL, IL-6<5.0 pg/mL, IL-1<5.0 pg/mL, IL-8<10.0 pg/mL, IL-10<5.0 pg/mL, IP-10<200 pg/mL) and monocyte HLA-DR (molecules per cell) either 24 hours before or 24 hours after systemic nTreg infusion, including two week follow-up for HLA-DR. No significant differences between groups for any assessed parameters. HLA=human leukocyte antigen; IFNγ=interferon γ; IL=interleukin; IP-10= urinary interferon induced protein 10; nTreg=natural regulatory T cell; TNFα=tumour necrosis factor α

fig S3). Weaning of immunosuppression to low dose tacrolimus monotherapy (trough blood levels <6 ng/mL) was achieved within 48 weeks for 10 of 11 patients receiving nTreg treatment (91%). One patient (C5058) was excluded from a switch to monotherapy as he developed a posterior reversible encephalopathy syndrome related to calcineurin inhibitor treatment early after kidney transplantation. Tacrolimus was extremely reduced (trough level 2-3 ng/mL) for more than six months to overcome this complication. When a protocol biopsy revealed acute cellular rejection Banff IB, the patient received antirejection treatment. He was kept on standard triple drug treatment with good three year graft function.

In two of the 10 patients who were weaned of immunosuppression, tacrolimus monotherapy failed: one patient (C5056) developed mixed acute cellular rejection Banff III, antibody mediated acute rejection, and donor specific antibodies shortly before the end of the 60 week study period. Graft function could be rescued by rejection treatment and reversal to triple drug immunosuppression with good three year function. In another patient (C5063) recurrence of the underlying renal disease (immunoglobulin A nephropathy) was observed in the protocol biopsy some weeks later that was associated with proteinuria. For safety reasons, immunosuppression was reversed to the triple drug regimen. Patient C5057 showed mild graft deterioration from uncontrolled type 2 diabetes mellitus and cardiac decompensation early on before protocol switch to monotherapy. He did not show any hints of alloreactivity or inflammation in the biomarkers and recompensation of heart failure and diabetes would probably have been sufficient to normalise kidney function. However, he received a short steroid bolus for safety reasons as the histological picture showed some cellular infiltrations (acute cellular rejection Banff IB). Later he was successfully weaned to tacrolimus monotherapy with excellent three year follow-up. During the three year follow-up, stable good graft function was observed in the eight patients from the nTreg group on low dose tacrolimus monotherapy, and in all three patients from the nTreg group who were kept on standard triple drug treatment. Figure S3 shows the individual patient courses.

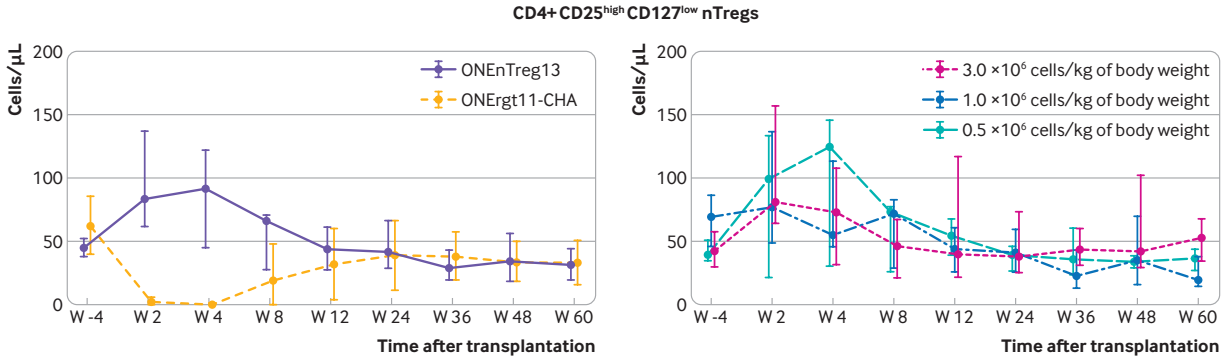
The consensus protocol of the ONE study consortium for the reference group was to aim for a steroid free dual immunosuppression after week 12 and to manage patients with standard triple drug regimens in case of impending complications (functional deterioration, proteinuria, de novo donor specific antibodies). At the end of the 60 week study period, five of nine patients were on dual immunosuppression; at the three year follow-up four of nine patients were on dual immunosuppression. The remaining patients were receiving triple drug immunosuppression (P=0.002 and P=0.001; table 2 and fig 2).

Exploratory biomarker analysis of nTreg treatment

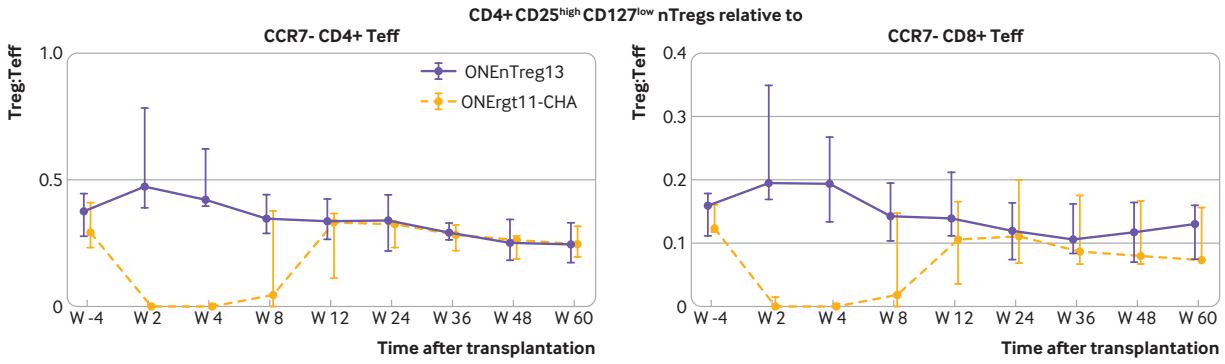
Analysis of a broad set of more than 100 exploratory biomarkers revealed only few significant differences between the two groups (table S6). Among the 59 immune cell subsets analysed by multiparameter flow cytometry, we observed significantly enhanced nTreg counts (CD4+ $CD25^{high}$ $CD127^{low}$ or CD4+ $CD25^{high}$ FoxP3+) with a favourable ratio of regulatory to effector T cells in the circulation for up to eight weeks after nTreg infusion with no apparent dose-response association (fig 5 and fig S7). By contrast, the reference group showed decreased nTreg levels compared with baseline for up to 12 weeks after kidney transplantation, partly owing to the basiliximab induction treatment,[33] which supports sequestration of nTreg and internalisation of CD25. The nTreg group developed significantly fewer activated HLA-DR+ CD4+ non-Treg conventional T cells (P=0.018) and the counts of activated CD25+ 127^{high} CD8+ conventional T cells did not change over the entire period in the nTreg group. However, in the reference group these cells were initially suppressed by basiliximab, but increased after week 12 in a rebound effect (P=0.012; fig S6 and table S6C-D). Additionally, in the reference group a shift was observed from the $CD56^{dim}$ to the more mature $CD56^{high}$ natural killer cell subset during the study period, while the ratio remained stable in the nTreg group (P=0.08). At the end of the 60 week study period, the nTreg group showed a slightly higher proportion of the marginal zone B cell subset in the circulation (P=0.045). All other immune

cell subsets were not significantly affected by nTreg treatment compared with the reference group (fig S6).

To track nTregs in vivo, we monitored the T cell receptor repertoire of ex vivo sorted Tregs from patients at different time points after kidney transplantation and compared them with the respective infused nTreg product (fig 6 and fig S5). As expected, expanded clones covering more than 0.1% or even more than 1% of the T cell receptor repertoire were rarely detectable or not detectable at all within the polyclonal nTreg product, respectively, and together reached less than 15% of the total repertoire. The peripheral blood nTreg samples from the ONEnTreg13 group showed a significant clonal expansion within a few weeks after infusion, which persisted for up to 60 weeks. Up to 80% of the total nTreg T cell receptor repertoire consisted of in vivo expanded nTreg clones at individual frequencies of more than 0.1%; strongly expanded nTreg clones of more than 1% clonal size covered up to 60% (P<0.01 *v* baseline). Most of these in vivo expanded clones were detectable at low frequency in the respective nTreg product of the individual patient. Analysis of a tolerance or rejection gene expression panel[30] in whole blood did not reveal significant differences, except for



Changes over time	ONErgt11-CHA	ONEnTreg13	ONErgt11 *v* ONEnTreg13
W -4 to W 60	***P <0.001	**P = 0.003	***P <0.001
W -4 *v* W 2	***P <0.001	**P = 0.002	***P <0.001
W -4 *v* W 4	***P <0.001	P = 0.781	***P <0.001



Changes over time (W -4 to W 60)	ONErgt11-CHA	ONEnTreg13	ONErgt11 *v* ONEnTreg13
Treg: CD4+ Teff	***P <0.001	**P = 0.003	***P <0.001
Treg: CD8+ Teff	***P <0.001	P = 0.062	***P <0.001

Fig 5 | Exploratory biomarker analysis of therapy response to nTreg treatment. More than 100 biomarkers were analysed in patients receiving nTregs, with a more comprehensive summary presented in figures S5-S6. Patients receiving nTregs were evaluated according to cell dose applied and compared with reference group. Transiently enhanced Treg levels and favourable Treg:Teff ratio. Upper panels: multiparameter flow cytometry profiling of nTregs in patient blood either comparing nTreg with reference group or showing the infused cell dose (0.5, 1.0, and 2.5-3.0×10^6 cells/kg of body weight); table shows significance levels. Lower panels: ratio between CD4+ CD25^{high} CD127^{low} Treg levels relative to either CCR7– CD4+ or CCR7– CD8+Teffs shown as representative result for almost 60 immune cell subsets shown in figure S6; table shows significance levels. nTreg=natural regulatory T cell; Teff= effector T cell; Treg=regulatory T cell

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the rejection associated gene TMEM176B (TORID), which was downregulated to almost undetectable levels for the first 12 weeks in the nTreg group but not the reference group (group difference P=0.023).

Discussion

We used kidney transplantation as a proof-of-concept disease model to address several challenges of nTreg cellular treatment for reshaping undesired immune reactions. We were able to show that GMP compliant production of nTreg products from patients with end stage renal disease who had several comorbidities is feasible, robust, and possible at reasonable costs (€17 500/product (£15 800; $20 500))—a prerequisite for trustworthy data from clinical adoptive cell treatment trials.[34-36] In line with the main trial objective, we also showed that infusion of autologous nTregs at the end of the first week after kidney transplantation was well tolerated. No evidence was found of cell infusion related short term or long term adverse effects, and we observed no signs of over immunosuppression. All patients in the nTreg group and the reference group had good graft function until the three year follow-up despite stepwise minimising immunosuppression within the first 60 weeks in the nTreg group. The data support the feasibility of Treg treatment for many medical indications in solid organ transplantation and many other diseases with immunopathologies.

Strengths and limitations of this study

We were able to establish a robust GMP process for the isolation and expansion of autologous polyclonal

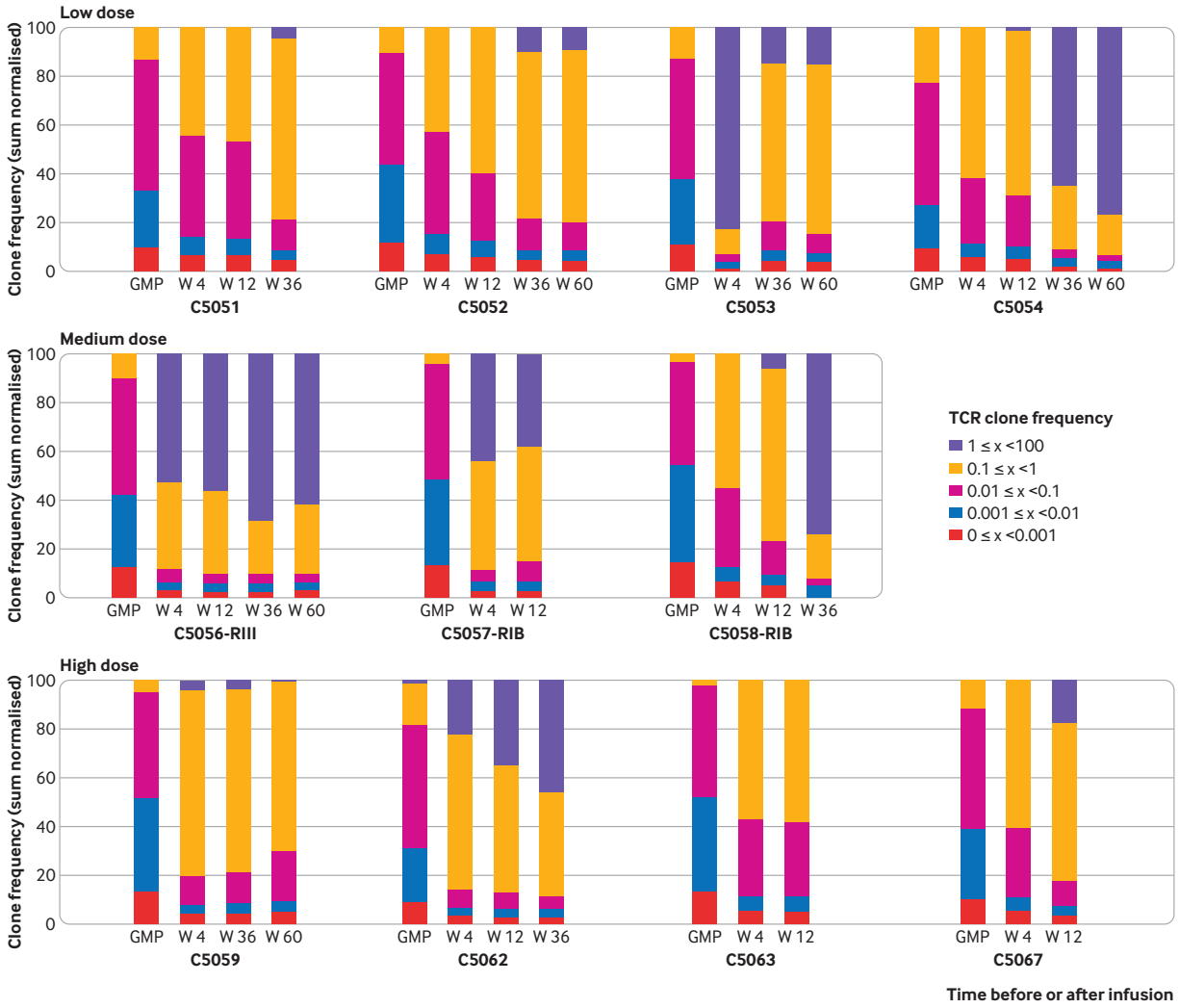

Fig 6 | Decreasing clonal diversity by oligoclonal nTreg expansion. Next generation sequencing based TCR repertoire analysis of individual nTreg GMP products before infusion (GMP columns) and ex vivo Tregs isolated at indicated patient visits (week 4, 12, 36, and 60, according to sample availability) for all 11 patients receiving nTregs, with a more detailed summary shown in figure S5. GMP=good manufacturing practice; nTreg=natural regulatory T cell; TCR=T cell receptor; Treg=regulatory T cell

nTregs with good purity and yield (table 3) from small peripheral blood samples of 40-50 mL. Starting sources normally use approximately 10 times this amount of blood or leukapheresis material.[37-40] Therefore, the use of nTregs is a more feasible treatment option and its applicability can be widened to different medical indications.

Our attempt to taper the standard triple or dual immunosuppression regimen to less toxic monotherapy with low dose tacrolimus showed stable three year success in 73% of the patients treated with nTregs. In the remaining 27% of patients, in whom tapering to tacrolimus monotherapy was not feasible or had to be reversed, we observed good graft function after three years of follow-up with a standard triple drug immunosuppression regimen. In contrast, reference patients remained on gold standard dual or triple immunosuppression as further tapering of immunosuppression in this group was not justifiable for safety reasons. Despite the limitation of low numbers of patients, the biomarker programme revealed five observations.

Firstly, the absence of any signs of inflammatory reaction after cell infusion, as seen typically after anticancer T cell treatment, underlines the lack of significant effector cell contamination of the nTreg product as shown by the product release test. Secondly, we did not observe any clinical (infections) or laboratory signs of over immunosuppression, previously discussed as a putative safety issue of polyclonal Treg treatment.[41] We suggest that without T cell receptor or CD28 stimulation, Tregs lose their activation within a few days or even die. Therefore, only Treg clones that are repeatedly antigen stimulated keep their suppressive capacity and can expand in vivo. Thirdly, we observed that the polyclonal T cell receptor repertoire of infused Treg products shifts in vivo over time to an oligoclonal pattern, suggesting an alloantigen driven selection process. Similarly, the replacement of polyclonal anti-CD3/28 monoclonal antibodies by alloantigen in vitro stimulation induces a biased T cell receptor repertoire within a few days,[22] which suggests specific immunoregulation even after administration of polyclonal nTregs.

Fourthly, nTreg infusion induced only a temporary increase in Treg counts. The drop in circulating Tregs after four weeks might be explained by nTregs homing to the inflamed graft or lack of lasting engraftment. Because we hypothesise that only alloantigen triggered Tregs survive as suggested by the oligoclonal expansion, the clonal size might not be sufficient to be visible at total Treg counts. Finally, the nTreg group expressed less conventional T cell activation, natural killer cell maturation, and downregulation of the rejection associated gene TORID, which might give an indication of the mode of action of nTreg in vivo.

The study has some further limitations. As typical for cell treatment phase I/IIa trials, the number of patients is low, which limits the power of statistical analyses. Therefore, interpretation of the data must be done carefully and on a case-by-case basis. However, the consistency of many parameters with the same message makes core statements plausible (eg, no over immunosuppression, stable graft function despite weaning, inhibition of conventional T cell activation). In two patients, tapering of immunosuppression was not successful. The first patient developed mixed acute cellular rejection Banff III, antibody mediated acute rejection, and donor specific antibodies shortly before the study endpoint. This was the only patient in both groups with high levels of pre-existing donor specific T cell immunity. After successful intense rejection treatment, immunosuppression could be reversed to the triple drug regimen. The second patient had recurrence of the underlying kidney disease, immunoglobulin A nephropathy, an event observed in 10-60% of patients after kidney transplantation. Because high immunosuppression by thymoglobuline and weaning of steroids correlates with decreased and enhanced risk of immunoglobulin A nephropathy recurrence, respectively, we decided to change treatment back to the triple drug regimen.[42 43] We have seen an improvement in proteinurea and stable graft function for more than three years in this patient. Therefore, in both patients treatment was successfully reversed to the triple drug regimen.

These data might suggest that nTregs together with low dose tacrolimus monotherapy cannot sufficiently control pre-existing pathogenic memory or effector immune cells. This hypothesis should be carefully studied in follow-up studies. The third patient on standard treatment in the nTreg group was not suitable for tapering because he developed tacrolimus related posterior reversible encephalopathy syndrome. We could speculate that the survival of his transplant with good three year function despite subtherapeutically low tacrolimus levels for several months (<2-3 ng/mL) might be partially because of nTreg mediated protection.

Comparison with other studies

Minimising the clinical need for chronic immunosuppression as early and as much as possible is a major task after transplantation because it offers the chance to diminish undesired immunosuppression associated comorbidities.[4] Empirical approaches with calcineurin inhibitor sparing regimens that minimise,[9] withdraw,[44] or avoid calcineurin inhibitors[45] have only been partially successful or have even failed to consistently show long term safety.[4 46] At first view, Chan and colleagues[47] reported promising data after temporary T cell or B cell depletion by alemtuzumab induction in patients who had kidney transplantation (73% on monotherapy after two years). However, several critical points should be noted: firstly, the Chan study reported a high rate of graft loss by graft deterioration or death within two years of follow-up (7.4%), whereas our trial reported 0% graft loss or death within three years of follow-up; secondly, the Chan study had a relatively high number of urinary tract infections (38%), whereas we observed only 10%; and thirdly, the target tacrolimus blood levels at the second half

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of the first year (and thereafter) were much higher in the Chan study than in our cell treatment group (mean approximately 9 ng/mL *v* <6 ng/mL tacrolimus). Other studies that used alemtuzumab induction showed higher rates of monotherapy failure, acute rejections, and infections.[46 48]

The challenge remains to find new approaches that enable sustainable reduction of immunosuppression, thereby diminishing undesirable treatment associated comorbidities.[4] This incentive formed the rationale for the ONEnTreg13 clinical trial because adoptive transfer of nTregs is a promising option to reshape undesired alloimmunity. Currently there are more than 50 active and completed early clinical trials that examine the safety and efficacy of Treg treatment for indications such as solid organ transplantation, inflammatory disease, and autoimmune diseases.[1 2 17] Recently, two Treg clinical trials have been reported in patients undergoing kidney transplantation. The Leventhal study treated nine patients in a dose escalating phase I/IIa trial with much higher cell doses (up to 15 times higher than our highest tier) at week 4 after kidney transplantation and patients were kept on full rapamycin or mycophenolate mofetil based immunosuppression, allowing only safety analysis. Similar to our observations, this study did not see any major dose dependent effects.[22] Chandran and colleagues treated three patients with subclinical rejection months after kidney transplantation without safety issues.[49] Our study adds several novel aspects: new, more widely applicable manufacturing processes; application of Tregs early after solid organ transplantation with concomitant tapering immunosuppression to low dose tacrolimus monotherapy; and extensive mechanistic analyses giving insights into the mode of action of nTreg application.

Conclusions

Administration of our nTreg product was safe, well tolerated, and met the primary and secondary objectives. To further improve the efficacy of this treatment and to reach stable low dose immunosuppression or even complete weaning in almost all patients, next generation Treg approaches are required to address more sustainable and functional engraftment of Tregs, redirected specificity, resistance to immunosuppressive drugs, control of pre-existing memory or effector T cell response, as well as randomised trials. We also need to have a better understanding of the optimal dose. Our robust GMP manufacturing process is a key enabling platform technology for the ongoing development of more advanced next generation genetically modified products, such as chimeric antigen receptor nTregs, to improve the efficacy of these highly promising adoptive cellular therapies.[34-36 50 51]

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Competing interests: All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: support from the European Union 7th EU Framework Programme and Horizon 2020 programme and the BMBF under grant agreement BCRT and the BIH for the submitted work; no direct funding or donations from private parties, including the pharmaceutical industry; PR, HDV, and SLK also received unrestricted research funding from the public sources for the project (see funding).

Ethical approval: The trials were conducted in accordance with the ethical principles expressed in the Declaration of Helsinki after written informed consent. The ONE study ONEnTreg13 (EudraCT No 2013-001294-24; NCT02371434) and the ONErgt11 Reference Group Trial (EudraCT No 2011-004301-24) are registered clinical trials and were approved by the local Berlin State Ethics Committee (LAGESO approval: 3 November 2014; file No 14/0168-EK12), the German national regulatory body (PEI approval: 16 April 2014; file No 2118/01), and the institutional review board of the Charité.

Patient confidentiality: Patient data are handled according to the European and German rules of health data protection. Pseudonymised (deidentified) individual participant data were analysed by the data compiling team and the biostatisticians. Participant identities remain known exclusively to the clinical study doctors (PR, GB, NMO, MC, TS) and study nurses (AS, CG). All biomarkers were analysis blinded.

Data sharing: The data from the clinical trial have already been reported to the regulatory body (Paul-Ehrlich Institute, Germany). Most of the data are presented in the main manuscript and the extensive supplementary tables and figures of the paper (open access); any additional data will be made available by the corresponding author on request (petra.reinke@charite.de).

The corresponding author affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

Dissemination to participants and related patient and public communities: The dissemination plan for the results allows for the involvement of patients and patient organisations as well as the public, and implementation has already begun: for example, presentation of initial data and introduction of patients in regular doctor-patient seminars at our transplant centre 2017/18, as well as at the RESTORE health by advanced therapies event at the European Parliament in Brussels, annual long night of science event for public in Berlin 2019, production of a film for the public and politics about advanced therapies with an example of nTreg therapy (www.restore-horizon.eu).

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Web appendix: Supplementary materials

Auf der Basis unserer positiven Erfahrungen bei Nierentransplantationspatienten behandelten wir zusätzlich drei Kinder, die nach einer allogenen Stammzelltransplantation an einer schweren therapierefraktären und lebensbedrohlichen Abstoßungsreaktion erkrankt waren. Im Rahmen eines individuellen Heilversuches erhielten diese Kinder *ex vivo* expandierte Tregs, welche wir aus peripherem Blut der ursprünglichen Stammzellspender hergestellt hatten.

2.6. Der adoptive Transfer von ex-vivo-expandierten regulatorischen T-Zellen verbessert das immunologische Engraftment und die therapierefraktäre chronische GvHD.

Der folgende Text wurde durch die Autorin übersetzt und entspricht im Wesentlichen dem Abstrakt der Arbeit

Landwehr-Kenzel S, Mueller-Jensen L, Kuehl JS, Abou-el-Enein M, Hoffmann H, Muench S, Kaiser D, Roemhild A, von Bernuth H, Voeller M, Schmueck-Henneresse M, Gruhn B, Stervbo U, Babel N, Volk HD, Reinke P. Adoptive transfer of *ex vivo* expanded regulatory T-cells improves immune cell engraftment and therapy-refractory chronic GvHD. Molecular Therapy, Juni 2022, Seiten 2298-2314, Band 30. [119]

Die Graft-versus-Host-Erkrankungen (GvHD) ist außerhalb von Rezidiven immer noch die wichtigste lebensbegrenzende Komplikation nach einer hämatopoetischen Stammzelltransplantation. Auch eine moderne pharmakologische Immunsuppression ist oft unzureichend und mit erheblichen Nebenwirkungen verbunden. Neue Behandlungsstrategien umfassen nun den adoptiven Transfer von ex vivo expandierten regulatorischen T-Zellen (Tregs), aber ihre Wirksamkeit bei chronischer GvHD ist unbekannt. Wir behandelten drei Kinder mit schwerer, therapierefraktärer GvHD mit polyklonal expandierten Treg-Produkten, welche aus dem ursprünglichen Stammzellspender generiert wurden. Die immunsuppressive Drittlinien-Therapie wurde kurz vor dem Zelltransfer auf Cyclosporin A und niedrig dosierte Steroide reduziert. Die regelmäßige Nachsorge umfasste die Beurteilung der subjektiven und objektiven klinischen Entwicklung, der Sicherheitsparameter und ein eingehendes Immunmonitoring. Alle Patienten zeigten eine deutliche klinische Verbesserung mit einer wesentlich reduzierten GvHD-Aktivität. Laboruntersuchungen zeigten eine

signifikante Verbesserung des immunologischen Engraftments, sowohl im Hinblick auf Lymphozyten als auch auf dendritische Zellen. Die Nachverfolgung adoptiv transferierter Tregs mittels Next-Generation-Sequencing zeigte eine klonale Expansion. Zusammenfassend zeigte sich, dass der adoptive Transfer von Tregs gut vertragen wurde und in der Lage war, eine etablierte unerwünschte T-Zell-vermittelte, alloreaktive Immunreaktion zu modulieren. Obwohl keine Anzeichen einer Überimmunsuppression zu beobachten waren, sollte die Behandlung von Patienten mit invasiven opportunistischen Infektionen mit Zurückhaltung erfolgen. Weitere kontrollierte Studien sind notwendig, um diese ermutigenden Ergebnisse zu bestätigen und schließlich den Weg für eine adoptive Treg-Therapie bei chronischer GvHD zu ebnen.

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Molecular Therapy
Original Article

Adoptive transfer of *ex vivo* expanded regulatory T cells improves immune cell engraftment and therapy-refractory chronic GvHD

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Graft-versus-host disease (GvHD) is still the major non-relapse, life-limiting complication after hematopoietic stem cell transplantation. Modern pharmacologic immunosuppression is often insufficient and associated with significant side effects. Novel treatment strategies now include adoptive transfer of *ex vivo* expanded regulatory T cells (Tregs), but their efficacy in chronic GvHD is unknown. We treated three children suffering from severe, therapy-refractory GvHD with polyclonally expanded Tregs generated from the original stem cell donor. Third-line maintenance immunosuppression was tapered to cyclosporin A and low-dose steroids shortly before cell transfer. Regular follow-up included an assessment of the subjective and objective clinical development, safety parameters, and in-depth immune monitoring. All patients showed marked clinical improvement with substantially decreased GvHD activity. Laboratory follow-up showed a significant enhancement of the immunologic engraftment, including lymphocytes and dendritic cells. Monitoring the fate of Tregs by next-generation sequencing demonstrated clonal expansion. In summary, adoptive transfer of Tregs was well tolerated and able to modulate an established undesired T cell mediated allo-response. Although no signs of overimmunosuppression were detectable, the treatment of patients with invasive opportunistic infections should be undertaken with caution. Further controlled studies are necessary to confirm these encouraging effects and eventually pave the way for adoptive Treg therapy in chronic GvHD.

INTRODUCTION

Chronic graft-versus-host disease (cGvHD) remains the most significant post-transplant non-relapse cause of morbidity and mortality after hematopoietic stem cell transplantation (HSCT), the standard treatment for multiple malignant and benign hematologic diseases.[1–4] Despite being increasingly target-specific, modern pharmacologic immunosuppression has only insufficiently decreased the burden of cGvHD and is associated with significant side effects, including toxicities or an increased risk of infections and malignancies.[5] During early phases, GvHD is usually triggered by chemotherapy or infection-induced tissue damage, which leads to a release of pro-inflammatory cytokines. This results in activation of

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antigen-presenting cells, which then present recipient-specific tissue antigens to alloreactive donor T cells.[5,6] In case of cGvHD, donor lymphocytes remain continuously activated and fail to establish a host-specific tolerance.[5] Therefore, concurrent with increasing indications for HSCT and driven by the medical need to combat cGvHD, the identification of thymic derived regulatory T cells (Tregs) has paved the way for the development of novel immunotherapeutic cell-based strategies.

Tregs are characterized as $CD4^+CD25^+$ cells expressing high levels of FoxP3,[7] but very low levels of the IL-7 receptor α chain[8] and have been demonstrated to play a critical role in the maintenance of immunological tolerance.[9] As physiologic counterplayers of conventional T cells, Tregs can control overactivation and expansion of conventional T cells[10] in various preclinical disease models.[11–17] Recently, clinical grade Tregs products became available and first data on safety and efficacy has been published for the preventive/pre-emptive use of Tregs for GvHD in adults.[18–23] Reports on the therapeutic use of Tregs to treat GvHD are limited to individual case reports, and to the best of our knowledge, their use has not yet been reported in children.

We now report on three children who suffered from therapy-refractory, life-threatening GvHD and therefore received adoptive transfer of *ex vivo* expanded Tregs that were generated from the original stem cell donor on an individual treatment basis. Concomitant pharmacologic immunosuppression was tapered to cyclosporin A (CSA) and low-dose prednisolone shortly before cell transfer. We further demonstrate by clinical and immunological follow-up data that adoptive transfer of *ex vivo* expanded Treg products is well tolerated and can modulate established T cell-mediated allo-responses, supporting immune cell engraftment and ameliorating GvHD activity.

RESULTS

Patient characteristics and clinical course

Patient 1, 8 years old, underwent peripheral blood stem cell transplantation from a 10/10 HLA related donor owing to transfusion dependent beta-thalassemia major (details listed in Table 1). Despite guideline-compliant GvHD prophylaxis, the patient developed acute GvHD 12 days after transplantation, which progressed to severe cGvHD. Skin and skin appendages were the main site of manifestation, but the gastrointestinal mucous membranes were also severely affected. Chronic pain, difficulties associated with food intake, contractures of the finger joints, dryness and burning of the eyes, and not least the disease-related stigma led to a significant impairment of the patient's quality of life (Figures 1A–1C and 1I–1O). The patient received first, second and third-line therapy (Table 1), including subcutaneous IL-2 applications as previously described by Koreth et al.[24] None of these approaches significantly improved the clinical symptoms. On the contrary, the patient developed major complications, including arterial hypertension and two episodes of severe sepsis. Owing to the lack of therapeutic alternatives, adoptive Treg therapy was performed day +639 after HSCT at a dose of 3.0×10^6 Treg/kg i.v. During the 93-week follow-up period, we observed a significant clinical improvement. The Treg product was well tolerated. No signs of acute or chronic toxicity were observed; no inflammatory cytokine storm (Figure S1) and no signs of embolism or other acute adverse reactions occurred. Sixteen weeks after Treg transfer the patient presented with abdominal pain; the clinical course was highly suggestive for a beginning bacterial infection. Upon initiation of i.v. antibiotic therapy, the acute symptoms promptly resolved. No relevant additional infectious complications were recorded, in particular no critical virus reactivation or infection, no fungal infection, no additional bacterial infection and no other adverse events occurred during the follow-up period. With respect to cutaneous GvHD manifestation, continuous improvement with substantial benefits in the skin and joints was documented as depicted in Figures 1D–1F. The general status of the patient substantially improved; the parents reported less pain, improved food intake and a significant increase in vitality and recreational activity. The daily prednisolone dose was reduced from 10 mg (0.45 mg/kg) before Treg therapy to 5 mg (0.22 mg/kg). Quantification of the clinical improvement showed amelioration of ocular symptoms including xerophthalmia, photosensitivity, foreign body sensation, and epiphora conjunctival injection (Figure 1K). Additionally, decreased scleroderma and inflammation of the mucosa were observed (Figures 1L–1O).

Patient 2, 11 years old, underwent stem cell transplantation and re-transplantation owing to myelodysplastic syndrome from a 10/10-ident unrelated donor (Table 1). The patient suffered from severe acute GvHD of the intestine starting 60 days after transplantation, which had progressed to cGvHD of the skin, the liver, the lung, and the intestine by day 103 after transplant. Signs of active cGvHD included facial erythema, multiple nummular skin lesions, severe oral (Figures 1G and 1H) and ocular, hepatic, and pulmonary manifestations. GvHD activity barely responded to first-, second- and third-line immunosuppressive regimens (Table 1), which induced various side effects, including severe osteonecrosis, which required regular pain medication. At the time when the patient was first presented to our team, he received quadruple immunosuppressive therapy with prednisolone, methotrexate, ruxolitinib, and weekly extracorporeal photopheresis. Owing to the lack of alternative treatment options, Treg product was generated from the original stem cell donor and the patient received 3.0×10^6 expanded Tregs/kg on days +736 and +675 after stem cell transplantation. The Treg product was well tolerated. No signs of acute or chronic toxicity were observed, nor was there an inflammatory cytokine storm, signs of embolism, or other acute adverse reactions. During the entire observation period, no infectious complications were recorded; in particular, no critical virus reactivation or infection, no fungal infection, no additional bacterial infections and no other adverse events occurred during this follow-up period. The patient's general condition substantially improved over time (Figure 1I). At time of Treg transfer, the patient required nightly high-flow oxygen supplementation; however, within 6 months after adoptive Treg transfer all oxygen supplementation could be terminated. Photophobia as well as foreign body sensation in the eyes and xerophthalmia (Figure 1K) and signs of cutaneous and mucosal inflammation decreased (Figures 1L–1O). Prednisolone

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Table 1. Clinical and transplantation related characteristics of patient 1, patient 2 and patient 3

	Patient 1	Patient 2	Patient 3
Sex	Male	Male	Male
Indication for HSCT	β-Thalassemia major	Myelodysplastic syndrome	Septic granulomatous disease
Age at HSCT	8 years	11 years	11 years
Conditioning	Busulfan Cyclophosphamide ATG-Fresenius	Fludarabin Thiothepa ATG-Fresenius	Fludarabin Busulfan** Alemtuzumab
Graft	PBSC	Bone marrow	Bone marrow
HLA match	10/10 MRD	10/10 MUD	9/10 MUD
Cell dose (/kg)	2.9×10^8 TNC 5.3×10^6 $CD34^+$ cells 1.2×10^8 $CD3^+$ T cells	4.1×10^8 TNC 3.4×10^6 $CD34^+$ cells 4.5×10^7 $CD3^+$ T cells	4.1×10^8 TNC 1.9×10^6 $CD34^+$ cells 5.1×10^6 $CD3^+$ T cells
GvHD prophylaxis	CSA, MTX, ATG	CSA, MTX	CSA, MMF, Prednisone
Retransplantation 2nd conditioning Graft	– – –	d +61 Thymoglobulin PBSC	d +531 $CD34^+$ PBSC
GvHD manifestation sites	Skin Mukosa GI tract	Skin Mukosa GI tract	GI tract Skin Liver
GvHD therapy	CSA Prednisolone MMF MTX Azathioprine Budenoside Low-dose IL-2 s.c.	CSA Prednisolone Methylprednisolone Tacrolimus Photopheresis Infliximab Methotrexate Ruxolitinib	CSA Prednisolone MMF MTX Budenoside ECP Imatinib MSC d+341, d+348, d+373, d+378
tTreg therapy post HSCT	d +639	d +736/+675	d +603
tTreg product characteristics			
Fold expansion rate	3 193	20,751	1 291
Viability	98%	98%	97%
$CD4^+CD25^+FoxP3^+$	95%	97%	94%
% IFNg producer	1.5%	1.3%	7.61%
tTreg dose	3×10^6/kg	3×10^6/kg	3×10^6/kg
Co-administered immunosuppression	CSA Low-dose prednisolone	CSA Low-dose prednisolone	CSA MMF

GI tract, gastrointestinal tract; MMF, mycophenolate mofetil. Conditioning: Busulfan**, only 75% of regular myeloablative busulfan i.v. dose (3 days instead of 4) with therapeutic drug monitoring for a targeted dose of 65,000 ng/h/mL; ATG-Fresenius$^+$, antithymocyte globulin (Grafalon). Grafts: BM, bone marrow; MRD, matched related donor; MUD, matched unrelated donor; PBSC, peripheral blood stem cells. Cell dose: TNC, dose of total nucleated cells per kilogram of body weight infused; MSC, mesenchymal stem cells.

could be tapered to 2.5 mg/d and the CSA trough levels were targeted at 80–100 μg/L. Radiologically, osteonecrosis did not progress further and partially improved; the patient did not require pain medication as of 6 months after Treg therapy. The overall clinical improvement continued for 15 months after Treg therapy until the patient suffered from primarily non-GvHD complications, including a urethral polyp and gastroesophagitis. Endoscopy of both sites was performed, but during microbiological, virological, and histological workup, no evidence of infectious triggers, GvHD, or malignancy was found. Nineteen months after Treg transfer, the patient suffered from sudden onset of hemoptysis. During bronchoscopy, the bronchial and pulmonary mucosa showed diffuse signs of bleeding without larger bleedings sources. Biopsies as well as microbiological and virological specimens were taken and thoroughly analyzed, but no signs of GvHD or infections were found. Owing to respiratory failure on mechanical ventilation the patient died 10 days after bleeding onset. Retrospectively, cGvHD-related coagulopathy seems possible.[25]

Patient 3, 11 years old, was stem cell transplanted owing to chronic granulomatous disease (a *de novo* mutation of the CYBB gene) from a 9/10 HLA-ident unrelated donor (Table 1). The patient developed mild aGvHD of the skin (day +10). Timely correlating with CSA trough levels below the target range aGvHD later progressed to severe cGvHD affecting the skin (moderately) and the gastrointestinal tract including the intestine and the liver (both severely). Multimodal immunosuppressive therapy promoted infectious complications with microbiological evidence of *Candida glabrata*, *Staphylococcus epidermidis*, *Lactobacillus rhamnosus*, *Aspergillus fumigatus*, BK

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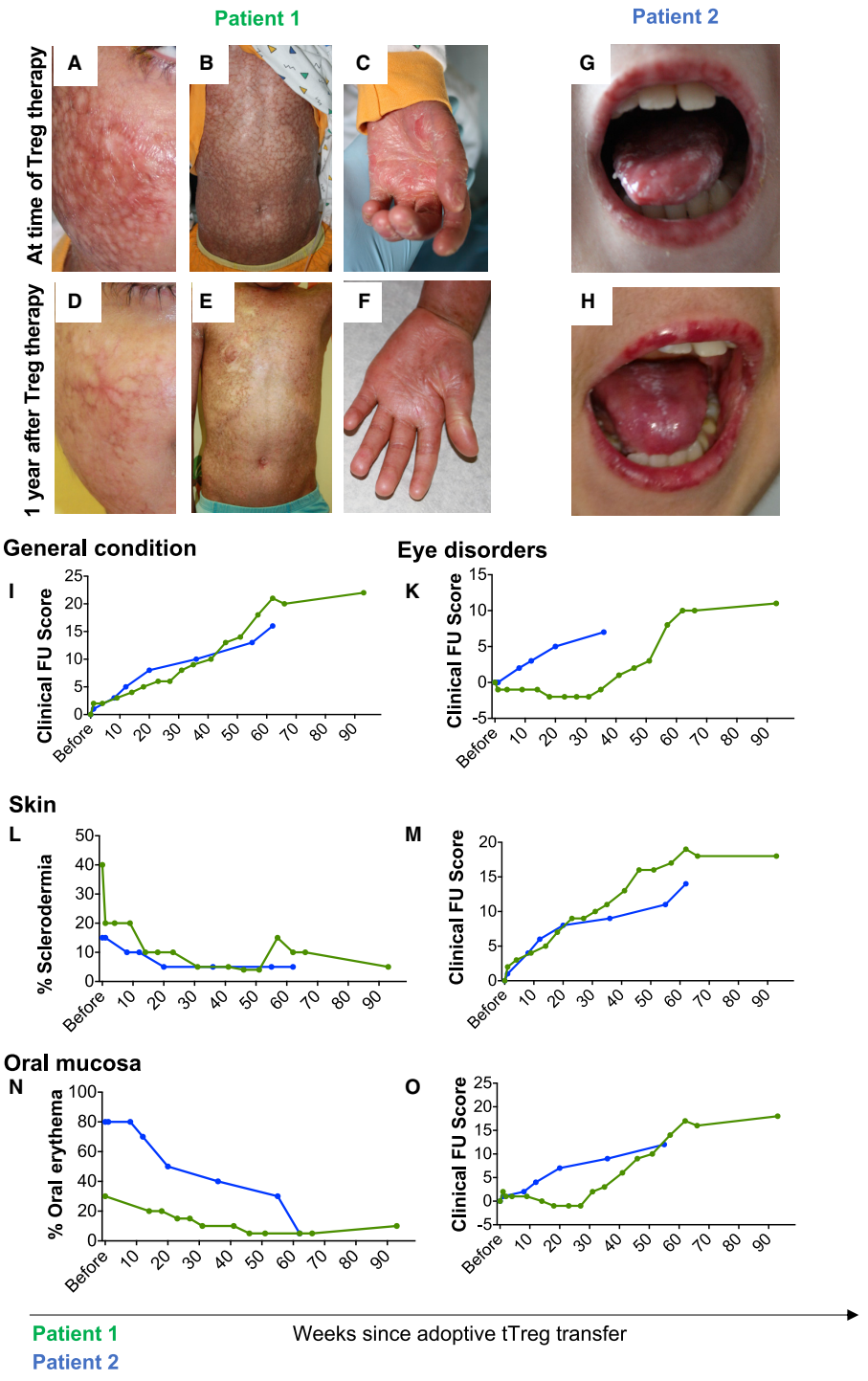

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virus, and adenovirus. Pulmonary computed tomography scans showed atypical infiltrates of the lung, as typically seen in pulmonary aspergillosis. Anti-infective therapy was escalated but options were limited by chronic kidney and liver insufficiency. Chronic GvHD treatment included first-, second-, and third-line treatment approaches including CSA, mycophenolate mofetil, prednisolone (max 5 mg/kg/day), methotrexate, budenoside, and extracorporeal photopheresis (Table 1). Owing to continuously uncontrolled intestinal GvHD the patient received four doses of mesenchymal stem cells between day +341 and day +378 (individual treatment approach). Subsequently, transplant failure occurred and a stem cell boost with CD34-selected stem cells of the initial donor became necessary on day +531. The clinical condition of the patient remained critical and cGvHD activity was persistently high. Finally, the patient received 3.0×10^6 expanded Tregs/kg as an individual treatment approach on day +603 after HSCT. Tregs were well tolerated without any signs of undesired cytokine release (Figure S1). The patient showed a remarkable clinical stabilization and improvement of GvHD symptoms. Steroids were reduced from a maximum of 20 mg (0.77 mg/kg) daily to a minimum of 10 mg (0.38 mg/kg) and the patient was mobilized. A computed tomography scan of the lung performed on day +19 after Treg transfer (day +622 after HSCT) showed declining, bilateral milk glass changes and infiltrates. Four weeks after Treg transfer, infectious parameters, fever, and GvHD activity increased again. The option of anti-infectious therapy escalation was limited by the pre-existing chronic liver and kidney disease. The clinical condition rapidly deteriorated and during an acute event, most likely a pulmonary hemorrhage owing to the underlying pulmonary aspergillosis, the patient died on day +35 after Treg therapy (day +638 after HSCT).

Improved T cell engraftment after Treg transfer

An analysis of the total T cell compartment (Figures 2A–2P, S2, and S3) by multicolor flow cytometry revealed distinct changes that together indicated substantial progress in T cell engraftment and normalization of T cell subset distributions. At the time of Treg transfer, all patients showed mild (patient 1) to severe (patient 2 and patient 3) $CD3^+$ lymphopenia (Figures 2A–2C), with particularly reduced $CD4^+$ T cell numbers (Figures 2D–2F) and reduced $CD8^+$ T cells (Figures 2K–2M). While total the $CD3^+$ and $CD8^+$ T cells remained largely stable throughout the observation periods (Figures 2A–2C), we observed a remarkable and long-term increase of $CD4^+$ T cells (Figures 2D–2F). To assess T cell differentiation states the expression of CD45RA and CCR7 were employed to identify naive ($CD45RA^+$ $CCR7^+$, T_N), central memory (CD45RA-$CCR7^+$, T_{CM}), effector memory ($CD45RA^-CCR7^-$, T_{EM}) and terminally differentiated $CD45RA^+$ ($CD45RA^+$ $CCR7^-$, T_{EMRA}) T cells. In addition to the increase in absolute $CD4^+$ T cell counts, we found, for the first time since transplantation, peripherally detectable numbers of T_N, which is a sign of intrinsic expansion and improved T cell engraftment (Figures 2G–2I). This was first detectable at 4–8 weeks after Treg transfer and continued throughout the observation periods reaching levels of approximately 50% of all Tregs in patient 1. Interestingly, in contrast to the robust induction of $CD4^+$ T cells, absolute numbers of $CD8^+$ T cells remained largely stable (Figures 2K–2M). However, addressing the proportional distribution of T cell maturation states in more detail we observed a substantial shift toward $CD8^+$ T_N while the frequency of T_{EM} and T_{CM} was reduced in patient 1 > patient 2 (Figures 2N–2P).

T cell expansion occurs within HLA-DR^- T cells

HLA-DR is expressed on activated T cells *in vivo* and *in vitro* and is associated with a significantly higher risk of acute and cGvHD. To monitor for T cell activation *in vivo* after Treg transfer, expression of HLA-DR was assessed on $CD4^+$ and $CD8^+$ T cells (Figures 3A–3M and S4). In line with the observation of naive T cell engraftment, we found an impressive increase of non-activated HLA-DR^-CD4^+ T cells in patient 1 and patient 2 (Figures 3A–3F). This effect was particularly strong within the $CD4^+$ T cell compartment but paralleled by an increase in HLA-DR^-CD8^+ T cells. At the same time, the absolute number of activated, HLA-DR^+ $CD4^+$ and $CD8^+$ T cells remained largely stable (Figures 3A–3C and 3G–3I). The expansion of HLA-DR^- T cells was further confirmed by the concurrent proportional decrease in the relative counts of activated HLA-DR^+ T cells in both the $CD4^+$ and $CD8^+$ T cell compartment (Figures 3D–3F and 3K–3M).

Engraftment of naive and expansion of memory Tregs

For monitoring the fate of Tregs, $CD3^+CD4^+CD25^{high}CD127^{low}$ Treg counts were assessed whenever a blood collection was justified for the evaluation of the patients' status (Figures 4A–4I). Interestingly, a biphasic increase of $CD3^+CD4^+CD25^{high}CD127^{low}$ Tregs was observed in all patients (Figures 4A–4C and S5). Owing to the short observation time, this increase is, however, difficult to interpret in patient 3 (Figure 4C). In patient 2, the absolute Treg numbers and Treg frequencies decreased again starting after week 40 post Treg transfusion (Figure 4B).

Figure 1. Adoptive Treg transfer ameliorates clinical symptoms of cGvHD in individual patients

(A–H) document the clinical extent of cutaneous and mucosal GvHD in patient 1 (A–F, green lines) and patient 2 (G and H, blue lines) at time of Treg transfer (A–C) and 1 year later (D–F). As depicted in (A–F), patient 1 suffered from severe signs of inflammation, hyper- and hypopigmentation and skin lesions as well as finger contractures (A–C). Patient 2 showed severe sings of mucositis with leukoplakia of the oral mucosa (G). One year after Treg therapy not only leukoplakia and mucositis was substantially reduced, but their reduced clinical burden also substantially increased quality of life (H). (I–O) The overall patient status (I) and GvHD symptoms of the eyes such as xerophthalmia, photosensitivity foreign body sensation, epiphora conjunctival injection (K), the skin (L and M) and the oral mucosa (N and O) were further quantified by a clinical follow-up score, which evaluated the clinical situation as compared with the previous visit (scoring depicted in Table 2). As documented by the clinical scoring, the general condition, ocular disorders and symptoms of both the skin and the mucosa continuously improved over time (I, K, M, and O). In line with these observations, the percentage of skin affected from scleroderma and oral erythroderma substantially decreased (L and N). Most impressively, skin softening, which cannot be depicted in the photographs, was also observed.

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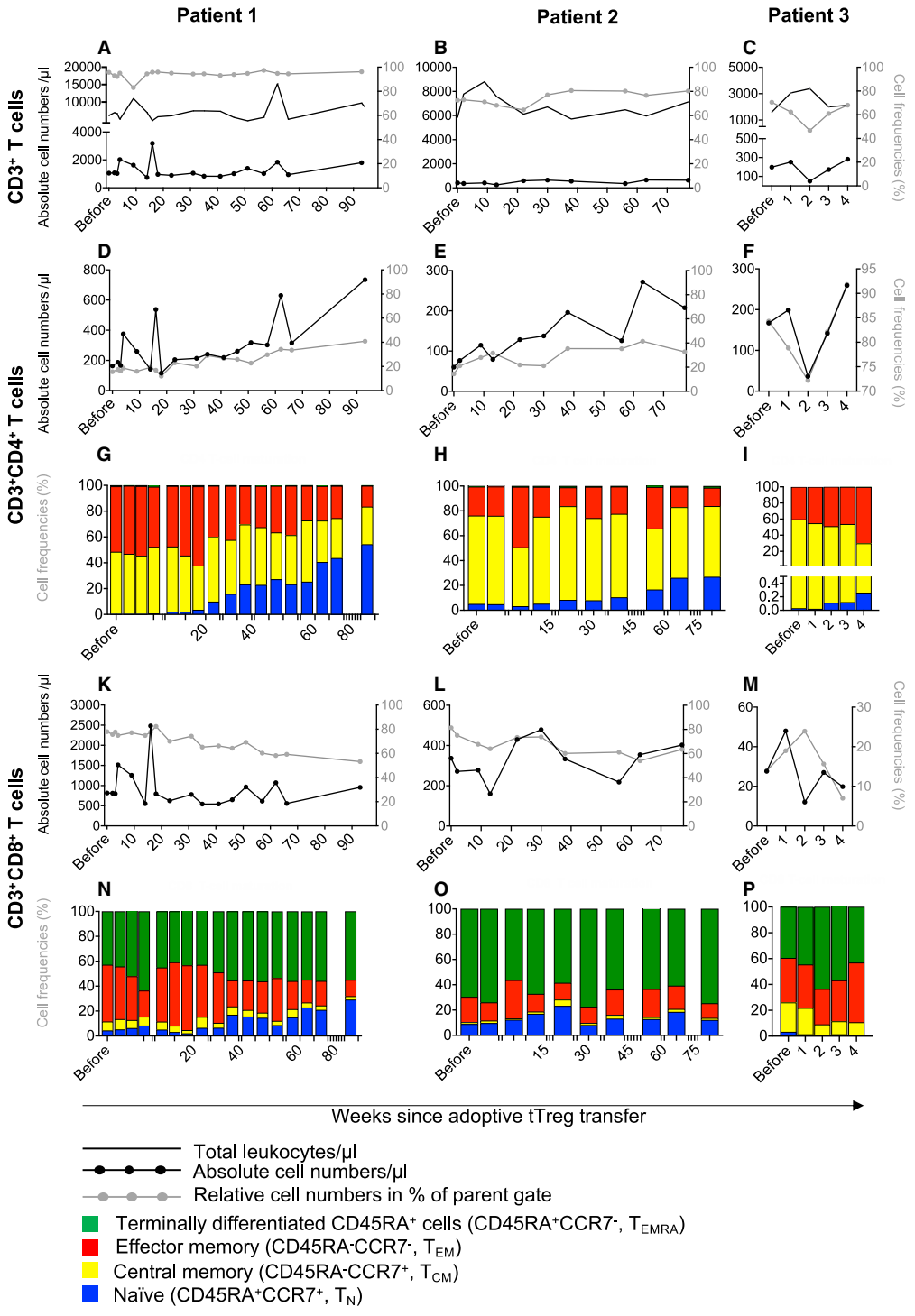

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To better understand Treg biology *in vivo,* we further analyzed Treg maturation states according to their expression of CD45RA, the characteristic marker for naive T cells (Figures 4D–4I). At the time of Treg transfer all three patients showed very low numbers of Tregs; naive Tregs were—if at all—only barely detectable (Figures 4D–4F). Starting at about 3 months after adoptive Treg transfer, both patient 1 and patient 2 showed a remarkable increase of naive Treg, which occurred for the first time since HSCT and may indicate intrinsic Treg engraftment (Figures 4D and 4E). At 65 weeks after Treg transfer, patient 1 reached the lower limit of normal Treg numbers (Figure 4D). The biphasic increase of total Tregs was also observed in circulating memory Tregs and might be explained as adoptively transferred Tregs in the first phase and *in vivo* Treg expansion and/or maturation in the second phase (Figures 4G–4I). Further underlining and paralleled by the observation of relative and absolute Treg expansion, the relative frequency of memory Tregs decreased (Figures 4G and 4H). In line with the early peak of memory Tregs observed in patient 1 and patient 2, we found comparably high numbers of memory Tregs in patient 3 (Figure 4I).

T cell receptor repertoire analyses

In line with our previously published reports,[26] our Treg products expressed a polyclonal T cell receptor (TCR) repertoire comparable with those of freshly isolated Tregs but distinct from non-Tregs (Figure 5). To track the fate of adoptively transferred Tregs, we compared the TCR repertoire of the respective Treg product with those of PBMC collected during follow-up of our patients. As shown in Figure 5, the donor-derived Treg product contains overlapping T cell clones with PBMC of the respective HSCT recipient, representing the few Treg cells within the PBMC. The number of distinct Treg TCRs overlapping PBMC samples rose from 256 (patient 1) and 281 (patient 3) before Treg infusion up to more than 1,000 in both patients within the first weeks. Treg TCR clones were detectable at high levels throughout the observation periods of both patients. Interestingly, in patient 1 there was a temporary decrease in the overlapping clone counts to 484 in association with a bacterial infection at 16 weeks after Treg therapy, which recovered fast after clearance of the infection. Of major importance, we found that the number of distinct Treg TCRs (Figures 5C and 5D) paralleled the frequency of total Tregs in the peripheral blood detected by flow cytometric analysis (Figures 4A and 4C). The biphasic increase of Treg engraftment described in Figure 4 was, however, not only represented by the dynamic of unique TCRs, but also detected when the total amount of overlapping Treg TCRs (Figures 5E and 5F) and frequencies of Treg TCRs within all T cells (Figures 5G and 5H) were assessed. In summary, these data support the hypothesis of long-term engraftment of adoptively transferred Tregs and their survival for the full follow-up time of more than 9 months.

B cell engraftment after Treg transfer

Long lasting B cell deficiency is a common phenomenon in HSCT patients suffering from cGvHD.[27,28] Deficient B cell development at early maturation states has previously been reported to be both a cause and consequence of active GvHD. Therapeutic approaches to combat acute and cGvHD frequently cause further delays to B cell engraftment. To assess the effects of adoptive Treg therapy on B cell development, the maturation state of B cells was analyzed, ranging from naive B cells to plasmablasts, as well as their engraftment (Figures 6A–6S and S6). Before Treg infusion, $CD19^+$ B cells were barely detectable in patient 1 and patient 3 and substantially decreased in patient 2 (Figures 6A–6C); these data were in line with very low numbers of naive B cell (Figures 6D–6F). Approximately 20 weeks after Treg transfer, we observed for the first time since transplantation a remarkable expansion within the naive B cell compartment (Figures 6D–6F), which also resulted in an absolute increase of in the total number of B cells (Figures 6A–6C). Transitional (Figures 6G–6I) and non-class-switched (Figures 6K–6M) B cell initially followed the same trend in expansion as naive B cells; however, this expansion effect was lost over time as depicted here for transitional (Figures 6G–6H), non-class-switched (Figures 6K–6M), class-switched (Figures 6N–6P) B cells and plasmablasts (Figures 6Q–6S).

Adoptive Treg transfer improves dendritic cell engraftment

The effect of adoptively transferred Tregs on innate immune cells critically involved in early infection control is largely unknown. To elucidate if Tregs can support the engraftment of innate immune cells as well as lymphocytes, we analyzed the number of granulocytes, monocytes, and dendritic cells (DC) (Figures 7A–7P and S7). After Treg transfer, granulocyte (Figures 7A–7C) and monocyte (Figures 7D–7F) numbers were largely stable, although we found both cell compartments to be transiently increased after Treg transfer with repetitive peaks at later stages. Impaired reconstitution of DC in patients suffering from GvHD has previously been described[29–31] and was also observed in our patients with cGvHD before Treg therapy (Figure 7). Serial follow-up, however, showed that adoptive Treg therapy seems to support DC engraftment (Figures 7G–7P). After Treg transfer, and in parallel with the engraftment of naive $CD4^+$ T cells, we observed an increase in $LIN^-HLA\text{-}DR^+CD11c^+$ DC (Figures 7G–7I), $CD16^+$ DCs (Figures 7K–7M), and mDC1 (Figures 7N–7P).

Figure 2. Engraftment of naive T cells after Treg therapy

(A–P) Peripherally collected blood was analyzed by multicolor flow cytometry. Cells were gated as singlets, $CD45^+$ leukocytes, lymphocytes, and the expression of $CD3^+$ (A–C), $CD3^+CD4^+$ (D–I) and $CD8^+$ (K–P). $CD3^+CD4^+$and $CD3^+CD8^+$ T cells were further analyzed for the expression of CD45RA and CCR7 (G–I and N–P). Subsequently, T cell maturation of the $CD4^+$ and the $CD8^+$ compartment was separately analyzed by the identification of naive as $CD45RA^+CCR7^+$ (T_N), central memory as $CD45RA^-CCR7^+$ (T_{CM}), effector memory as $CD45RA^-CCR7^-$ (T_{EM}), and terminally differentiated $CD45RA^+$ T cells as $CD45RA^+CCR7^-$ (T_{EMRA}). Depicted are total leukocytes numbers per microliter as a continuous line in (A–C). All cells subsets are shown before Treg administration and in the weeks after Treg transfer (x axis). (A–F and K–M) Results are depicted as black solid lines in absolute cell numbers per microliter and assigned to the left y axis. Relative cell frequencies of the parental gate are depicted as gray lines in percent and assigned to the right y axis. (G, H, and N–P) $CD4^+$ and $CD8^+$ compartments were analyzed for their composition of T_N, T_{CM}, T_{EM}, and T_{EMRA} cells are depicted in relative frequencies.

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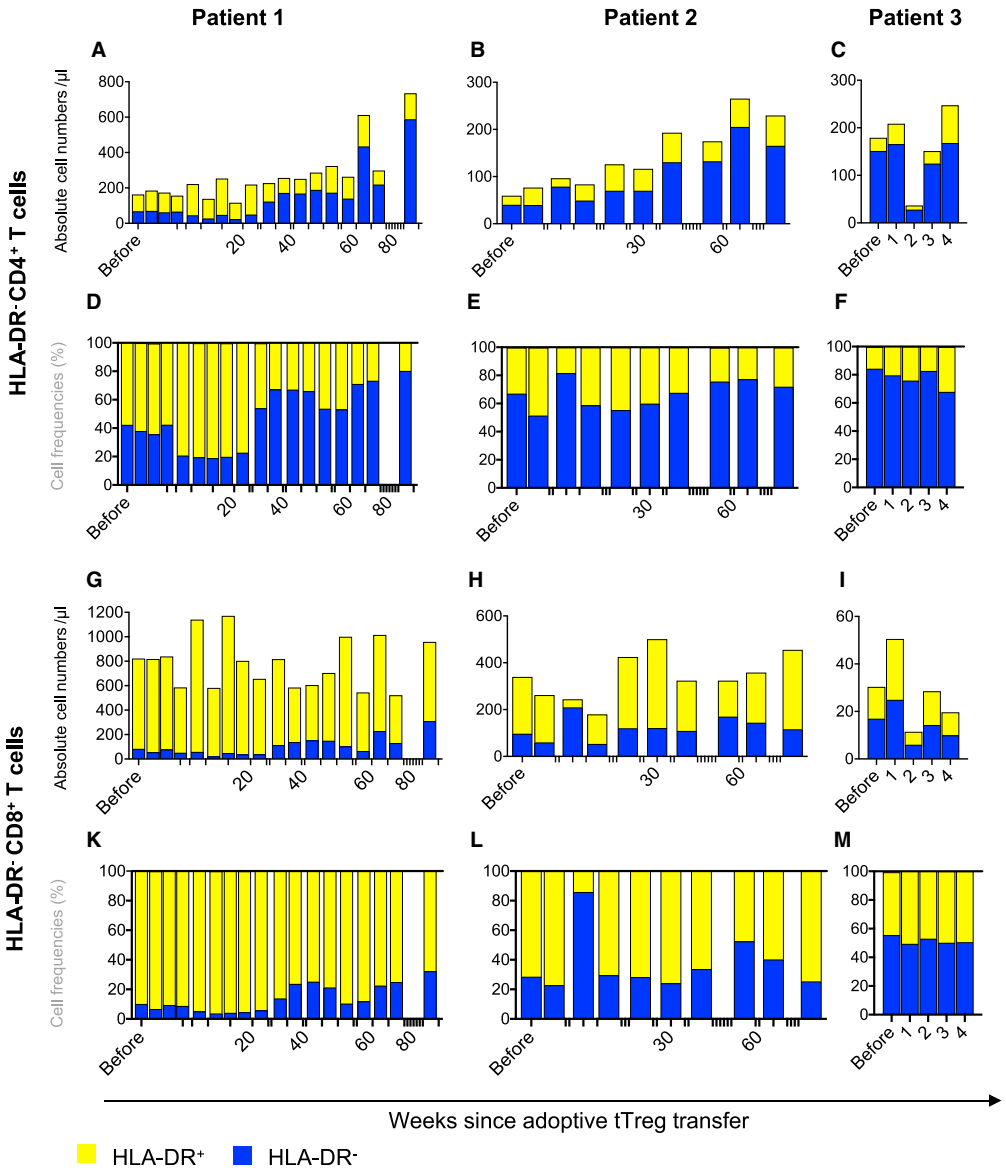

Figure 3. T cell engraftment is not accompanied by increased HLA-DR$^+$ cells

(A–M) CD4 and CD8 T cell compartments were identified from singlets, CD45$^+$ leukocytes, lymphocytes, CD3$^+$ cells and analyzed for the expression if HLA-DR$^+$ by multicolor flow cytometry. (A–C and G–I): Depicted are absolute cell numbers of HLA-DR expressing CD4$^+$ (A–C) and CD8$^+$ (G–I) T cells. (D–F and K–M): Relative distribution of HLA-DR$^+$ (yellow) and HLA-DR$^-$ (blue) cells within the CD4$^+$ and CD8$^+$ compartment are depicted in (D–F and K–M).

DISCUSSION

Our data suggest that the adoptive transfer of polyclonal *ex vivo* expanded Tregs from the original stem cell donor at a single dose of 3×10^6/kg body weight is well tolerated and can improve the clinical condition in children suffering from therapy-refractory cGvHD. We did not observe adverse effects directly related to Treg transfer, such as allergic reactions, cytokine storm, transfusion-related lung injury, or pulmonary embolism. All three severely ill patients showed fast improvement of cGVHD symptoms and engraftment of naive lymphocytes and DC. In two patients this effect lasted more than

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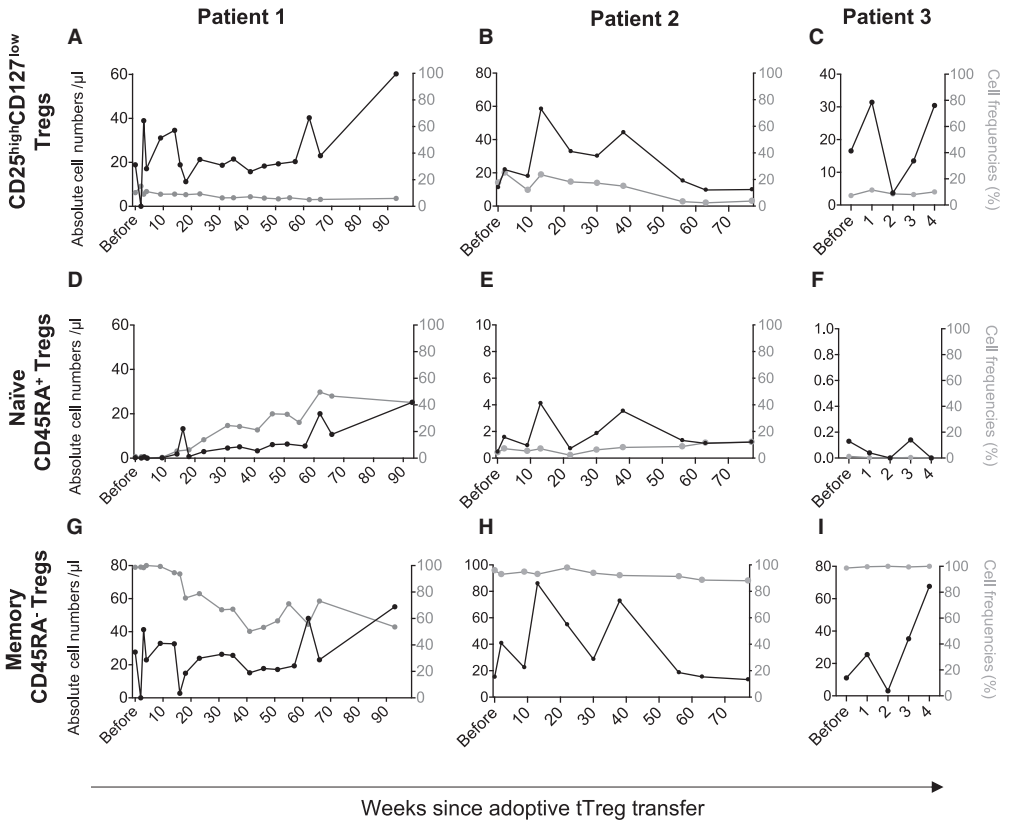

Figure 4. Substantial engraftment of naive and memory Tregs

Numbers and frequencies of peripherally circulating Tregs were assessed by multicolor flow cytometry. Tregs were gated as singlets, $CD45^+$ leukocytes, lymphocytes, $CD3^+CD4^+$ T cells and subsequently identified as $CD25^{high}CD127^{low}$ expressing cells. (A–C) Total Tregs were further sub-analyzed as (D–F) naive $CD45RA^+$ and (G–I) memory $CD45RA^-$ Tregs. Results are depicted as black solid lines in absolute cell numbers per microliter and assigned to the left y axis. Relative cell frequencies of the parental gate are depicted as gray lines in % and assigned to the right y axis. The s axis shows time from Treg transfer.

1 year. One patient died of an unexpected pulmonary hemorrhage 19 months after Treg transfer; however, no infectious cause or active pulmonary GvHD could be detected. The third patient could not be followed long term because he died of a pre-existing invasive aspergillus infection at day 35 after Treg treatment.

The encouraging results on the course of cGvHD are even more pronounced considering the tapering of maintenance third-line immunosuppression in all three patients to CSA and a low-dose steroid treatment just before Treg transfer to allow Treg survival and functionality. The improvement of cGvHD symptoms that allowed further tapering of immunosuppression, underlines the efficacy of Treg transfer. Furthermore, the reduction in immunosuppression had the knock-on effect of decreasing drug-related side effects such as organ toxicity, osteonecrosis and the incidence of clinically relevant infection episodes. There are several reports that calcineurin inhibitors may decrease Treg survival and function *in vivo*;[32–36] however, our recent data in a GvHD mouse model rather suggests that low-dose CSA is beneficial for Treg functionality, whereas steroid treatment should be avoided.[37] The long-lasting increased levels of Tregs and the sustainable amelioration of GvHD in our low-dose CSA-treated patients supports our previous findings.

The only other case reports describing a therapeutic approach of adoptive Treg transfer in cGvHD were published by Theil et al.[23] (five patients) and Trzonkowski et al.[19] (one patient) with cell doses ranging from 1×10^5/kg to 4.5×10^6/kg. In line with our approach, Theil et al. administered Treg products that were produced from the original stem cell donor after Treg isolation by magnetic bead separation (CliniMACS technology). In contrast, however, unstimulated leukapheresis products were used as the starting material. Further, while our products were expanded for 3 weeks and reached a purity of greater than 94% at an average fold expansion rate of 8,411, Theil et al.[23] conducted Treg expansion over 12 days only and reached an

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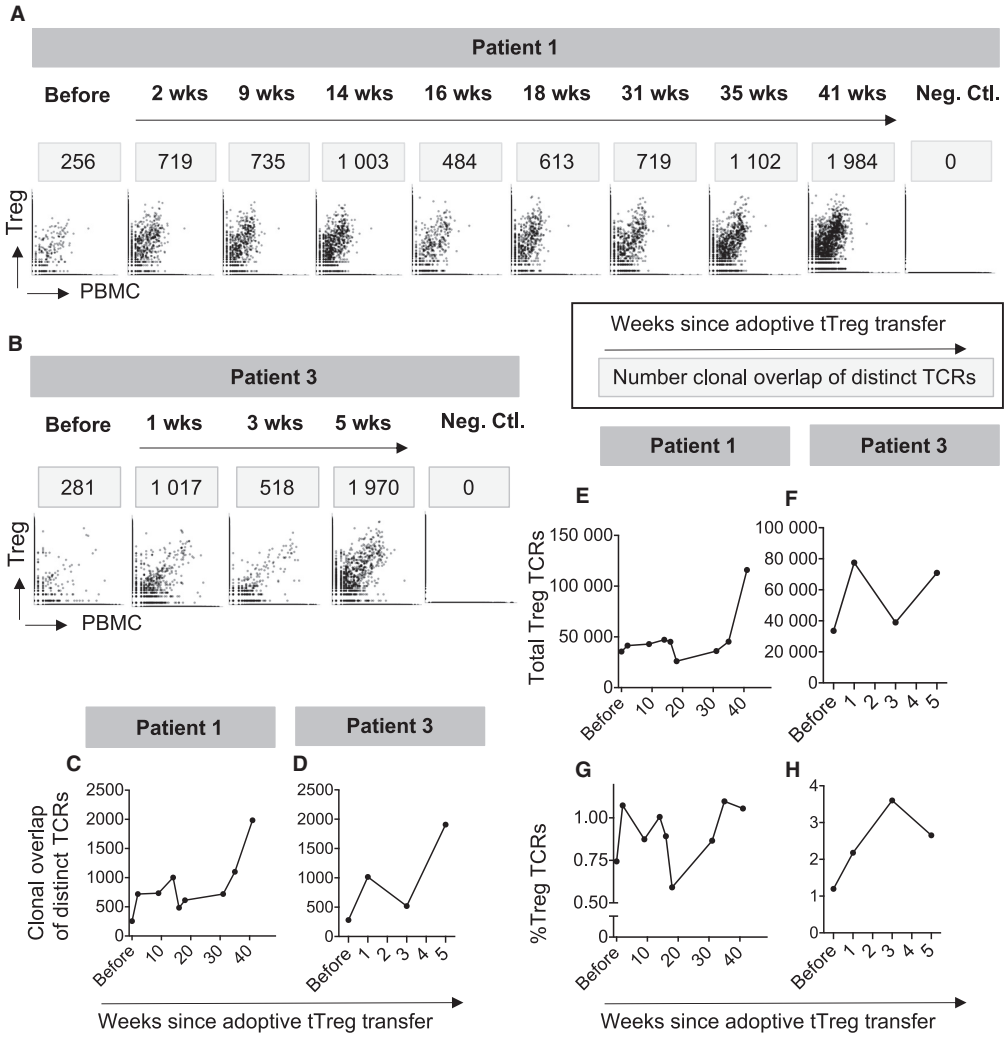

Figure 5. Adoptively transferred Tregs expand *in vivo* and show long-term survival

(A–H) A clonotype analysis was performed from an aliquot of the adoptively transferred Treg product. The clonotype analysis was further conducted in peripherally collected PBMC before Treg therapy and serially thereafter. Overlapping clonotypes were assessed and allowed cellular tracking of the Treg product in the peripheral patient blood. (A and B) TCR clones detected in the Treg product are depicted on the y axis, TCR clones detected in the peripheral PBMC preparations are depicted on the x axes. Overlapping clones, present in the Treg product and the peripheral blood, are depicted between both axes. As a control, Treg product clones of patient 1 were analyzed in PBMC preparations of patient 3 and vice versa. The number of distinct TCRs present in both the Treg product and PBMCs are indicated above each panel. (C and D) Number of distinct TCRs present in Treg products and patient samples. (E and F) Depicted are total numbers of Treg product TCRs detected in PBMC samples. (G and H) Depicted are frequencies of different Treg TCRs relative to all TCRs detected in each PBMC sample.

average of 4.5-fold expansion with 78% purity. The patient with cGvHD described by Trzonkowski et al.[19] received Tregs that were produced from a buffy coat of the original family stem cell donor. $CD4^+$ cells were isolated by negative immunomagnetic sorting (StemCell Technologies) and subsequently sorted using the FACSARIA sorter (BD Biosciences) for $CD3^+CD4^+CD25^{high}CD127^-$doublet$^-$lineage$^-$dead$^-$. Tregs were cultured for 2 weeks to reach sufficient cell numbers at a purity for FoxP3 of 90%.[19] In both studies, co-administered GvHD immunosuppressive treatments were also tapered before Treg infusion.[19,23] In the cohort published by Theil et al., patients received between 0.97 and 4.45 $\times$ 10^6 Tregs/kg body weight; one patient received a second Treg dose, and three of

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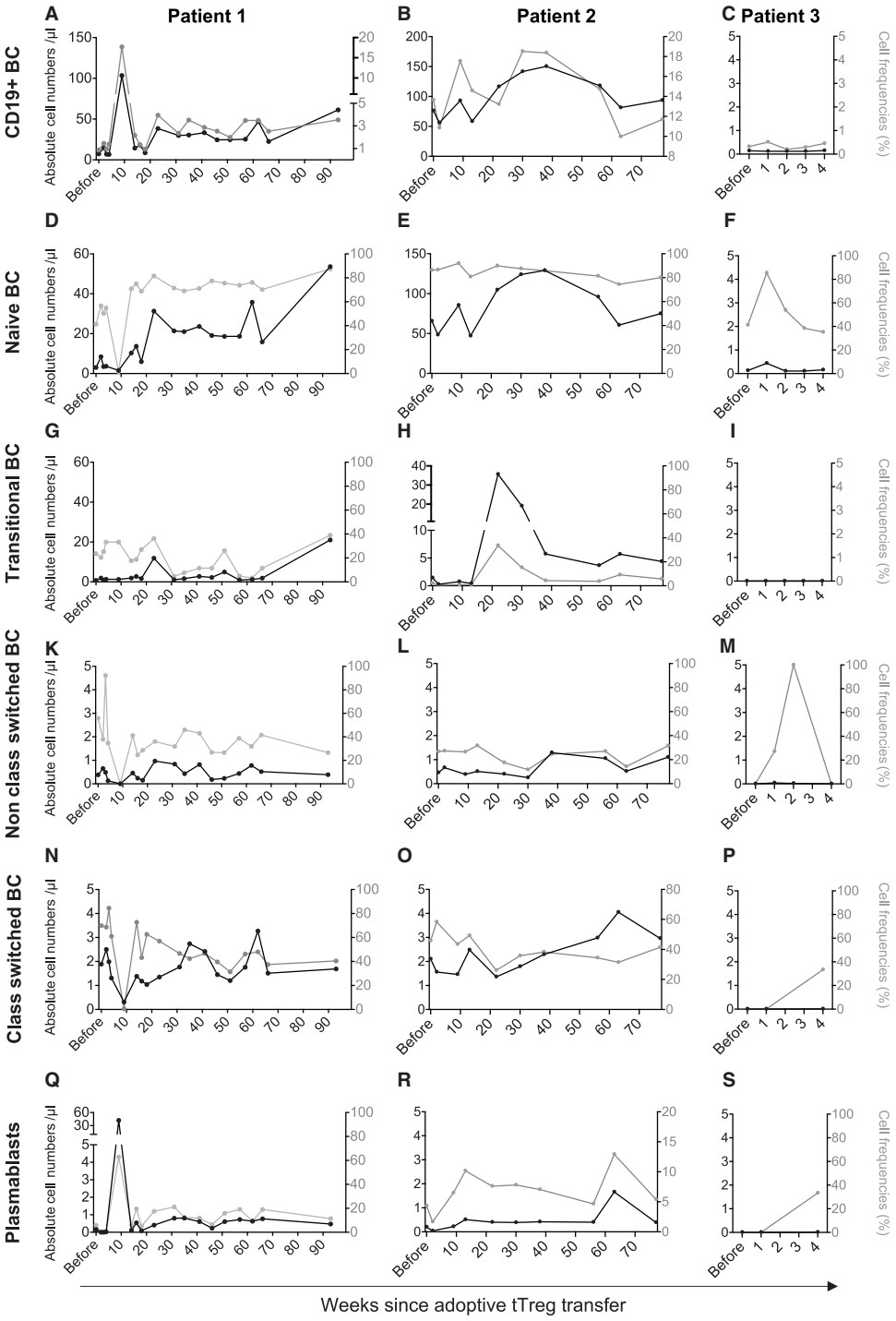

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five patients received concomitant low-dose IL-2 applications, which complicates the differentiation between Treg transfusion related and direct IL-2 related impacts on the clinical course.[23] The patient treated by Trzonkowski et al.[19] received 1×10^5 Treg/kg body weight. In line with our data, both groups reported partial response or stable disease within the limited follow-up time of 5–23 weeks after Treg infusion. Similar to one patient with cGvHD described by Trzonkowski et al.,[19] patient 2's symptoms of pulmonary fibrosis unexpectedly but substantially improved over time. The underlying pathophysiology for the diffuse mucosal hemorrhage in patient 2 remains unexplained as there were no direct or indirect indicators of GvHD or infections found.

From clinical approaches in humans, we know that it is very difficult to describe correlations between the dose and the effect of adoptively transferred Tregs in the transplant setting. Multiple clinical trials for various clinical indications are currently ongoing and registered at www.clinicaltrials.gov. Our group conducted a phase I/IIa study (NCT02371434 (ONEnTreg13) and EudraCT:2011-004,301-24 (ONErgt11) treating living donor kidney transplant patients with escalating doses of Tregs (3 + 3 design, n = 11, 0.5, 1.0, or 2.5–3.0×10^6 cells/kg body weight) while tapering standard immunosuppressive comedication. This study was conducted as part of the ONE study consortium, which aimed to investigate the feasibility and safety of different cell therapeutic approaches. Study patients were compared with a reference group, in which patients received standard of care. Although it seems counterintuitive that higher Treg doses are not superior to lower Treg doses, we did not find any dose-response association for any of the parameters investigated.[38] These observations were in line with a parallel study conducted in two centers in the UK.[39] Harden et al.[39] found that a dose-dependent effect can be observed on an immunological but not on a clinical level, even if Tregs were administered at a dose as high as 10×10^6 per kg. Reports on Treg therapy in human patients suffering from GvHD are heterogeneous with respect to cell dose, Treg source, and patient characteristics. The administered Treg doses varied between 1×10^5 and 5×10^6, but no clear dose-response correlation can be derived from these data.[19–23] Last but not least, one should bear in mind that the production process of Tregs varies from center to center and may lead to substantially different product characteristics; comparability is, therefore, limited and should be treated with caution. In summary, we chose the dose of 3.0×10^6 cells/kg body weight based on our clinical experience from the ONE study, in which we learnt that 3.0×10^6 cells/kg are safe, at least in adult patients, and after intensive discussions in a multidisciplinary team. The life-threatening condition of all patients made the treatment approach necessary, and the dose was chosen that was most likely to be safe and, more important, hopefully effective.

Concerns that the immunosuppressive properties of polyclonal Treg infusion could increase susceptibility to infections may have hampered the progressive development of broad Treg applications. In autoimmune and solid organ transplant recipients, however, there is no evidence of an increased risk, and immune monitoring has not revealed signs of over-immunosuppression.[25] In 23 HSCT patients treated with Tregs as a GvHD prophylactic approach, Brunstein et al.[40] showed a higher cumulative density (accounting for multiple infections in one individual patient per 1,000 patient-days) of opportunistic viral infections caused by human herpesvirus 6, CMV and parainfluenza compared with historical controls. However, the cumulative incidence remained comparable. We closely monitored all patients for viral infections or reactivation and found viral loads continuously at or below the lowest limit of detection, indicating sufficient control. Only patient 2 showed a short-term, low-level Epstein-Barr virus reactivation, which was promptly controlled by CSA tapering.

Di Ianni et al.[20] reported in 2011 on 4 of 28 patients who underwent HLA-haploidentical HSCT and were prophylactically treated with Tregs but died from acute exacerbation of an pre-existing invasive aspergillosis. After Treg therapy, the authors, however, observed an improved immune reconstitution of protective T cells toward different pathogens, including opportunistic infections, most likely mediated by early T cell reconstitution after.[20] Accordingly, if the pre-transplant conditioning regimen caused the loss of infection control and how Treg infusion may have additionally contributed to the exacerbation remains unresolved. Our results, in addition to previously published data, suggest that the overall low incidence of opportunistic infections, including aspergillosis, after Treg therapy in immunocompromised patients demonstrates a favorable safety profile. However, in the case of preexisting, active invasive fungal disease, Treg therapy might not be beneficial.

GvHD and the associated therapeutic interventions are known to substantially hamper T cell[41–46] and B cell[47–49] engraftment and maturation. Accordingly, patients with cGvHD suffer from combined immunodeficiency. In addition, the cellular phenotype in cGvHD is not only characterized by low total lymphocyte counts, particularly of naive lymphocytes, but also by an imbalance between regulatory and effector T cells with persistently decreased frequencies of Tregs, and a shift toward a pro-inflammatory environment.[42,50] A positive impact of Treg therapy on bone marrow functions, but limited to erythrocyte formation only, was first reported by Trzonkowski

Figure 6. Treg transfer induces engraftment of circulating B cells in early maturation states

(A–C) Peripherally circulating B cells were identified in freshly collected blood as singlets, $CD45^+$ leukocytes, lymphocytes, and the expression of CD19. (D–F) Naive B cells were detected as $CD27^-IgD^+$ within $CD19^+$ cells. (G–I) Cells expressing $CD38^{high}CD24^{high}$ of $CD19^+IgM^+CD27^-$ B cells were defined as transitional B cells. (K–P) $CD19^+IgD^-IgM^+$ cells were further analyzed for the expression of CD27 and CD38 to define non-class-switched B cells as $CD27^+CD38^{high}$ and class-switched B cells as $CD27^+CD38^{low}$. (Q–S) Plasmablasts were identified as $CD19^+IgD^-IgM^-$ and $CD27^{int}CD38^{high}$ cells. All results are depicted as black solid lines in absolute cell numbers per microliter and assigned to the left y axis. Relative cell frequencies of the parental gate are depicted as gray lines in percent and assigned to the right y axis. The x axis shows time from Treg transfer. A more detailed description of the gating protocol was previously described by Streitz et al.[24]

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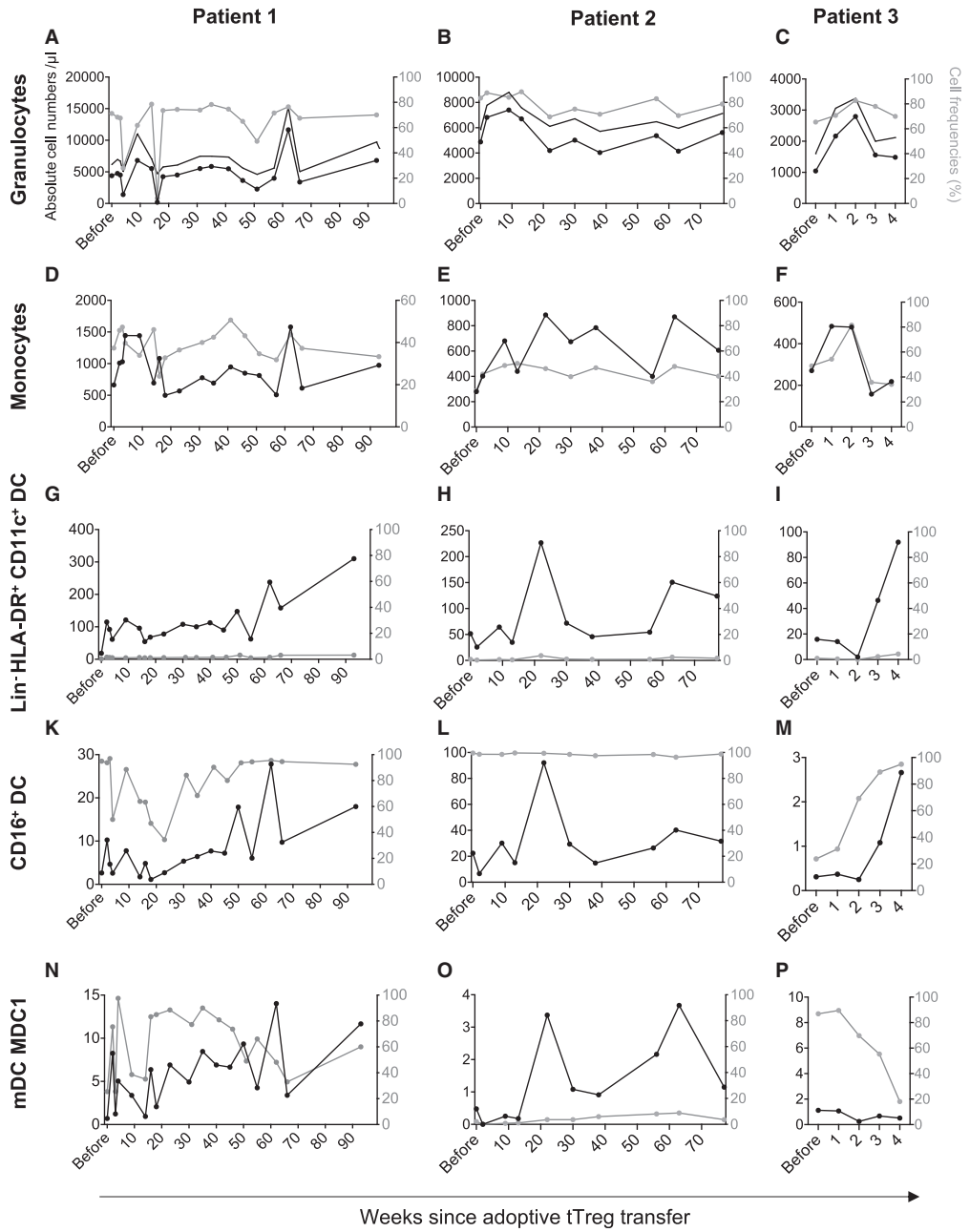

Figure 7. Engraftment after Treg therapy is not restricted to the lymphocyte compartment but similarly observed in DC

Possible alterations within the innate immune system were monitored using the DURAClone IM Phenotyping Basic (A–F) and the DURAClone IM Dendritic Cells (G–P) kits using peripherally collected blood. (A–C) Granulocytes were defined as a distinct population in the side scatter from singlet $CD45^+$ leukocytes. (D–F) Within the non-granulocytic population, monocytes were defined as $CD14^+$ cells. (G–P) $Lin^-HLA^-DR^+CD11c^+$ myeloid DC (mDC) were gated within the HLA-DR^+ DC population of $CD45^+$ leukocytes. $CD16^+$ monocytic DC were identified as $CD16^+Clec9^-$, while mDC1 were defined as $CD1c^+CD16^-$. All results are depicted as black solid lines in absolute cell numbers per microliter and assigned to the left y axis. Relative cell frequencies of the parental gate are depicted as gray lines in percent and assigned to the right y axis. The x axis shows time from Treg transfer. (A–C) In addition to absolute and relative granulocyte numbers, total leukocyte numbers are depicted in black without symbols.

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et al.,[19] showing stabilized hemoglobin levels after Treg infusion in cGvHD. In our patients, we found a remarkable and lasting increase in circulating Tregs after cell infusion. The expansion of naive T and B cells is of particular importance and, thus, a good marker for the reconstitution of primary immune organs and related immunocompetence after transplantation. The TCR clonotypic analyses during the follow-up confirmed the long-lasting expansion of Tregs in the patients. We observed a very low frequency of clonal overlap between the patients' blood and the products before Treg therapy, despite long-lasting contact with the recipients' alloantigen and active GvHD releasing inflammatory triggers. Clearly, intrinsic HSCT-derived donor Tregs were not sufficiently activated in the host. Further, an increased expansion of donor Treg clonotypes with shared clones with the Treg product is paralleled by the increase of absolute Treg counts and relative Treg frequencies, suggesting the *in vivo* expansion of the Treg preactivated during manufacturing process. Additionally, the biphasic increase in circulating Treg numbers most likely reflected (i) adoptively transferred memory Tregs early after cell infusion, while (ii) at later stages the increase in circulating Tregs seems to be an additive effect of both (a) expansion of adoptively transferred Tregs and (b) the detection of naive Tregs, indicating intrinsic engraftment and expansion of this compartment. Indeed, naive Tregs and the increase of conventional naive $CD4^+$ T cell, $CD8^+$ T cell, and B cell subsets were detected after adoptive Treg transfer, even in the absence of IL-2 administration.

This observation is in contrast with finding from Theil et al.,[23] who demonstrated slightly increased frequencies of naive Tregs only in patients who received combined IL-2/Treg therapy, suggesting that naive Treg induction might be IL-2 mediated.[24] Another interesting observation was the increased number of DC after Treg infusion, underlining the improved competence of the adaptive immune system. Granulocytic and monocytic functions were not hampered after Treg therapy. These findings are of particular importance, since none of the previously investigated immunosuppressive drugs have been demonstrated to improve engraftment of naive B and T cells or DC to this extent, indicating true *de novo* synthesis from the bone marrow.

When interpreting these results, the following limiting factors should be considered: (i) we report on the individual case treatments of just three patients, and (ii) the patients suffered from cGvHD for a mean time of more than 2 years and received first-, second-, and third-line immunosuppressive therapies without satisfactory efficacy before Treg infusion, thus there may already have been irreversible tissue destruction in affected organs. As there is no standard for second and third-line or therapy-refractory GvHD therapy, our patients had received a heterogeneous treatment regimen before adoptive Treg therapy. Thus, the inter-individual comparability between the three treated patients is limited. As all patients with this severity of cGvHD have a very poor prognosis with a high short-term mortality risk, historical controls do not exist. For ethical reasons, we did not routinely perform comparable immunomonitoring in patients who did not receive Treg therapy on an individual treatment basis. However, since time since transplantation exceeded 600 days we consider the likelihood of improvement by spontaneous events or owing to alternative third-line treatment options very unlikely. Therefore, we consider the clinical and immunological of course each patient themselves as their matched historical control.

Based on this experience, a clinical trial is currently being planned with a standardized clinical protocol for the administration of adoptive Treg therapy in patients with cGvHD. Whether complete remission of cGvHD or, alternatively, control of chronically established GvHD and prevention of further progress is a realistic goal is debatable. Adoptive Treg therapy, will have to be compared to alternative immunomodulatory therapy approaches, e.g., the administration of the JAK1/2 inhibitor ruxolitinib[51–53] and adoptive transfer of mesenchymal stem cells (MSC),[54] for which promising results in the prevention and treatment of GvHD recently became available. Although beneficial effects were demonstrated for ruxolitinib, considerable side effects seem to limit the long-term administration in both adults and children.[51,55] In contrast, despite the clinical efficacy reported for MSCs by some authors, data are contradictory and based on a recent Cochrane analysis further data are necessary to prove MSCs to be effective in preventing and/or treating GvHD.[54] Based on our experience, we believe that, in addition to its good tolerability, the adoptive transfer of Tregs may be effective even in long-lasting cGVHD, but will most likely improve the overall outcome more effectively at a stage when the patient is (i) not yet critically ill, (ii) has suffered from less organ toxicity, and (iii) is at a lower risk for acute infection exacerbation.

MATERIALS AND METHODS

Study design

Three children with therapy-refractory or steroid-dependent GvHD were treated by adoptive transfer of *ex vivo* expanded Tregs on an individual treatment basis. All patients and their parents provided written informed consent after receiving detailed oral and written information. Before adoptive Treg therapy, maintenance immunosuppression, initiated before making the decision to undergo Treg therapy, was tapered to CSA (target trough level 80–100 μg/L) and the lowest clinically possible dose of prednisolone before cell therapy. A single dose of 3×10^6 HSCT donor-derived Treg/kg was administered by intravenous infusion. Single dose antipyretic and antihistamine was given 30 min before Treg application. Clinical and laboratory follow-up was performed using standardized clinical evaluation sheets as well as a laboratory work-up, including biochemistry, CSA trough levels, and hematologic, immunologic, and microbiologic parameters. Clinical follow-up was performed in accordance with the recommendations summarized by the National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: IV. The 2014 Response Criteria Working Group Report.[56]

To ensure a high validity of our data we used a combination of consistently having the same physician visiting all patients at every study visit and a systematic, validated documentation system. To quantify

Table 2. Clinical follow-up score

			Significantly	Moderately	Slightly		Slightly	Moderately	Significantly
	None	CR	Improved			Equal	Worse		
General			+3	+2	+1	0	−1	−2	−3
Skin			+3	+2	+1	0	−1	−2	−3
Oral mucosa			+3	+2	+1	0	−1	−2	−3
GI tract			+3	+2	+1	0	−1	−2	−3
Eyes SCORE (sum)			+3	+2	+1	0	−1	−2	−3

To monitor and quantify the clinical course of GvHD symptoms, the general condition as well as the status of the skin, mucosa, eyes and the GI-Tract were documented. Symptoms were scored, as compared to the previous visit, by +1 to +3 points for improvement of symptoms, while worsening of disease activity was scored by −1 to −3. Scores were cumulatively added up throughout the entire follow-up period. CR, complete remission; GI, gastrointestinal.

clinical changes the clinical follow-up score depicted in Table 2 was used to evaluate the patient's current status as compared the previous visit (Table 2).

Treg product manufacturing

The clinical grade Treg products were manufactured at our GMP facility, Berlin Center for Advanced Therapies, as recently described in detail.[38] The manufacturing procedure has been authorized by the regional and national regulatory authorities (LAGeSo-Berlin and Paul-Ehrlich Institute, respectively). Briefly, 50 mL peripheral blood was collected from the original hematopoietic stem cell donors. Tregs were isolated by depletion of $CD8^+$ and enrichment of $CD25^+$ cells using CliniMACs technology (Miltenyi Biotech). Subsequently, Tregs were stimulated by Treg-expansion beads (Miltenyi Biotech) and cultured for 23 days in the presence of IL-2 and a mammalian target of rapamycin inhibitor. The robust manufacturing process demonstrated high expansion rates, high purity, and high viability (Table 2). All products met our release criteria and complied with the safety-relevant parameters (mycoplasmas, endotoxins, sterility). The end-product was resuspended in 50 mL 0.9% saline and administered within 8 h after filling at a rate of 2 mL/min.

Immune monitoring

Safety and hematologic data were monitored at each clinical follow-up visit by routine measures. On a voluntary basis, additional clinical and laboratory parameters were assessed to monitor safety and pharmacokinetics/pharmacodynamics in more detail. Specifically, peripherally collected blood counts and standardized multi-color flow cytometry were used to assess more than 60 immune cell subsets as described by Streitz et al.[57] Briefly, freshly collected blood (EDTA Vacutainers, BD) was stained using the DURAClone IM Phenotyping Basic, IM T cell Subsets IM Treg, IM TCRs, IM B Cell, and IM DC kits according to the manufacturer's protocol (Beckman Coulters). All samples were analyzed on CE-labeled 10-color Navios flow cytometers (Beckman Coulter). Exemplary plots of all gating strategies of subsets described in this manuscript are depicted in Figures S2–S7. The T cell repertoire was assessed in all Treg products by using next-generation sequencing, as well as in peripheral whole blood samples collected during follow-up of Treg-treated patients as described elsewhere.[58]

DATA AND MATERIALS AVAILABILITY

Most of the data are presented in the text, figures, and legends. Any additional data will be made available upon request by the corresponding author (Landwehr-kenzel.sybille@mh-hannover.de).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2022.02.025.

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DECLARATION OF INTERESTS

Authors declare that they have no competing interests.

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3. Diskussion

In den letzten Jahrzehnten konnten in der Transplantationsmedizin erhebliche Erfolge erzielt werden und sowohl die hämatopoetische Stammzelltransplantation als auch die solide Organtransplantation etablierte sich für viele Patienten zur einzigen kurativen Therapieoption. Auch wenn das kurzfristige Überleben von Transplantationspatienten durch den Einsatz moderner immunsuppressiver bzw. immunmodulatorischer Medikamente substantiell verbessert werden konnte, können wir chronische Abstoßungsprozesse bis heute nur unzureichend beeinflussen und die langfristige Morbidität und Mortalität bleiben dadurch auf nicht zufriedenstellendem Niveau zurück [30–35]. Die Entwicklung neuartiger Therapien eröffnete in den letzten beiden Jahrzehnten neue Perspektiven. Mit dem schnell wachsenden Verständnis über die immunologischen Prozesse und den technischen Möglichkeiten wurden Tregs als neue Generation einer immunmodulatorischen Therapieoption identifiziert. Insbesondere im Hinblick auf die Transplantationsmedizin ist der Einsatz von regulatorisch wirksamen Zellen ein vielversprechender Ansatz. Die in dieser Arbeit vorgestellten Untersuchungen verfolgen das gemeinsame Ziel, *ex vivo* expandierte Tregs für die klinische Anwendung bei Transplantationspatienten nutzbar zu machen.

3.1. Entwicklung regulatorischer T-Zell-Produkte

Von der Identifikation von Tregs 1995 durch Sakaguchi und Kollegen [43] bis zur Publikation der ersten Fallberichte über den therapeutischen Einsatz von Tregs [120–123] sind etwa 15 Jahre vergangen. Heute, weitere 10 Jahre später, stecken wir trotz zahlreicher Fortschritte noch immer mitten im Translationsprozess. Stand (04/2022) hat keine einzige klinische Studie die Phase 3 erreicht (Quelle www.clinicaltrials.gov), obwohl publizierte Daten Treg-Produkten sowohl ein gutes Sicherheitsprofil als auch eine gute Wirksamkeit im Kontext von Organtransplantationen [118,124–126] und bei Autoimmunerkrankungen [127–129] attestieren. Die Gründe für den schleppenden Translationsprozess sind vielfältig. Einerseits führen die biologischen Eigenschaften von Tregs dazu, dass sich die Etablierung eines stabilen und reproduzierbaren Herstellungsprozesses herausfordernd gestaltet. Auf der anderen Seite erfordert die Neuartigkeit dieses Therapieansatzes von Wissenschaftlern, Behörden, Anwendern und schließlich auch Patienten ein verantwortungsvolles Abwägen zwischen

sicherheitsrelevanten Aspekten und dem drängenden medizinischen Bedarf insbesondere für Patienten, für die sich etablierte Therapieverfahren bereits als erfolglos dargestellt haben. Neben der Zulassung eines stabilen Herstellungsprozesses sind Dosisfindungsprozesse und die Evaluation einer geeigneten Begleitmedikation Gegenstand der Arbeiten unserer Gruppe.

Isolation und Expansion von tTregs

Eine der größten Hürden im Herstellungsprozess eines Treg-Produktes stellt **die Isolation** dar. Dies liegt einerseits daran, dass tTregs nur einen sehr geringen prozentualen Anteil der T-Zellen im peripheren Blut stellen; in gesunden Spendern liegt dieser innerhalb der CD4+ T-Zellen bei 5-10 %, und bei 0,1-0,7 % der Leukozyten. Gleichzeitig sind die unter GMP zu Verfügung stehenden Isolationstechniken limitiert. Treg-Produkte unterliegen wie alle sog. Arzneimittel für neuartige Therapien (Advanced Therapy Medicinal Products, ATMPs) auf Grund der Komplexität in der Herstellung und Anwendung besonderen Regularien, die in Deutschland durch das Paul-Ehrlich-Institut und auf europäischer Ebene durch die European Medical Association (EMA) definiert und umgesetzt werden. Technisch stehen die Bead-basierte Magnetisolation (MACS-Technologie) oder die im Translationsprozess befindliche fluoreszenz-aktivierte Zell-Sortierung (FACS) zur Verfügung. Die erste Herstellung eines klinischen Treg-Produktes wurde 2006 durch Hoffmann und Kollegen beschrieben, denen es gelang mittel MACS-Isolation unter GMP-Bedingungen eine ausreichende Anzahl an Tregs aus einem Leukapheresat zu erzielen [130]. Damit war der Grundstein für die weitere Entwicklung von Treg-Produkten für die klinische Anwendung gelegt [118,131,132]. Durch eine äußerst sorgfältige Materialaufarbeitung und die kontinuierliche Verbesserung des nachfolgenden Produktionsprozesses, entwickelten wir ein Protokoll, das ohne Leukapherese auskommt und sowohl bei Gesunden als auch bei Patienten in einer ausreichenden Zellzahl für klinische Anwendungen resultiert [114–116,118,119]. Alternative Protokolle ermöglichen die Isolation von Tregs aus Nabelschnurblut [121,133–136]. Obwohl der Einsatz von Tregs, welche aus Nabelschnurblut generiert wurden, einige Vorteile mit sich bringen mag (Availability, Diversity, Storage), haben sich im Hinblick auf klinische Studien, Protokolle die Tregs von erwachsenen Spendern sowohl im autologen wie auch im allogenen Setting verwenden durchgesetzt. Die in Deutschland etablierte und zugelassene MACS-Technologie zur Anreicherung von Tregs wird in einem geschlossenen System durchgeführt und erlaubt durch eine Aneinanderreihung mehrerer Depletionsschritte in Kombination mit einer Positivselektion eine ausreichende Reinheit in der

Zielpopulation. Zur Treg-Isolation werden hierbei im ersten Schritt $CD8^+$ T-Zellen depletiert und anschließend CD25+ Zellen angereichert, so dass man ein $CD4^+CD25^+$ Ausgangsprodukt erhält. Der entscheidende Vorteil dieser Technologie ist, dass während des gesamten Isolationsprozess ein geschlossenes System die Anfälligkeit für Kontaminationen minimiert. Im Gegensatz dazu erlaubt die FACS-basiert Zell-Isolation beispielsweise durch die Definition von $CD4^+CD25^{high}CD127^{low}$ Zellen zwar eine noch bessere Definition der Zielpopulation, befindet sich unter GMP-Bedingungen allerdings noch im Entwicklungs- und Zulassungsprozess. Auf Grund der aktuell geltenden EU-Richtlinien (EU Directive 2003/94/EC incl. Annex 2) sind die Hürden für eine regulatorische Zulassung und letztendlich auch die Umsetzung in Routineprozessen hoch. Auch wenn eine durchflusszytometrische Isolation uns in absehbarer Zeit zur Verfügung stehen wird, müssen wir bis auf weiteres die Limitation in der Auswahl der Marker und die damit einhergehend begrenzte Reinheit der Treg-Produkte direkt nach Isolation in Kauf nehmen. Da wir die Reinheit des Treg-Produktes im Rahmen des nachfolgenden Expansionsprozess jedoch optimieren konnten [114,116,118], stellt der mäßige Kontaminationsgrad durch non-Tregs am Ende des Isolationsprozesses keine Einschränkung für die abschließende Produktqualität dar.

Auf Grund der großen Diversität des T-Zell-Kompartiments ist die Applikation relativ großer Zellzahlen notwendig, um eine ausreichende Anzahl (allo)antigenspezifischer T-Zellen zu erreichen. Dem gegenüber steht die verhältnismäßig niedrige Anzahl an Tregs im peripheren Blut. Um eine ausreichende Anzahl an Tregs zu erhalten, besteht die Möglichkeit, Tregs GMP-kompatibel ***ex vivo* zu expandieren**. Hierzu kann eine Stimulation der Tregs mit Bead-basierter, CD3/CD28-vermittelter Stimulation zum Einsatz kommen. Wir entwickelten ein Expansionsprotokoll, das nach repetitiver Stimulation in IL-2 angereichertem Medium zu einer 100- bis 10.000-fachen Expansion und damit zu ausreichenden Zellzahlen für die therapeutische Anwendung aus 50 ml peripher entnommenem Blut erzielte [114,118]. Ein weiterer Vorteil der Treg-Expansion ist, dass durch eine Optimierung der Kulturbedingungen mit einem Selektionsvorteil für Tregs das Wachstum und Überleben kontaminierender Effektor-Zellen unterdrückt werden kann. Hierbei erwies sich die Zugabe des mTOR-Inhibitors Rapamycin zur Zellkultur bei uns [114,115,115,116,118] und anderen [137–148] als effektiv und führte zusätzlich zu einer verbesserten Expansion, Stabilität und Funktion von Tregs [139,149,150]. Neben der TZR-abhängigen aber antigen-unspezifischen Stimulation mittels CD3/CD28-Beads haben wir [114]

und andere [151–160]zwischenzeitlich das Ziel der Herstellung eines allospezifischen Treg-Produktes verfolgt. Dabei entwickelten wir ein Protokoll, das es uns ermöglicht, über eine allospezifische B-Zell-vermittelte Treg-Stimulation ein allospezifisches Treg-Produkt zu generieren [114]. Mittels TZR-Sequenzierung untersuchten wir die Klonotypen von Treg-Produkten unterschiedlicher Expansionsprotokolle und konnten nachweisen, dass eine polyklonale Expansion mittels CD3/CD28-Beads zu polyklonalen Treg-Produkten führt, während eine allospezifische Stimulation zur Expansion einzelner Treg-Klone führt und eine allospezifische Oligoklonalität im Treg-Produkt induziert [114]. Ähnliche Ansätze zur allospezifischen Treg-Expansion wurden von anderen Gruppen beschrieben [154,158,160–162]. Funktionell scheinen allogenspezifische Tregs Abstoßungsprozesse tatsächlich besser zu verhindern als polyklonal expandierte Zellprodukte [114,157,159,163,164]. Eine alloantigenspezifische Expansion von Tregs ist jedoch mit dem Risiko behaftet, dass kontaminierende pro-inflammatorisch wirkende T-Zellen expandieren [149,165,166] oder das Tregs ihren regulatorischen Phänotyp verlieren [75,167–169], dass eine sehr starke TZR-abhängige Stimulation im Rahmen der *in vitro*-Expansion zu einer Überstimulation einzelner Klone führt, die sich epigenetisch verändern bzw. maligne entarten können [170] oder dass die Überstimulation – wie für Effektor-T-Zellen bereits beschrieben – zu einer funktionellen Erschöpfung der Tregs führt [171–173]. In der Summe fällt die Risiko-Nutzen-Abwägung daher unter diesen Bedingungen zugunsten von polyklonalen Treg-Produkten aus. Mit den neuerdings zur Verfügung stehenden technischen Möglichkeiten, wird sich die Nutzen-Risiko-Bewertung in Zukunft jedoch zu Gunsten von allogenspezifischen Treg-Produkten ändern.

Die **Freigabe eines ATMP** erfordert – zu Recht – ein hohes Maß an Qualität hinsichtlich Zuverlässigkeit und Reproduzierbarkeit, die sicherheitsrelevante Merkmale des Produktes zum Zeitpunkt der Produktfreigabe abbilden. Da alle uns zu Verfügung stehenden Tests, durch technische Gegebenheiten sowie Treg-spezifische biologische Merkmale limitiert sind und eine hohe Variabilität zwischen Herstellungsstandorten besteht, ist eine Kombination von Freigabeuntersuchungen und viel Erfahrung in der Interpretation der erlangen Ergebnissen essentiell [174,175]. Insgesamt hat sich die Kombination von rein phänotypischen Markern und funktionellen Assays als sinnvoll erwiesen. Eine gleichzeitige Analyse der bekannten Treg-Marker ($CD3^+CD4^+CD25^{high}FoxP3^+$ oder $CD3^+CD4^+CD25^{high}CD127^{low}$) sowie eine Darstellung von möglicherweise kontaminieren Zellen haben wir für unsere Herstellungsprotokolle etabliert

und validiert. Ergänzend können funktionelle Nachweise von pro-inflammatorischen Zytokinen (IFNγ, Interleukinen) nach starker T-Zell-unabhängiger Stimulation Hinweise auf eine Kontamination mit non-Tregs liefern und wurden von uns begleitend validiert [114–118]. Als nicht praktikabel für die GMP-gerechte funktionelle Analyse hat sich die Durchführung von Suppressions-Assays erwiesen [176–180], insbesondere da sie durch zahlreiche technische Schwierigkeiten belastet sind und die funktionelle Kapazität von Tregs *in vivo* nur unzureichend abbilden [178,181].

Die Qualität eines Treg-Produktes unterliegt auch bei Anwendung eines **standardisierten Herstellungsprotokolls** hohen interindividuellen Schwankungen, die in der Natur des spenderabhängigen Ausgangsmaterials liegen. Spenderspezifische Charakteristika, die die Produktcharakteristik beeinflussen, können unter anderem das Alter des Spenders, das den natürlichen Reifungsgrad von T-Zellen erheblich beeinflusst, oder chronische Grunderkrankungen sein. Daher war es für uns von größter Wichtigkeit, Treg-Produkte altersabhängig zu analysieren und Treg-Produkte von Patienten, die möglicherweise für eine autologe Treg-Therapie in Frage kommen zu charakterisieren. Die Tatsache, dass mit steigendem Alter eine zunehmende Reifung von T-Zellen und damit eine Verschiebung von naiven Tregs hin zu Memory-Tregs mit $Treg_{CM}$- oder $Treg_{EM}$- und $Treg_{EMRA}$-Phänotyp beschrieben ist, und die Beobachtung, dass naive Tregs im Vergleich zu Memory-Tregs ein höheres suppressives Potential aufweisen könnten, warf die Frage auf, ob das Spenderalter bei der Planung einer Treg-Therapie Berücksichtigung finden sollte [182–184]. Daher untersuchten wir zunächst, wie hoch der Anteil an Memory-Tregs innerhalb einer Treg-Population, die nach o.g. Protokollen isoliert wurde, ist. Im nächsten Schritt analysierten wir, wie gut aus peripherem Blut isolierte (Memory-) Tregs expandiert werden können. Hierbei zeigte sich in Vollblutanalysen, dass der Anteil an $Treg_{CM}$ im peripheren Blut mit dem Alter insgesamt deutlich zunimmt und mit zunehmendem Alter innerhalb des Treg-Kompartiments an Dominanz auch gegenüber $Treg_{EM}$ gewinnt [115]. Nach Isolation fanden wir innerhalb der $Treg_{CM}$-Population ein TSDR-Demethylierungsanteil von fast 100%, was auf eine hohe Reinheit an tTregs hinweist. Next-Generation-Sequencing-Analysen der einzelnen Treg-Fraktionen zeigten, dass $Treg_{CM}$ eine große klonale Überlappung mit $Treg_N$ aufweisen, während die klonale Gemeinsamkeiten mit Effektor-T-Zellen deutlich geringer sind [115]. Auf Grund der Tatsache, dass klonale Gemeinsamkeiten innerhalb zellulärer Kompartimente über

verschiedene Reifungsstufen erhalten bleiben, unterstreichen diese Ergebnisse, dass die $Treg_{CM}$ aus dem naiven Treg-Pool stammen und – wenn überhaupt – nur zu einem sehr geringen Anteil als iTregs aus dem Effektor-T-Zell-Kompartiment entstanden sind [98,115,185,186]. Übereinstimmend mit den Daten aus präklinischen Tierstudien zeigte sich in unseren Untersuchungen, dass aktivierte $Treg_{CM}$ eine höhere suppressive Kapazität aufweisen und damit für die klinische Anwendung vielversprechende Charakteristika innehaben [187–189]. Nach Expansion zeigte sich, dass der Anteil $Treg_N$ über den Expansionszeitraum zugunsten des $Treg_{CM}$ abnimmt, während $Treg_{EM}$ *ex vivo* nicht expandierten. Übereinstimmend dazu wurde berichtet, dass die Expression von CD62L für die klinische Besserung der akuten GvHD im Mausmodell essentiell ist [190]. Unklar war lange Zeit zudem, ob und inwiefern diese Daten auf Patienten mit vorbestehender Grunderkrankung oder Immunsuppression übertragbar sind. Im Hinblick auf unseren Schwerpunkt, die Transplantationsimmunologie und unser Ziel, neben Patienten nach hämatopoetischer Stammzelltransplantation auch Patienten nach solider Organtransplantation zu behandeln, konzentrierten wir uns neben der Analyse von gesunden Spendern auf Patienten, die auf Grund einer terminalen Niereninsuffizienz eine chronische Hämodialyse erhielten. Hierbei zeigte sich, dass weder die Niereninsuffizienz *per se* noch die Hämodialyse zu Einschränkungen in der Herstellung eines qualitativ hochwertigen Treg-Produktes führt. Daher sind keine Modifikationen im Herstellungsprozess notwendig, so dass diese Patientengruppe im Hinblick auf eine bevorstehende Nierentransplantation für eine autologe prophylaktische oder präemptive Treg-Therapie geeignet erscheint [116].

3.2. Translation adoptiver Treg-Therapien in die klinische Anwendung

Daten aus präklinischen Studien deuteten bereits frühzeitig darauf hin, dass eine Monotherapie mit regulatorischen T-Zellen zur Kontrolle von Abstoßungsreaktionen nicht ausreichend bzw. ein Transfer von Zellzahlen notwendig ist, der in präklinischen Tierexperimenten zwar etabliert, bei Translation in die klinische Situation jedoch nicht erreichbar sein wird. Darüber hinaus haben wir bislang nur unzureichend verstanden, welchen Einfluss zusätzliche Faktoren wie die immunsuppressive Begleitmedikation bzw. eine chronische, allogene Stimulation des Immunsystems auf das Verhalten von adoptiv transferierten Treg haben [191–194]; die publizierte Datenlage ist teilweise widersprüchlich [195–198]. Während mTOR-Inhibitoren (z. B. Rapamycin, Everolimus) [199], Histon-Dacetylase-Inhibitoren

[200,201], niedrig dosiertem IL-2 [202–209] sowie Anti-Thymozyten-Globulin [210,211] eine günstige Wirkung auf die Reifung, die Funktion und das Überleben von Tregs zugeschrieben wurde, schienen Calcineurin-Inhibitoren (CNI) und Tacrolimus [197,212–221] sowie eine Blockade von IL-2 [222] und CTLA-4 [223] die Treg-Entwicklung und -Reifung zu hemmen. Schwer beurteilbar erscheint auch die Wirkung von Steroiden [224,225] und MMF [217], den beiden am häufigsten in der Transplantationsmedizin verwendeten Medikamenten zur Behandlung einer Abstoßungsreaktion. Die steroidvermittelte Hemmung der Transkriptionsfaktoren AP-1 und NF-κB suggerierte, einen quantitativen Anstieg von Tregs zu induzieren [224,226–231]. In unseren eigenen Querschnittsuntersuchungen an etwa 150 nierentransplantierten Patienten, die bereits ein bis 20 Jahre eine immunsuppressive Dauermedikation erhalten, zeigte sich jedoch für keines der genannten Medikamente einen Effekt auf intrinsische *in vivo* zirkulierende Tregs [116]. Problematisch bei der Interpretation aller Untersuchungsergebnisse an Transplantationspatienten und vielen Tiermodellen ist jedoch die Tatsache, dass in der Regel immunsuppressive Medikamente als Kombinationspartner eingesetzt werden und der individuelle Einfluss dieser Medikamente auf intrinsische aber auch adoptiv transferierte Tregs nicht bestimmbar ist. Daher haben wir in einem NOD/SCID/IL2Rgamma$^{-/-}$ GvHD-Mausmodell Tregs in Kombination mit einzelnen immunsuppressiven Medikamenten eingesetzt. Hierbei zeigte sich, dass die Kombination des adoptiven Transfers von *ex vivo* expandierten Tregs mit CSA – und in etwas geringerem Maß auch mit MMF – das klinische Outcome substanziell verbessert [117]. Gleichzeitig zeigte sich, dass CSA die Rekrutierung von Tregs in inflammatorische Areale von Leber und Lunge verstärkt, während die Infiltration von anderen lymphozytären Zellen und Entzündungsprozesse im Gewebe reduziert werden [117]. Im Gegensatz dazu führte die Kombinationstherapie von Tregs mit Steroiden zu einer erhöhten Mortalität [117]. Unterschiede unserer Daten bezüglich des Einsatzes von Steroiden zu Teilen der Ergebnisse anderer Gruppen könnten in der unterschiedlichen Definition von Tregs und dem Fokus auf quantitative Analysen in vormals publizierten Ergebnissen liegen. So zeigte sich beispielsweise, dass die steroidvermittelte Induktion von FoxP3^{+} Zellen zwar zu einem (vermeintlich) nummerischen Treg-Anstieg führt, dass dieser Anstieg jedoch nicht mit der funktionell suppressiven Kapazität dieser Zellen korreliert [232]. Möglicherweise handelt es sich hierbei um induzierte Tregs, die bei Erreichen eines alloreaktiven Milieus erneut pro-inflammatorische Eigenschaften annehmen und lokale Entzündungsprozesse unterstützen. MMF, das als Pro-Drug verabreicht und enteral sowie hepatisch zur bioaktiven

Mycophenolsäure umgewandelt wird reduziert durch eine selektive Hemmung der Inosinmonophosphat-Dehydrogenase die Guanin-Synthese in T- und B-Zellen. Unter dem Vorbehalt, dass MMF leitliniengereicht in der Transplantationsmedizin als Kombinationspartner mit CSA und/oder Steroiden eingesetzt wird und auch in präklinischen Modellen häufig kombiniert wird, weisen publizierte Daten darauf hin, dass MMF einen positiven Einfluss auf Reifung, Überleben und die funktionellen Kapazitäten von Tregs hat [217,219,233,234]. Mittels unserer Untersuchungsergebnisse konnten wir diese Hinweise bestätigen und erstmalig nachweisen, dass MMF und adoptiv transferierte Tregs zur Verhinderung von Abstoßungsprozessen direkt synergistisch wirken können [117].

Da CSA als klassisches T-Zell-supprimierendes Agens entwickelt wurde und durch eine Hemmung von Calcineurin TZR-abhängige Signalwege, die Aktivierung und Proliferation von T-Zellen spezifisch hemmt, erscheint es zunächst naheliegend, dass diese Wirkung von CSA auch auf regulatorische T-Zellen übertragbar ist. Zudem wurde berichtet, dass die CSA-vermittelte Hemmung der IL-2 auch die Entwicklung allogenspezifischer Tregs unterdrückt [235] und die suppressive Funktion von Tregs sowohl über eine quantitative Reduktion der Zellzahl als auch qualitativ über eine Einschränkung der suppressiven Eigenschaften hemmt [219,220,233]. Nur wenige Berichte stellten diesen Treg-hemmenden Effekt von CSA substanziell in Frage [235–239]. Für die Beurteilung der *in-vivo*-Wirkung von CSA auf *ex vivo* expandierte und adoptiv transferierte Tregs ist daher eine sehr genaue und dosisabhängige Betrachtung der möglichen Interaktionswege notwendig. In unserem Mausmodell verzichteten wir daher auf immunsuppressive Kombinationstherapien, um den individuellen Effekt von CSA auf Tregs untersuchen zu können. Vormals publizierte *in-vitro*-Daten zeigten, dass die CNI-vermittelte Immunsuppression durch CSA einen Serumspiegel von > 50 ng/ml erfordert, wobei die maximale Hemmung bei 1.000 ng/ml zu beobachten ist. Aufgrund seiner Toxizität kann diese Dosis bei Patienten jedoch nicht eingesetzt werden. Entsprechend wird CSA im klinischen Umfeld meist in submaximaler Dosis (100-200 ng/ml Blutspiegel) in Kombination mit anderen immunsuppressiven Arzneimitteln verwendet, die auf verschiedene Signalwege gerichtet und dabei synergistisch ausreichend wirksam sind, beispielsweise mit Kortikosteroiden und/oder MMF [240]. Aus präklinischen *in-vivo*-Studien ist uns bekannt, dass Nagetiere eine ähnliche Pharmakokinetik und Pharmakodynamik für diese Medikamente aufweisen; dennoch werden immunsuppressive Medikamente hier vielfach in deutlich höherer Dosierung eingesetzt, was

die direkte Translation erschwert. Im Rahmen unseres Translationsprozesses konzentrierten wir uns daher auf Modelle, die therapeutische Medikamentenspiegel erreichen und der klinischen Situation möglichst ähnlich sind [117,212]. Dosisfindungsstudien an Ratten zeigten, dass bei 1 mg/kg i. m. CSA Serumspiegel erreicht werden, die leicht unter den Zielwerten liegen, während 8-10 mg/kg CSA pro Tag Werte von bis zu 2.098 + 57 ng/ml erreichen und damit den humanen Zielbereich deutlich überschreiten [241,242]. Entsprechend verwendeten wir in unseren Ratten- und Maus-Organtransplantationsmodellen 3 bis 4 mg/kg CSA (zweimal täglich per os oder einmal täglich iv) [117,212]. Unsere Dosisfindung für den Einsatz von MMF und Prednisolon führten wir ebenso evidenzbasiert durch und setzen in unseren Tierstudien MMF in einer Dosierung von 0,5 mg/kg ein, was bei einem Mycophenolat-Serumspiegel von 2 bis 3 mg/L den therapeutisch eingesetzten Bereich erzielt [116,243]. Der positive Effekt, der sich für CSA und MMF, nicht jedoch für den Einsatz von Steroiden in Kombination mit adoptivem Treg-Transfer zeigt, bildete damit die Basis für unsere weiteren Schritte auf dem Weg in die klinische Anwendung.

Dosisfindungsprozesse hinsichtlich der optimalen Treg-Dosis stellten uns vor noch größere Herausforderungen, da systematische Dosisfindungsstudien für die Treg-Therapie weder bei chronischer GvHD noch im Kontext solider Organtransplantationen bislang nicht publiziert wurden. Aus den bisherigen klinischen Ansätzen wissen wir jedoch, dass es sehr schwierig ist, Korrelationen zwischen der Dosis und der Wirkung von adoptiv übertragenen Tregs im Rahmen einer Transplantation zu definieren. (Einzelfall-) Berichte über die Treg-Therapie bei erwachsenen Patienten mit GvHD sind hinsichtlich Zelldosis, Treg-Quelle und Patientencharakteristika heterogen. Die verabreichten Treg-Dosen variieren zwischen $1x10^5$ und $5x10^6$ pro kg Körpergewicht; eine eindeutige Dosis-Wirkungs-Korrelation kann daraus nicht abgeleitet werden [120–123,244]. Hinsichtlich des Einsatzes von Tregs bei soliden Organtransplantationen ist die Datenlage ähnlich dünn. Als eine der ersten Gruppen führten wir in den letzten Jahren nun eine Phase-I/IIa-Studie (NCT02371434 (ONEnTreg13) und EudraCT:2011-004301-24 (ONErgt11)) durch, in der Lebendspende-Nierentransplantationspatienten mit steigenden Treg-Dosen behandelt wurden (3+3-Design, n=11, 0,5, 1,0, oder 2,5-3,0×106 Zellen pro kg Körpergewicht) [118]. Gleichzeitig wurde die immunsuppressive Komedikation, die dem protokollgerechten Therapiestandard entsprach schrittweise ausgeschlichen. Diese Studie wurde im Rahmen des ONE-Studienkonsortiums

durchgeführt und hatte zum Ziel, die Machbarkeit und die Sicherheit verschiedener zelltherapeutischer Ansätze zu untersuchen. Studienpatienten wurden mit einer Referenz-Gruppe vergleichen, in der die Patienten die Standardbehandlung erhielten. Obwohl es kontraintuitiv erscheint, dass höhere Treg-Dosen niedrigeren Treg-Dosen nicht überlegen sind, fanden wir für keinen der untersuchten Parameter eine Dosis-Wirkungs-Beziehung [118]. Diese Beobachtungen stimmten mit den Ergebnissen einer von Harden und Kollegen parallel dazu durchgeführten Studie überein [245]. Harden et al. fanden heraus, dass zirkulierende Tregs aber auch Marginalzonen-B-Zellen in Korrelation mit der verabreichten Treg-Dosis auch langfristig erhöht waren, dass dies jedoch keinen Einfluss auf den klinischen Verlauf hatte, selbst wenn Treg-Dosen von bis zu $10x10^6$ pro kg verabreicht wurden [245]. Bei Patienten mit Autoimmunerkrankungen kann die Situation anders sein. Die derzeit veröffentlichten Daten reichen jedoch nicht aus, um evidenzbasiert eine ideale Treg-Dosis abzuleiten. Zusammenfassend halten wir die Dosis von $3,0\times10^6$ Zellen pro kg Körpergewicht basierend auf unserer klinischen Erfahrung aus der ONE-Studie und nach intensiven Diskussionen in einem multidisziplinären Team für sinnvoll.

Basierend auf diesen Ergebnissen und auf Grund der medizinischen Dringlichkeit behandelten wir schließlich drei Kinder, die nach einer allogenen Stammzelltransplantation an einer schweren, therapierefraktären, chronischen und damit lebensgefährlichen GvHD litten, erfolgreich mit *ex vivo* expandierten Tregs [246]. Auf Grund der medizinischen Dringlichkeit erfolgte die Behandlung jeweils im Rahmen eines individuellen Heilversuches nach ausführlicher Aufklärung und mit dem Einverständnis der Eltern. Das Treg-Produkt wurde entsprechend der Herstellungserlaubnis aus 50 ml peripherem Blut der ursprünglichen Stammzellspender generiert. Klinisch zeigte sich bei allen Patienten ein erfreulicher Verlauf mit deutlicher Besserung der GvHD in allen Organsystemen. Immunologisch konnte erstmalig nach der Transplantation ein Engraftment von naiven T-Zellen, naiven B-Zellen und naiven Tregs sowie dendritischen Zellen dokumentiert werden, ohne dass klinisch oder immunologisch Zeichen einer schießenden Immunsuppression zu beobachten waren [246]. Adoptiv transferierte Treg-Klone expandierten *in vivo* und waren auch über einen Zeitraum von mehr als 9 Monaten nachweisbar. Alle Patienten erhielten als Komedikation CSA mit einem Zielspiegel von 80-100 ng/ml und einer niedrigen Dosis Prednisolon, welches über die Zeit weiter reduziert werden konnte. Besonders wichtig erscheint uns die Beobachtung, dass

anders als bislang angenommen [244,247] der adoptive Transfer von Tregs auch ohne zusätzlichen Einsatz von IL-2 zu einer Expansion adoptiv transferierer Treg-Klone führt [211]. Damit konnten wir erstmalig zeigen, dass Patienten mit chronischer GvHD nicht nur klinisch, sondern auch immunologisch nachweisbar von einer Treg-Therapie profitieren. Es scheint tatsächlich möglich, auch chronisch etabliert Prozesse günstig zu beeinflussen oder gar zum Stillstand zu bringen.

3.3. Ausblick

Im Rahmen der hier präsentierten Arbeiten sind wir im Translationsprozess der adoptiven Treg-Therapie ein großes Stück vorangekommen. Doch trotz der erreichten Meilensteine auf dem Weg in die klinische Anwendung ist der Einsatz von Tregs nach wie vor Einzelfällen vorbehalten. Es bliebt daher unsere dringliche Aufgabe, systematische klinische Studien durchzuführen und gleichzeitig offene Fragestellungen begleitend zum Translationsprozess zu bearbeiten. Ein besonderer Fokus wird dabei neben der Sicherheit auf der Therapieoptimierung liegen. Die Bestimmung einer indikationsabhängig sicheren und wirksamen Treg-Dosis sowie die Evaluation, ob wiederholte Treg-Gaben vorteilhaft sind, ist relativ kurzfristig umsetzbar. Neuere Technologien erlauben zusätzlich eine genetische Modifikation von Tregs, die die Chance für eine weitere Optimierung von Treg-Produkten bietet. Hier konnte bereits gezeigt werden, dass sowohl transgene TZR [248–251] als auch chimäre Antigen-Rezeptoren (CAR) [155,252–256] T-Zellen eine neue Antigen-Spezifizität verleihen können [257,258]. Lenti- oder retro-virale sowie elektroporative Transfektionen für den Transfer von DNA oder CRISPR-Cas [259–263] können hierfür ebenso zum Einsatz kommen wie ein *gene editing* mittel Zink-Finger-Nukleasen oder TALENs (transcription activator-like effector nucelases) [264–266]. Darüber hinaus können diese Technologien eingesetzt werden, um T-Zellen bezüglich unerwünschter pharmakologischer Einflüsse, beispielsweise gegenüber Glukokortikoiden oder Tacrolimus, resistent zu machen [267–271]. Bevor diese Ansätze jedoch die klinische Anwendung erreichen wird, müssen die einzelnen Schritte der Translation einschließlich der präklinischen Modelle sowie die Zulassung erneut durchlaufen werden. Da hierbei zu Lasten der Patienten kostbare Zeit vergeht, sollte der Fortschritt mehrgleisig erfolgen. Einerseits sollten bereits erfolgreich angewandte Konzepte zeitnah im Rahmen größerer klinischer Studien weiter untersucht werden, damit bald möglichst viele Patienten, denen wir aktuell

keine erfolgversprechenden alternativen Therapieoptionen bieten können, von einer Treg-Therapie profitieren können. Andererseits müssen parallel hierzu vorliegende Ergebnisse auch im Hinblick auf eine Indikationserweiterung, die u.a. schwer behandelbare Autoimmunerkrankungen wie Diabetes mellitus Typ 1, chronisch entzündliche Darmerkrankungen, sowie rheumatische Erkrankungen einschließen, geprüft werden. Gleichzeitig bleibt es unsere fortwährende Aufgabe, die Qualität und Effektivität von Treg-Produkten zu verbessern und diese aufbauend auf bewährten Translationsstrategien frühzeitig in klinische Studien zu überführen.

4. Zusammenfassung

Die Transplantation von soliden Organen oder hämatopoetischen Stammzellen hat sich über die letzten Jahrzehnte für viele Patienten zu einer kurativen Therapieoption entwickelt. Der langfristige Transplantationserfolg ist jedoch wesentlich von der Kontrolle chronischer Abstoßungsreaktionen abhängig. Die Behandlungserfolge im Falle einer chronischen Abstoßungsreaktion sind trotz des Einsatzes moderner immunsuppressiver Medikamente bis heute leider nicht zufriedenstellend. Der therapeutische Einsatz von regulatorischen T-Zellen, welche anti-inflammatorisch wirken und die Immunhomöostase erhalten oder nach Inflammationsprozessen wiederherstellen, entwickelte sich in den letzten Jahren zu einem vielversprechenden Therapiekonzept. Hierbei werden regulatorische T-Zellen (Tregs) dem Patienten selbst oder dem ursprünglichen Stammzellspender entnommen, angereichert und anschließen intravenös verabreicht. Trotz der technischen Möglichkeiten und der klinischen Erfahrungen, die sowohl die Sicherheit als auch die Wirksamkeit von Tregs belegen, stagnieren die Entwicklungen in frühen Phasen klinischer Studien. Im Rahmen unserer Arbeiten haben wir in den letzten Jahren ein eigenes Protokoll entwickelt, das es uns ermöglicht, mittels *ex-vivo*-Expansion von Tregs aus 50 ml peripherem Vollblut – ohne die Notwendigkeit einer Leukozytapherese – ein sehr reines und funktionell wirksames Treg-Produkt zu generieren und dafür die Herstellungserlaubnis zu erlangen. Wir untersuchten zudem den Einfluss von konventionellen immunsuppressiven Medikamenten auf adoptiv transferierte Tregs, um Synergien zu nutzen und antagonistische Effekte zu vermeiden. Hierbei zeigte sich, dass Calcineurin-Inhibitoren wie Cyclosporin A und Mycophenolat Mofetil das Überleben und die Funktion adoptiv transferierter Tregs unterstützen, während Glukokortikoide in hoher Dosierung vermieden werden sollten. Mit diesem Wissen gelang uns die Translation *ex vivo* expandierter Tregs: im Rahmen einer Phase I/IIa-Studie konnten bereits Patienten, die eine Lebendspende-Nierentransplantation erhalten haben erfolgreich behandelt werden. Zusätzlich behandelten wir erstmalig drei Kinder, die nach allogener Stammzelltransplantation eine schwere, therapie-refraktäre chronische Abstoßungsreaktion erlitten hatten. Bei allen drei Patienten zeigte sich eine deutliche Besserung des klinischen Bildes und wir konnten zudem ein immunologisches Engraftment von naiven T-Zellen, naiven B-Zellen und dendritischen Zellen nachweisen. Angesichts dieser Ergebnisse und der übereinstimmend vielversprechenden Erfahrungen anderer Gruppen weltweit bleibt es unsere

gemeinsame Aufgabe, die Sicherheit und Wirksamkeit von Treg-Therapien zeitnah im Rahmen größerer klinischer Studien weiter untersuchen. Gleichzeitig ist es essentiell, neue Erkenntnisse und innovative technische Möglichkeiten der Produktmodifikation fortlaufend in die Weiterentwicklung von Treg-Produkten einfließen zu lassen. Damit ist die Treg-Therapie auf absehbare Zeit zwar noch immer Einzelfällen vorbehalten, aber weitere wesentliche Meilensteine auf dem Weg in die breite klinische Anwendung werden nach und nach erreicht werden.

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„Wir können der Tatsache nicht ausweichen,
dass jede einzelne Handlung, die wir tun,
ihre Auswirkung aufs Ganze hat.“
Albert Einstein (1879 – 1955)

7. Erklärung

§ 4 Abs. 3 (l) der HabOMed der Charité

Hiermit erkläre ich, dass

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- mir die geltende Habilitationsordnung bekannt ist.

Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

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