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Orientadores: Prof. Doutor João Forjaz de Lacerda
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Tese especialmente elaborada para obtenção do grau de Doutor em Medicina, Hematologia

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Júri
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Para os meus pais,

mestres de determinação e perseverança

Para a Camila,

como exemplo de determinação e perseverança

Climb a mountain not to plant your flag,

but to embrace the challenge, enjoy the air,

and behold the view.

Climb mountains not so the world can see you,

but so you can see the world.

David McCullough, Jr

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ABBREVIATIONS

αβ	alpha/beta
aGvHD	acute Graft-*versus*-Host Disease
Alo-TPH	Transplante Alogénico de Progenitores Hematopoiéticos
Allo-HSCT	Allogeneic Hematopoietic Stem Cell Transplantation
Ag	Antigen
AICD	Activation-Induced Cell Death
APCs	Antigen Presenting Cells
ATG	Anti-Thymocyte Globulin
β	beta
Bcl-x	Bcl 2 like 1 protein
Bcl-2	B-cell lymphoma protein 2
Bim	Bcl-2-interacting mediator of cell death
BM	Bone Marrow
BSA	Bovine Serum Albumin
BWH	Brigham and Women's Hospital
CsA	Cyclosporine A
cGvHD	Chronic Graft-*versus*-Host Disease
CD	Cluster of Differentiation
CTLA-4	Cytotoxic T-lymphocyte-Associated protein 4
DC	Dendritic Cells
cDEcH	Doença de Enxerto contra Hospedeirio crónica
DFCI	Dana-Farber Cancer Institute
DN	Double Negative
DP	Double Positive
FMUL	Faculdade de Medicina da Universidade de Lisboa
Foxp3	Forkhead box P3
γC	gamma chain

GITR	Glucocorticoid-Induced Tumor necrosis factor Receptor
GvHD	Graft-*versus*-Host Disease
GvT	Graft-*versus*-Tumor
HD	Healthy Donors
HLA	Human Leukocyte Antigen
HSCs	Hematopoietic Stem Cells
HSM	Hospital de Santa Maria
HP	Homeostatic Proliferation
IFNγ	Interferon gamma
IL	Interleukin
IL-R	Interleukin Receptor
iMM-JLA	Instituto de Medicina Molecular João Lobo Antunes
IR	Immune Reconstitution
ITIM	Immunoreceptor Tyrosine-Based Inhibitory Motif
iTreg	Induced $CD4^+$ regulatory T-cell
LN	Lymph Node
LIP	Lymphopenia-Induced Proliferation
µl	Microliters
mL	Milliliter
mAbs	Monoclonal Antibodies
MAC	Myeloablative Intensity Conditioning
MFI	Mean/Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
mHA	Minor Histocompatibility Antigen
MMF	Mycophenolate Mofetil
MTX	Metotrexate
PBMC	Peripheral Blood Mononuclear Cell
PBSC	Peripheral Blood Stem Cells
PBS	Phosphate-Buffered Saline
pTregs	Peripheral-derived $CD4^+$ regulatory T-cells
RIC	Reduced Intensity Conditioning

SCID	Severe Combined Immunodeficiency
SIR	Sirolimus
SLOs	Secondary Lymphoid Organs
SN	Single Negative
SR-cGvHD	Steroid dependent/Refractory cGvHD
SP	Single Positive
spMHC	self-peptide/Major Histocompatibility Complex
TAC	Tacrolimus
TCR	T-cell Receptor
TREC	T-cell Receptor rearrangement Excision DNA Circles
TSDR	Treg-Specific Demethylated Region
Tcon	$CD4^+$ conventional T-cell
Treg	$CD4^+$ regulatory T-cell
tTregs	Thymic-derived $CD4^+$ regulatory T-cells
T_{CM}	Central Memory T-cell
T_{EMRA}	Terminal Effector Memory Re-expressing RA
T_{EFF}	Effector T-cell
T_{EM}	Effector Memory T-cell
T_{RTE}	Recent Thymic Emigrant T-cell
TGF-β	Transforming Growth Factor β
pT_N	Peripheral Naïve T-cell/True Naïve
TNF	Tumor Necrosis Factor
T_{SCM}	T Memory Stem Cell/Stem Cell Memory
vs.	*versus*

SUMMARY

Following allogeneic hematopoietic stem cell transplantation (allo-HSCT), the homeostasis of the T-cell pool is deeply challenged, requiring years to recover. Frequently, this recovery is dysfunctional, accompanied by loss of T-cell tolerance and subsequent development of chronic Graft-*versus*-Host Disease (cGvHD), leading to significant morbidity and mortality. With this work we investigated the mechanisms involved in the restoration of peripheral T-cell homeostasis following allo-HSCT, particularly the role of $CD4^+$ regulatory T-cells (Tregs), critical mediators of peripheral T-cell tolerance.

We performed a comprehensive analysis of the reconstitution of peripheral blood $CD4^+$ and $CD8^+$ T-cells in patients undergoing allo-HSCT for the treatment of hematologic malignancies. We took advantage of being able to study three distinct transplant settings, differing primarily in the conditioning regimen, GvHD prophylaxis and HLA (Human Leukocyte Antigen) matching. We consistently found a significantly lower number of Tregs at nine months post-transplant in patients developing cGvHD, coinciding with decreased Treg:Tcon (conventional T-cells) and Treg:CD8 ratios. This unbalance resulted from significantly delayed proliferation of naïve Tregs, and increased expansion of memory Tcon and $CD8^+$ T-cell, observed in all cohorts. The expansion of memory subsets, likely involved in cGvHD pathogenesis, together with impaired thymic generation of *de novo* naïve $CD4^+$ T-cells, resulted in significantly decreased diversity of the $CD4^+$ TCR repertoire in cGvHD patients.

Importantly, we were able study for the first time the contribution of true naïve and naïve-derived T memory stem cells (T_{SCM}) to the development of cGvHD in humans. An early and transient increase of naïve T-cells was observed in two cohorts, anticipating the development of cGvHD, while the sustained increase of T_{SCM} CD8 was observed in the cohort where allo-HSCT was performed with concomitant T-cell depletion . The heterogeneity of these results, reflect the effect of the different transplant settings,

particularly in the recovery of donor-infused naïve T-cells and their progeny, in the early post-transplant period.

In the final part of this work, we sought to determine the mechanisms involved in cGvHD amelioration after the administration of low-dose rIL-2 (interleukin 2) to patients with steroid dependent/refractory cGvHD. In this setting, the expansion of novel or low frequency Treg clones was the only variable associated to cGvHD improvement. This finding enhances the relevance of antigen specificity for adequate Treg suppression and induction of tolerance post allo-HSCT.

Overall, the data obtained reiterates that achieving an adequate balance between effector and regulatory T-cells post allo-HSCT is of critical importance to avoid the development of cGvHD in the post transplantation period. Hence, we not only confirm the association between impaired Treg homeostasis and cGvHD, but further validate the contribution of naïve T-cells to the initiation of cGvHD. In addition, we describe for the first time, a role for T_{SCM} in cGvHD pathogenesis in humans, and stress the importance to clarify the role of antigen-specificity to efficient Treg function.

Keywords: $CD4^+$ regulatory T-cells; T-cell homeostasis; Immune T-cell reconstitution; Allogeneic Hematopoietic Stem Cell Transplantation; Chronic Graft-*versus*-Host Disease.

SUMÁRIO

O Transplante Alogénico de Progenitores Hematopoiéticos (alo-TPH) condiciona uma alteração profunda na homeostasia dos linfócitos T, cuja reconstituição é lenta, podendo requerer anos até normalizar. A reconstituição disfuncional, associada à perda da tolerância imune T, resulta num desequilíbrio homeostático que se traduz clinicamente em doença de enxerto contra hospedeiro crónica (cDEcH), que se associa a elevada morbilidade e mortalidade. Este trabalho, teve como objetivo principal investigar os mecanismos envolvidos na recuperação da homeostasia linfocitária T periférica após alo-TPH. Em particular, no contributo dos linfócitos T $CD4^+$ reguladores (Tregs), dado o seu papel primordial na indução e manutenção da tolerância imune.

Procedemos à análise detalhada da reconstituição linfocitária T, $CD4^+$ Treg, $CD4^+$ T convencional (Tcon) e $CD8^+$, no sangue periférico de doentes submetidos a alo-TPH por doença hematológica. De salientar que nos foi possível estudar três grupos de doentes sujeitos a alo-TPH sob regimes de condicionamento, profilaxia da DEcH e compatibilidade HLA distintos. O trabalho desenvolvido demonstrou que os doentes com cDEcH se distinguiam pela redução significativa no número de linfócitos Treg e concomitante redução do rácio Treg:Tcon e Treg:CD8, aos nove meses após transplante. Este desequilíbrio entre linfócitos T reguladores e linfócitos T efetores associou-se a um atraso significativo na proliferação das linfócitos naïve Treg e a um aumento significativo na proliferação de linfócitos Tcon e $CD8^+$ de memória. Além disso, observou-se ainda uma diminuição significativa na diversidade do repertório do recetor das células T (TCR) dos linfócitos T $CD4^+$, em todos os doentes com cDEcH, independentemente do grupo de estudo. Esta redução na diversidade do repertório TCR associou-se a uma expansão dos subtipos de memória, provavelmente envolvidos na patogénese da cDEcH, e à diminuição da produção tímica de linfócitos naïve $CD4^+$.

Estudámos, pela primeira vez, o contributo dos linfócitos T *true naïve* e *stem cell memory* (T_{SCM}), na fisiopatologia da cDEcH em humanos. Os resultados demonstraram um aumento significativo, embora precoce e temporário, do número de linfócitos T naïve em dois grupos de estudo, antecipando o desenvolvimento de cDEcH. O aumento das T_{SCM}

foi observado em doentes submetidos a alo-TPH com algum nível de depleção de linfócitos T, sendo este aumento mantido ao longo do tempo. A heterogeneidade dos resultados obtidos reflete o efeito das variáveis clinicas, que distinguem os três grupos de estudo, na reconstituição imune após transplante, nomeadamente nas linfócitos T naïve infundidas do dador.

Por último, pretendeu-se esclarecer quais os mecanismos envolvidos na melhoria dos sintomas de cDEcH em doentes tratados com Interleucina 2 (rIL-2) em baixa dose. A única variável relacionada com a melhoria clínica foi a expansão de novos clones de linfócitos Treg. Este resultado veio reforçar a importância da especificidade antigénica para a correta função dos linfócitos T reguladores, e consequente indução da tolerância imune após alo-TPH.

Em suma, os resultados obtidos com este trabalho reiteram a importância dos mecanismos homeostáticos para o estabelecimento de um equilíbrio adequado entre linfócitos T com função efetora e linfócitos T com função reguladora, após alo-TPH. Desta forma, os dados confirmam que a ocorrência de alterações da homeostasia Treg se relaciona com o desenvolvimento de cDEcH, e validam o contributo dos linfócitos T naïve para a fisiopatologia desta doença. Além disso, os resultados sugerem, pela primeira vez, a participação dos linfócitos T_{SCM} na patogénese da cDEcH em humanos, e alertam para a necessidade de clarificar o papel da especificidade antigénica dos linfºocitos Treg para a sua função reguladora.

Palavras Chave: Linfócitos T reguladores $CD4^+$; Homeostasia linfocitária T; Reconstituição imunológica T; Transplante alogénico de progenitores hematopoiéticos; Doença do enxerto contra hospedeiro crónica.

SUMÁRIO EXTENSO

O Transplante Alogénico de Progenitores Hematopoiéticos (alo-TPH) é uma opção terapêutica frequentemente utilizada no tratamento de diversas patologias, nomeadamente de neoplasias malignas do foro hematológico. O potencial curativo do alo-TPH decorre do efeito alo-reactivo dos linfócitos do dador e da substituição do sistema imunitário do doente. A eficácia desta terapêutica é limitada pela imunossupressão profunda e prolongada e pelo desequilíbrio grave da homeostasia T, que a longo prazo pode levar à perda da tolerância imunológica com subsequente desenvolvimento de DEcH crónica.

A cDEcH é a principal complicação tardia do alo-TPH, estando associada a uma elevada morbilidade e mortalidade. A sua incidência, de cerca de 40% aos 12 meses, teve um aumento progressivo na última década, devido a variáveis como a maior utilização de dadores com disparidade HLA, pares dador-recetor com idade superior, e o uso de sangue periférico como fonte de progenitores hematopoiéticos. Esta tendência, aliada à ausência de uma estratégia preventiva e/ou terapêutica eficaz, reforçam a necessidade de aprofundar os mecanismos imunológicos que estão implicados na patogénese da cDEcH.

Apesar dos avanços científicos nas últimas décadas, a complexidade que define a fisiopatologia da cDEcH continua a ser um dos principais desafios clínicos e terapêuticos na área da transplantação. Estudos pré-clínicos e clínicos realizados no âmbito da cDEcH demonstraram consistentemente um desequilíbrio entre os mecanismos reguladores que mantêm a tolerância imune e os mecanismos efetores que causam a doença. Os linfócitos T $CD4^+$ reguladores (Tregs) desempenham um papel crucial na tolerância imune periférica e na homeostasia linfocitária T, sendo este papel particularmente relevante na reconstituição imunológica após transplante alogénico. Este facto foi demonstrado em vários estudos que descrevem não só a reconstituição anómala de Tregs nos doentes com cDEcH, mas também a redução na incidência de doença com a infusão profilática de Tregs e a melhoria clínica aquando da terapêutica com interleucina 2 (IL-2), a principal citocina envolvida na indução e manutenção de Tregs.

Este trabalho foi desenvolvido com o objectivo de aprofundar o conhecimento sobre a homeostasia linfocitária T após alo-TPH, com um ênfase particular no papel dos linfócitos T $CD4^+$ com função reguladora neste processo. Para tal, estudámos a reconstituição linfocitária T periférica num período de dois anos após alo-TPH, utilizando um painel fenotípico e funcional para identificar e caracterizar as subpopulações naïve e memória das linfócitos T $CD4^+$ reguladores, T $CD4^+$ convencionais (Tcon) e $CD8^+$. O estudo simultâneo destas subpopulações e da respetiva expressão de marcadores de proliferação celular (Ki-67), apoptose (Bcl-2 e CD95) e produção tímica (CD31) permitiram, pela primeira vez, uma caracterização detalhada da homeostasia linfocitária T após alo-TPH.

Estudámos inicialmente um grupo de doentes submetidos a alo-TPH após condicionamento de intensidade reduzida (RIC), sem depleção de linfócitos T. Neste grupo observámos um atraso significativo na reconstituição de linfócitos Treg naïve, sendo este atraso coincidente com a reduzida proliferação e génese tímica deste subtipo celular. O mesmo não se observou para as populações Tcon e CD8, confirmando assim a existência de mecanismos de regulação da homeostasia específicos para cada subpopulação, tal como sugerido em estudos anteriores. Os doentes que desenvolveram cDEcH distinguiram-se aos 9 meses após transplante por desequilíbrios nas razões Treg:Tcon e Treg:CD8, decorrente da redução significativa do número total de Tregs e do aumento do número total de Tcon. Num período mais precoce, aos 3 meses, estes doentes apresentavam um predomínio significativo de subpopulações naïve Treg, Tcon e $CD8^+$, o que sugere um papel dos linfócitos T naïve na biologia da doença.

Os linfócitos T naïve correspondem a linfócitos indiferenciados/imaturos, com origem tímica, que são mantidos por proliferação homeostática. Esta subpopulação circula no sangue periférico num estado quiescente e após reconhecimento antingénico, são ativados e diferenciam-se em subpopulações com capacidade efetora e/ou de memória. Em modelos animais, os linfócitos T naïve apresentam um maior potencial alo-reativo quando comparados com linfócitos T memória. Em humanos, observou-se que a depleção de linfócitos T naïve do enxerto diminui significativamente a incidência de cDEcH. Estudos recentes de permitiram distinguir duas subpopulações linfocitárias que expressam marcadores de superfície naïve, os linfócitos T verdadeiramente naïve (pT_N) e os linfócitos *T-stem cell memory* (T_{SCM}). Estes últimos, distinguem-se pela elevada

expressão de CD95 e por atributos funcionais únicos, nomeadamente a capacidade de auto-renovação e persistência por períodos prolongados, bem como a capacidade de diferenciação em diferentes subtipos T de memória. Com o intuito de esclarecer o contributo dos linfócitos T naïve na patogénese da cDEcH, caracterizámos em pormenor o sangue periférico de dois grupos clínicos distintos.

No grupo de doentes submetidos a alo-TPH após RIC com depleção das linfócitos T com globulina antitimócitos (ATG), observámos um aumento precoce e significativo dos linfócitos pT_N e T_{SCM} nas populações Treg, Tcon e CD8, nos doentes que desenvolveram cDEcH. Esta expansão inicial foi transitória para as populações Treg e Tcon, mantendo-se durante todo o período de estudo para os linfócitos T $CD8^+$, em particular para as T_{SCM} CD8. Estes resultados poderão traduzir a expansão inicial de linfócitos pT_N do dador, infundidas num meio fortemente linfopénico, que após proliferação induzida pela linfopénia (LIP) e/ou após exposição alo-antigénica se diferenciam em $T_{SCM.}$ A capacidade de auto-renovação das T_{SCM} poderá explicar a expansão deste subtipo celular, embora não sejam claros quais os mecanismos que justificam a expansão preferencial das T_{SCM} CD8. Estudos prévios no contexto da transplantação demonstraram a importância das T_{SCM} na reconstituição imune e na ontogenia dos linfócitos T. No contexto da cDEcH, este é o primeiro estudo que sugere um papel para as T_{SCM} CD8 na fisiopatologia da doença.

No grupo de doentes submetidos a alo-TPH após condicionamento mieloablativo, sem depleção de linfócitos T, observámos um atraso significativo na reconstituição pT_N e T_{SCM} Treg nos doentes com cDEcH, sem que se fossem observadas diferenças na reconstituição Tcon e CD8. Este atraso foi observado desde o período precoce após transplante até ao diagnóstico da cDEcH. Foi ainda observada, uma expansão preferencial de linfócitos T-*effector memory* (T_{EM}) CD8. Estes resultados reforçam o papel essencial dos linfócitos Treg na recuperação da homeostasia T periférica após transplante e sugerem o envolvimento do subtipo T_{EM} CD8 na fisiopatologia da cDEcH.

A disparidade dos resultados obtidos relativamente ao contributo dos linfócitos T naïve na patogénese da cDEcH reflete o efeito de variáveis, tal como o tipo de condicionamento, a classe de fármacos imunossupressores e a compatibilidade HLA na reconstituição linfocitária T após transplante. Apesar da heterogeneidade entre os três

grupos clínicos, observámos de forma consistente uma associação entre cDEcH e a redução na produção tímica de linfócitos T $CD4^+$ e na diversidade do repertório TCR dos linfócitos T $CD4^+$, Estes resultados salientam o papel do timo na recuperação da homeostasia T e na tolerância imune após alo-TPH.

Por fim, com vista a complementar o conhecimento sobre os mecanismos de indução de tolerância imune mediados pelos linfócitos Treg, que levam à melhoria clinica, estudámos um grupo de doentes com diagnóstico de cDEcH resistente/refratária a corticosteróides, sob terapêutica com IL-2 em baixa dose. Em todos os doentes incluídos neste estudo observou-se expansão de Tregs, sendo observada melhoria clínica significativa em apenas 50-60% dos doentes . Após o estudo detalhado da homeostasia linfocitária T, do estado funcional de Tregs, da função supressora das linfócitos Treg e da diversidade do repertório TCR dos linfócitos T, concluímos que a única variável relacionada com melhoria clinica foi a expansão de novos clones de linfócitos Treg. Este resultado veio reforçar a importância da especificidade antigénica para a função supressora dos linfóctios Treg, e da consequente indução da tolerância imune após alo-TPH.

Globalmente, os resultados deste trabalho têm potenciais implicações para o desenho de terapêuticas dirigidas para a recuperação da homeostasia linfocitária T, não apenas no contexto da reconstituição imunológica após alo-TPH, mas também em contextos de auto-imunidade, linfopénia prolongada e depleção linfocitária por doença oncológica.

General Introduction

1. Overview of the αß T-cell compartment in humans

In healthy humans the T-cell compartment is characterized by a diverse set of populations capable of mounting efficient immune responses to immunological challenges, while preventing unwanted immunopathology[1,2]. Almost 80% of circulating human T-cells express the alpha/beta (αβ) T-cell receptor (TCR)[3], together with CD4 and CD8 molecules, identifying $CD4^+$ and $CD8^+$ T-cell populations[4]. Classically, each population is divided into naïve and memory subpopulations/subsets[5], according to T-cell differentiation, activation and functional status[2].

Most of our understanding of T-cell subsets derives from mouse studies and the extrapolation of this data to humans is hampered by significant differences between the two species[6,7]. Furthermore, the vast majority of studies in human T-cells are confined to sampling of peripheral blood, which contain a very small fraction (~2%) of the total body T-cells [2][8,9], and are likely not representative of the total immune compartment[10]. Despite this, a picture detailing the characteristics of human naïve and memory T-cell subsets in health and disease has emerged from several preclinical and clinical studies.

This Section provides an overview of the current knowledge on the human naïve and memory peripheral αβ T-cell compartment.

1.1. True Naïve T-cells

Naïve T-cells correspond to resting/quiescent, Ag-inexperienced and undifferentiated cells, that mature in the thymus and continuously recirculate between Secondary Lymphoid Organs (SLOs) and the blood[11]. Once naïve T-cells encounter cognate Ag, presented by Ag-Presenting Cells (APCs) in SLOs, they are activated and undergo clonal expansion with further differentiation into effector (T_{EFF}) and memory T-cells. During T-cell differentiation, the expression of surface molecules that participate in TCR signal transduction (CD45 isoforms), T-cell migration to SLOs (CD62L/CCR7/CD31) and T-

cell activation (CD28/CD27)[5], is dynamic and allow the phenotypic distinction between naïve and memory subsets (Table 1).

Table 1. Surface molecules used to identify and distinguish T-cells subsets

T-cell surface molecule	Description
CD45RA and CD45RO isoforms[12][13].	Transmembrane protein tyrosine phosphatase; involved in TCR signal transduction. CD45 can be expressed in six different isoforms by alternative splicing of exons that comprise the extracellular domain of this protein. The low molecular weight RO isoform replaces RA after naïve T-cell stimulation; RA can be re-expressed on terminally differentiated memory cells (T_{EMRA})
L-selectin (CD62L)[14]	Adhesion molecules; allow the binding to proteins on the surface of specialized high endothelial venules in SLOs. Expressed on naïve and Central Memory T-cells (T_{CM}).
C-C chemokine receptor 7 (CCR7)[15]	
CD27[16]	Co-stimulatory molecules; participate in T-cell generation, activation and maintenance. CD27 and CD28 bind to APC ligands CD70 and CD80/86, respectively. Expressed on naïve, T_{CM}, and T_{EMRA} T-cells,
CD28[16]	
CD31 (Platelet Endothelial Cell Adhesion Molecule 1, PECAM-1)[17]	ITIM-containing receptor; expressed on platelets, endothelial cells and leukocytes. On peripheral blood T-cells, CD31 mediates cell adhesion and migration, and inhibits TCR activation [17][18]. The co-expression with CD45RA identifies $CD4^+$ Recent Thymic Emigrants (T_{RTE})[18][19].

Based on the expression of surface markers, naïve T-cells are classically defined as $CD45RA^+CD45RO^-CCR7^+CD62L^+CD28^+CD27^+$ cells, which include the Recent Thymic Emigrants (T_{RTE}) and the peripheral naïve T-cells (pT_N), both Ag-inexperienced/ true naïve T-cells[11]. Recent studies have established the existence of a small subpopulation that phenotypically fall within the traditional naïve T-cell compartment, but display functional characteristics of effector and memory subsets, termed T Memory Stem Cell (T_{SCM})[11][20]. These are considered an early stage of the memory T-cell pool (described in Section 1.2).

Recent Thymic Emigrants (T_{RTEs}) correspond to naïve T-cells that have recently emerged from the thymus after positive and negative selection (described in Section 2.1), and have undergone limited proliferation[21].

Human T_{RTEs} are predominant in the neonatal T-cell compartment. With ageing, the thymic involution leads to a decline in T_{RTE} output, resulting in lower numbers of T_{RTE} in adulthood[22,23].

Functionally, RTE have an increased response to cytokines, such as interleukin (IL)-7[21][24] and an ability to synthesize IL-8[25], a cytokine with pro-inflammatory function that mediates the innate immune response in neonates. T_{RTE} can be distinguished from pT_N, by the high concentration of T-cell receptor excision circles (TRECs)[26]. TRECs are circular DNA fragments generated during TCR gene rearrangement[26]. The deletion of the δ locus, that takes place at a late stage of αβ T-cell development is considered to be the most accurate signal joint (sj)TREC, occurring in ~70% of αβ T-cells[26,27]. As sjTRECs do not replicate during cell division, they have been used as a tool to distinguish naïve T-cells that recently emerged from the thymus *versus* pT_N, which TREC content is diluted. However, in situations of high cell turnover due to homeostatic or Ag-induced proliferation, the identification of RTE through TREC content should be made with caution[28]; this is particularly relevant under lymphopenic conditions, such as following allogeneic Hematopoietic Stem Cell Transplantation (allo-HSCT)[29–31].

The fact that TREC data might be hampered by certain clinical factors has encouraged the study of other surrogate markers of T_{RTE}, leading to the identification of CD31[17][32]. $CD4^+$ naïve T-cells expressing CD31 are characterized by a broad TCR repertoire[21] and a high sjTREC content[22,23][33], and rapidly decline following thymectomy[34] or ageing[22,23], suggesting a recent thymic origin. CD31 is commonly used to distinguish $CD4^+$ T_{RTE} from $CD4^+$ pT_N, since its expression is lost with cell activation and proliferation[22][35]. However, in certain clinical settings, such as ageing and severe lymphopenia, the numbers of $CD4^+$ naïve T-cells expressing CD31 may not correlate with sjTREC levels. This can be explained by the effect of cytokines, such as IL-7 that induce naïve T-cell proliferation without the loss of CD31 surface expression[24].

Finally, recent studies have identified PTK7 as a novel marker for immature human $CD4^+T_{RTE}$[36]. PTK7 is a subclass of the receptor tyrosine kinase family, involved in cell survival, that is highly expressed on thymocytes during T-cell development[37]. PTK7 expression on $CD31^+CD4^+$ naïve T-cells is lost after cytokine-induced expansion, while CD31 persists[36], suggesting that $PTK7^+CD31^+CD4^+$ naïve T-cells correspond to the most immature RTEs.

Unlike $CD4^+T_{RTE}$, $CD8^+$ T-cells seem to retain the expression of CD31 during proliferation and differentiation[17][38], thus CD31 is not used to identify $CD8^+{}_{RTE}$. Results from a single center have suggested CD103 as a good surrogate marker for $CD8^+{}_{RTE}$[39]. CD103 is mostly expressed on intraepithelial T-cells of the skin and gut, but is also found in developing $CD4^-CD8^+$ thymocytes[40] and in a small percentage of human circulating naïve $CD8^+$ T-cells[41], which contain a high sjTREC content and decline after thymectomy, suggesting a thymic origin[39].

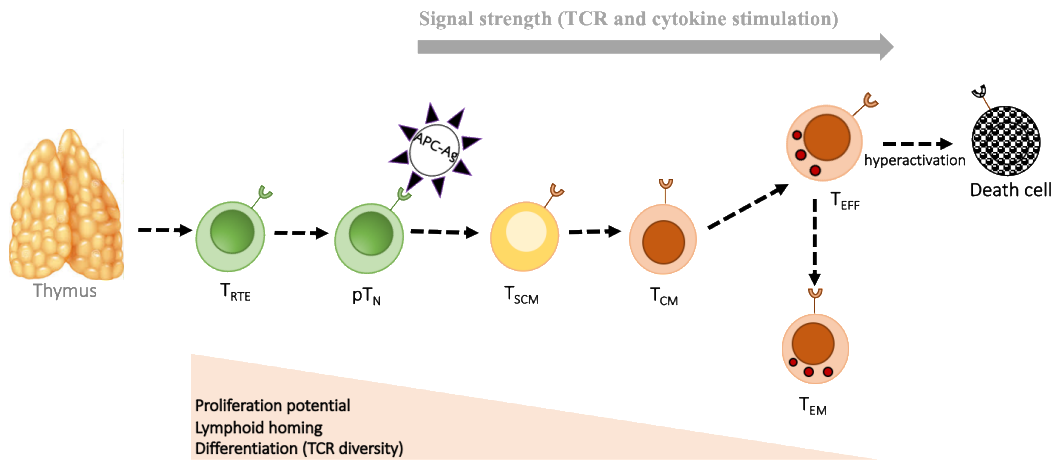
Peripheral naïve T-cells (pT_N) correspond to the majority of T-cells in the adult naïve compartment[11]. They differentiate from RTE, following homeostatic proliferation[42], which leads to down-regulation of CD31[33][42]. The maintenance of pT_N relies on peripheral mechanisms, such as slow homeostatic proliferation and extended survival (described in Section 2.2), that enable the stability of naïve T-cell numbers throughout adult life, despite thymic involution.

1.2. Effector and memory T-cells

Following Ag exposure in SLO, activated naïve T-cells proliferate and differentiate into memory T-cells, that can either be short-lived **effector T-cells (T_{EFF}),** committed to rapidly eliminate the pathogen, or long-lived memory T-cells. Memory T-cells have been conventionally divided into **Central Memory (T_{CM}),** that circulate between the blood and SLO, and **Effector Memory (T_{EM}),** which can migrate from the blood into non-lymphoid tissues and provide extended immune protection against reinfection[43].
The regulation of T-cell fate after a primary response is highly heterogeneous and the pathways of T-cell differentiation remain controversial[44,45]. The suggested models for T-cell differentiation in humans may follow a linear, progressive or asymmetric

pathway[46]. The linear model implies that activated pT_N expand and differentiate into T_{Eff}, that either die by activation-induced cell death (AICD) after pathogen elimination in the peripheral tissue, or differentiate into memory T-cells[47–49]. Although this has been the dominant model for $CD8^+$ T-cell differentiation, more recent studies suggest a progressive differentiation model for both $CD8^+$ and $CD4^+$ T-cells. According to the progressive model, the fate of activated pT_N is heterogeneous and relies primarily on the strength of TCR signaling and cytokine signals received during Ag contact (Figure 1)[50,51]. Thus, low TCR stimulation results in an intermediate state of differentiation characteristic of T_{CM}, whereas high TCR and cytokine stimulation results in T_{Eff} differentiation, with loss of lymph-node-homing capacity and migration to inflamed peripheral tissues. T_{Eff} may enter the memory pool as T_{EM}, or die by AICD in case of hyperactivation[51]. Recent studies hypothesize that the transition from pT_N to T_{CM} includes an intermediate subset, the T_{SCM}, which have the capacity to persist in the host in the absence of Ag, and to generate a pool of memory specific T-cells [52].

Figure 1. Progressive model for T-cell differentiation



	T-cell subsets	RTE	TpN	SCM	CM	EM
Surface T-cell markers	CD45RO /CD45RA	-/+	-/+	-/+	+/-	+/-
	62L/ CCR7	+	+	+	+	-
	CD28/ CD27	+	+	+	+	-
	CD31	+ (CD4)	-	-	-	-
	CD95 (FAS)	-	-	+	+	+

Schematic representation of the progressive-differentiation model and the corresponding surface antigens. The different subsets are categorized based on the current consensus as T_{RTE} (recent thymic emigrant); pT_N (peripheral naïve); T_{SCM} (stem cell memory); T_{CM} (central memory); T_{EM} (effector memory), and T_{EMRA} (terminal effector memory re-expressing CD45RA). Green cells represent naïve T-cells, orange cells are memory and effector T-cells, yellow cell represent the intermediate stage. Adapted from Yolanda D. Mahnke *et al.* [45] and Antonio Lanzavecchia and Federica Sallusto[53].

Effector T-cells (T_{EFF}) comprise short-lived $CD4^+$ and $CD8^+$ T-cells with specific trafficking proprieties (tissue-homing-receptors) and effector functions, which allow the migration from SLO to peripheral tissues. After Ag recognition naïve antigen-specific $CD4^+$ and $CD8^+$ T- cells proliferate and differentiate into T_{EFF}, which produce cytokines and/or cytolytic molecules that will enhance the activity of other immune cells responsible for pathogen clearance.

Based on the homing receptors and cytokine secretion profiles, $CD4^+$ T_{Eff} can be classified as T-helper (Th)1, Th2, Th17, T-follicular helper cell (TfH), and induced regulatory T-cell (iTreg)[54,55]. Th1 express CXCR3 and mediate immune responses against intracellular pathogens through the secretion of interferon gamma (IFNγ) and Tumor Necrosis Factor (TNF), which in turn activate macrophages and $CD8^+$ T-cells, respectively. Th2 express CCR3 and mediate immune responses against parasites through the secretion of cytokines, such as IL-4, IL-5, IL-13, which in turn promote eosinophil and mast cell activation, and IL-10, which suppress Th1. Th17 express CCR6 and produce mostly IL-17, which enhances neutrophil activity against extracellular bacteria and fungi. TfH express CXCR5 and produce IL-21 after engaging with Ag specific B cells, leading to the production of high-affinity antibodies. iTreg mediate specific suppression of T-cells through a variety of mechanisms discussed in Section 3. Finally, $CD8^+$ T_{Eff} eliminate intracellular pathogens, such as virus, mostly through the production of cytokines, such as IFN-γ and TNF-α, and/or trough cytolysis mediated by perforin, granzymes A/B, and CD95L[56].

Memory T-cells comprise long-lived Ag-specific cells and represent the dominant population in the peripheral blood of human adults[43]. Memory subsets are classically identified as $CD45RA^-CD45RO^+$ T-cells, which include T_{CM} and T_{EM}, distinguished by the expression of homing receptors CD62L/CCR7, and the proliferative or effector functions[57–59]. More recently, a subset of T-cells with memory and stem cell-like properties (T_{SCM}) has been identified. Despite the co-expression of antigens typically associated to naïve cells, T_{SCM} exhibit functional characteristics of memory and effector cells, thus being included within the memory compartment.

T_{CM} are $CD45RO^+CD45RA^-$ and have a high expression of the lymph-node-homing molecules CD62L and CCR7, which enable the homing to the T-cell area in SLOs. Here,

upon Ag re-exposure, T_{CM} become rapidly activated and capable of proliferating and secreting high levels of IL-2, which promotes the differentiation into T_{EFF}[58][60].

T_{EM} are also $CD45RO^{+}CD45RA^{-}$, but lack CD62L and CCR7 expression. They are memory cells that survive the apoptotic contraction of T_{EFF} after a primary immune response, and traffic to sites of infection in peripheral non-lymphoid tissues, where they provide an immediate effector response upon reinfection[61,62].

The relative proportions of T_{CM} and T_{EM} in healthy peripheral blood varies in $CD4^{+}$ and $CD8^{+}$ compartments, with a predominance of T_{CM} within $CD4^{+}$ T-cells and T_{EM} within $CD8^{+}$ T-cells[58]. The second most prevalent $CD8^{+}$ T-cell subset correspond to T_{EM} that re-express CD45RA ($CD62L^{-}CCR7^{-}CD45RA^{+}$), named **Terminal Effector Memory Re-expressing CD45RA (T_{EMRA})**[63]. T_{EMRA} arise particularly after a viral infection and exhibit characteristics of terminal differentiation, such as enhanced expression of effector and senescence molecules, high sensitivity to apoptosis and short telomeres[58][64]. T_{EMRA} cells within $CD4^{+}$ T-cells are less frequent, and their role remains to be clarified.

T_{SCM} is a rare subset, accounting for approximately 2-4% of the total $CD4^{+}$ and $CD8^{+}$ T-cells in human peripheral blood[65]. **T_{SCM}** can be identified by the co-expression of naïve molecules, such as CD45RA, CD62L, CCR7 and CD27, and memory molecules, such as CD95, IL-2Rβ (CD122), C-X-C Motif Chemokine Receptor 3 (CXCR3) and integrin CD11a[52][65]. CD95 is a member of the TNF receptor family which promotes apoptosis[66], being used as a "death-receptor" as described in Section 2.3; it is upregulated in activated T-cells and persists in all memory cells. CD122 is the common β chain of IL-2 and IL-15 receptors and is involved in the survival of memory T-cells[67]. CXCR3 and CD11a regulate lymphocyte migration and homing to inflammatory sites and SLOs, respectively[65].

Functionally, T_{SCM} are distinguished by their extensive proliferation, with high expression levels of the intracellular marker Ki-67, their capacity for self-renewal and the rapid differentiation into memory subsets (multipotency) upon antigenic or homeostatic stimulation[52]. These characteristics provide a potential reservoir for memory T-cells throughout life.

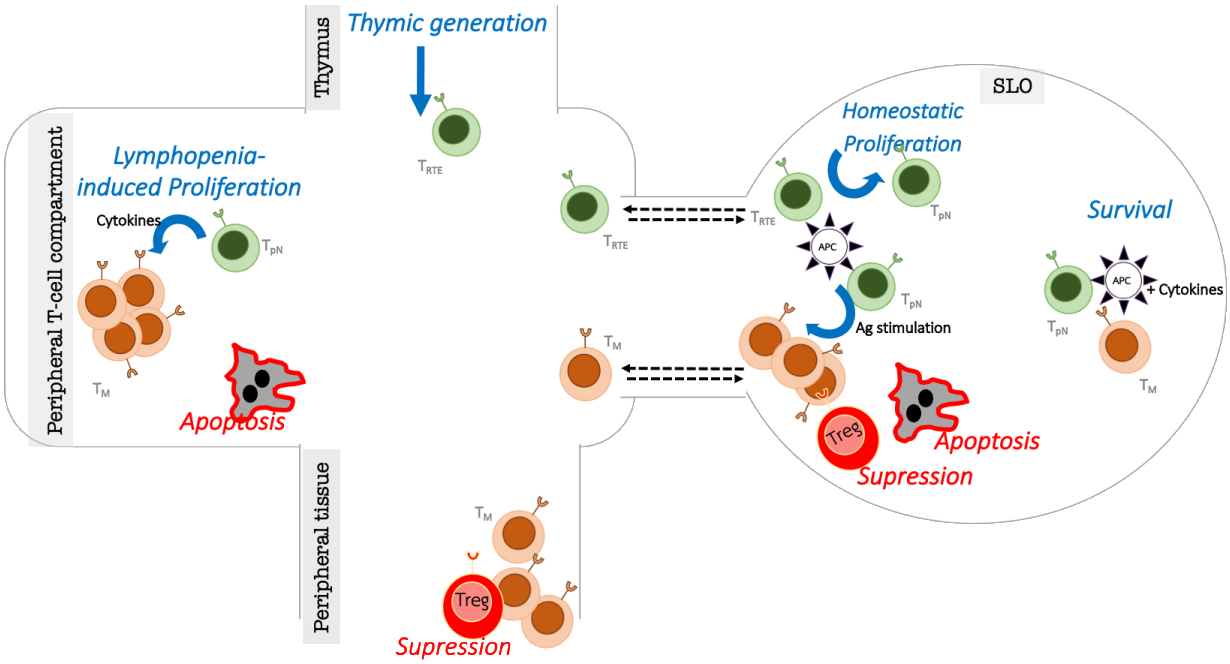
T_{SCM} were originally described in mouse models of human chronic Graft-*versus*-Host Disease (cGvHD)[68], where host-reactive $CD8^+$ donor T-cells were identified in transplant recipients with ongoing GvHD. T_{SCM} were shown to be able to undergo self-renewal and to generate all memory and effector T-cells. In humans, the stem-cell-like proprieties of T_{SCM} have been initially demonstrated *in vitro*[65] by Gattinoni *et al.*, and further confirmed *in vivo* in haploidentical-HSCT patient groups using post-transplant cyclophosphamide[69–71]. For both groups, T_{SCM} prevailed in the early post-transplant period, with an increased proliferation rate, maintaining their pool size for long periods after transplant. The proliferation of T_{SCM} correlated with the rapid recovery of both T_{EFF} and memory subsets, fulfilling the criteria for memory stem cells. This observation was validated by results from *in vivo* fate mapping, suggesting a linear differentiation path in which naïve T-cells give rise to T_{SCM}, which in turn differentiate into T_{CM} and T_{EM} subsets[72–74].

The capacity of T_{SCM} to induce lethal cGvHD in mouse models[68], the association with autoimmune diseases[75,76], and the capacity to generate and sustain T_{EFF} and memory cells suggest a potential role for this subset in the development of cGvHD in humans, which still remains to be elucidated.

2. Peripheral T-cell homeostasis in healthy adults

The peripheral αβ T-cell compartment remains stable throughout life in spite of constant immune disturbances, such as infections[77,78]. This stability is achieved through homeostasis, a term used to define the tendency and ability to resist change and preserve internal balance[77,79]. Although the aim of homeostasis is to achieve stability, its nature is not static but rather dynamic, ensuring a continuous adjustment to changing conditions[77]. Within the T-cell pool, homeostasis is controlled by sensing and feedback mechanisms that regulate T-cell generation, maintenance, and death (Figure 2)[78][80,81].

Figure 2. Overview of the main mechanisms of peripheral T-cell homeostasis in humans



Peripheral T-cell homeostasis reflects a precise balance between mechanisms of T-cell generation and maintenance (indicated in blue), and mechanisms of T-cell contraction (indicated in red). The generation of naïve T-cells occurs predominantly in the thymus and is maintained through mechanisms of cell survival and proliferation. Memory T-cells are generated either from primed naïve cells in SLOs, or through mechanisms of proliferation. Following an immune response, T-cell expansion is controlled by mechanisms of apoptosis and cell-mediated suppression by $CD4^+$ regulatory T-cells (Treg)[82]. Green and orange cells represent naïve and memory T-cells, respectively. Adapted from Bains *et al.*[30].

Despite the tightly regulated network of homeostatic mechanisms within the T-cell pool, certain immune challenges are too disruptive and require an exaggerated homeostatic response in order to allow for the return to steady-state conditions. A clear example is the drastic reduction in T-cell numbers that occurs after cytoreductive chemotherapy or T-cell depletion in allo-HSCT, as described in Section 4[83].

This Section provides an overview of the homeostatic mechanisms required for the maintenance of a stable peripheral T-cell compartment in humans, under steady state conditions.

2.1. T-cell generation

2.1.1. Thymus: the primary site of T-cell development

The thymus microenvironment supports and guides the generation of a functional and diverse repertoire of self-restricted and self-tolerant T-cells[84–86].

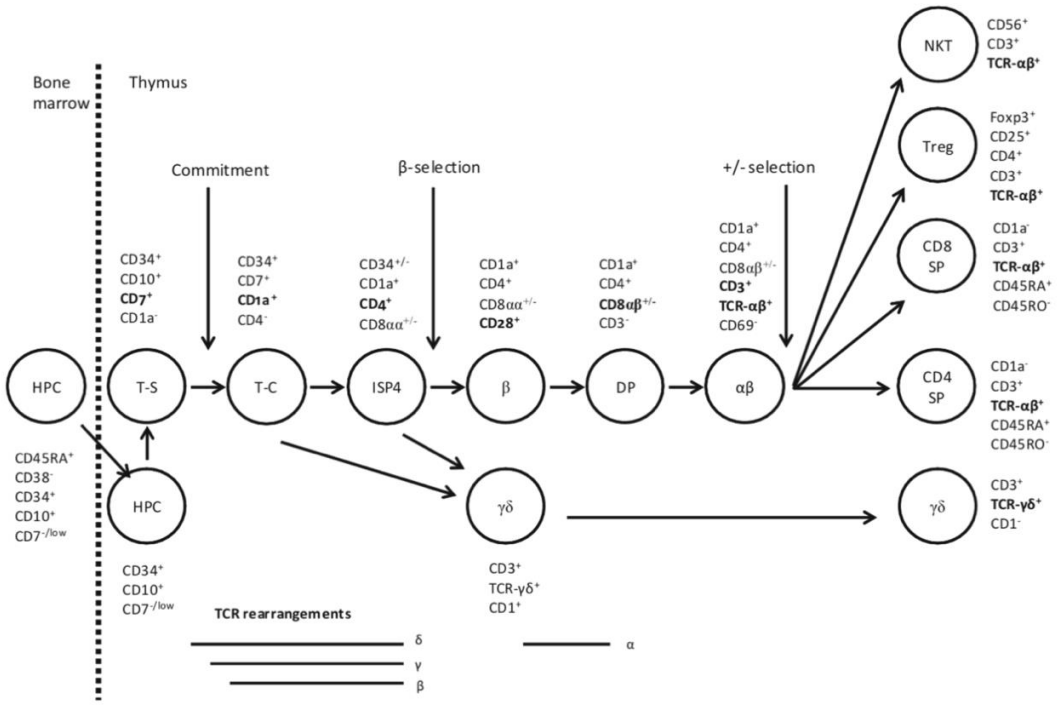
T-cell ontogeny starts in the embryonic period of fetal development, with the entry into the thymus of Early T-cell lineage Progenitors (ETPs) derived from Bone Marrow (BM) Hematopoietic Stem Cells (HSCs) (Figure 3)[87]. Within the thymic cortex, ETPs gradually develop , through a dynamic process primarily regulated by T-cell receptor (TCR) stimulation and cytokine signaling, namely IL-7[88]. Current understanding of this process in humans stems mostly from *in vivo* and *in vitro* assays with humanized mice models[4][34][89]. During T-cell development, thymocytes undergo the sequential expression of TCR, CD3 molecule, and co-receptors CD4 and CD8[85]. These latter molecules are used to identify the different maturation stages of thymocytes in the cortex, from the least differentiated double-negative (DN; $CD4^-CD8^-$) to the most differentiated single positive (SP; $CD4^+CD8^-$ or $CD4^-CD8^+$). TCRβ gene selection leads to generation of double positive (DP) cells, expressing both CD4 and CD8 co-receptors. At this stage, TCRδ, γ and α locus undergo sequential rearrangements, culminating either in αβ or γδ TCR[90].
Once αβ TCR is fully expressed at the surface of the thymocyte, the selection process that will shape the peripheral T-cell repertoire begins[84,85]. The diversity of the TCR repertoire reflects the ability of the immune system to recognize and fight a wide variety of antigens[91].

In the thymus, diversity is generated by somatic recombination. The constant (C) and variable (V) domains of the α and β chains are assembled via imprecise binding of the V and joining (J) regions along with random nucleotide additions and deletions at the V-D(diversity)-J junctions[92]. Most of the variability lies in the third complementary determining regions (CDR3), encoded by the V(D)J junction, which bind to the self-peptide/Major Histocompatibility Complex (spMHC) on the APC[93].

TCR repertoire generation is followed by TCR-lineage commitment, in which DP thymocytes with a TCR specificity for MHC class I retain CD8, while those with specificity for MHC class II retain CD4[94]. $CD4^+$ T-cell fate decision towards clonal deletion (also called negative selection) or the diversion into $CD4^+$ regulatory T-cells (Treg) or $CD4^+$conventional T-cell (Tcon) lineage is dictated by TCR affinity and/or avidity for self-peptides presented by thymic APC[95]. Hence, weak TCR signals support survival/positive selection[95], whereas strong/high-affinity signals support the apoptotic elimination/negative selection of potentially autoreactive thymocytes, also known as central tolerance[96–98]. While positive selection occurs in the thymic cortex, negative selection occurs mainly in the thymic medulla, where APCs express a gene known as autoimmune regulator (AIRE) that controls the expression of a diverse array of tissue-restricted Ag[99,100].

Within the thymic medulla, $CD4^+CD8^-$ thymocytes that upregulate the transcription factor Forkhead Box P3 (Foxp3) have been identified[101–104]. These correspond to natural/thymic Ag-specific $CD4^+$ Tregs (tTreg)[105]. Despite some controversy, several studies support TCR signaling strength (high affinity or extended ligand exposure) and signaling from gamma chain (γ_C) cytokines, particularly IL-2 and IL-15, as major mechanisms involved in Treg lineage commitment in the human thymus[106–109]. The fundamental role of $CD4^+$ Tregs and IL-2 in immunity and tolerance are described in Section 3.

Figure 3. The different stages of human T-cell development: a schematic overview



Within the thymus, thymocytes undergo a multiple-step maturation process, from TCR gene rearrangement to T-cell selection, providing a highly diverse, antigen-specific, and self-tolerant naïve T-cell repertoire. HPC: hematopoietic progenitor cell; T-S: T-lineage specified progenitor; T-C: T-cell committed progenitor; ISP4: immature single positive CD4$^+$; β: T-cell receptor-β chain; DP: double positive; αβ: αβ T-cell receptor; NKT: natural killer T-cell; Treg: regulatory T-cell: CD8 SP: CD8$^+$ single positive, CD4 SP: CD4$^+$ single positive, γδ: γδ T-cell receptor. From Van de Walle *et al.* [87].

2.1.2. Quantification of thymic function

The contribution of the thymus to the T-cell pool in humans has been extensively studied through the quantification of CD31, PTK7 and TRECs, surrogates of T_{RTE} as summarized in Section 1.1. These studies have shown that the thymus is active until puberty, then progressively declines with ageing[110–113]. This decline is associated with a reduction in the diversity of T-cell repertoire, resulting in decreased immune responses to vaccination, increased vulnerability to infections, and increased risk of autoimmunity and cancer in elderly individuals[114,115].

In addition to thymic involution that occurs with ageing, damage to the thymic microenvironment may also be caused by external factors such as infection[116,117],

radiation, immunosuppressive therapy, and GvHD following allo-HSCT[111][118,119]. In these settings, the thymus has the remarkable capacity to regenerate, restoring thymopoiesis and naïve T-cell numbers[117]. This regenerative capacity depends on factors such as patient´s age[86][118]. Thus, in children and young adults a compensatory enlargement of the thymus, known as rebound thymic hyperplasia, may occur[120,121]; while in adults, the regenerative capacity of the thymus is reduced and imprecise, contributing to the development of autoimmunity and immunodeficiencies[122,123].

Despite the decreased number of T_{RTE} with ageing , the size of the naïve T-cell pool remains stable, suggesting the existence of post-thymic mechanisms to maintain T-cell numbers[124]. Indeed, in humans, contrary to mice, the naïve T-cell pool is maintained almost exclusively through mechanisms of peripheral T-cell survival and proliferation, as will be further addressed[125,126].

2.2. T-cell maintenance: survival and proliferation

The current understanding of the mechanisms underlying T-cell maintenance in humans relies mostly from the analysis of T-cell telomere length and levels of deuterium incorporated by T-cells *in vivo*[43][127]. These data have shown that, under steady state conditions, naïve T-cells have increased survival and lower proliferation rate compared to memory cells, suggesting an independent homeostatic control of naïve and memory subsets.

This Section describes the mechanisms of homeostatic survival and proliferation within the naïve and memory subsets.

Naïve T-cells recirculate through SLOs for long periods of time, relying on continuous survival mechanisms[126][128]. Upon Ag contact, naïve T-cells are activated and differentiate into memory cells, thus shrinking the naïve T-cell pool, which can be restored through *de novo* thymic production and homeostatic/cytokine driven proliferation[129,130]. Both the long-term survival and proliferation of naïve T- cells are sustained by homeostatic signals, including IL-7[131] and spMHC contact on the surface of APC[132–134].

IL-7 is a member of the common γ_C family of cytokines, that includes IL-2, IL-4, IL-15 and IL-21, and plays a central role on $CD4^+$ and $CD8^+$ T-cell development [24][88][129] and maintenance[135]. The IL-7 receptor (IL-7R) is composed of the common γ_C subunit (CD132)[136,137] and the IL-7Rα (CD127)[138], mostly expressed on naïve Tcon[139]. IL-7 biding induces several pro-survival pathways[140,141], particularly through the activation of the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, that up-regulates the anti-apoptotic Bcl-2 protein[141]. Treatment with IL-7 in humans has been tested in different lymphopenic conditions, where it mostly favors the expansion of T_N and $T_{SCM,}$ Tcon and $CD8^+$ T-cells, while the effect on naïve Tregs is still questionable[74][142,143].

The propensity of naïve T-cells to undergo survival or proliferation depends on the strength of TCR signaling and the plasma levels of IL-7, which inversely correlate with T-cell counts[139][144]. Therefore, under lymphoreplete conditions, limiting IL-7 signaling and weak TCR-spMHC engagement impose limits on proliferation, as determined by the low expression of Ki-67[2][81]. Conversely, under lymphopenic conditions, increased IL-7 availability amplifies the TCR/spMHC interaction, resulting in naïve T-cell increased proliferation, primarily within T-cons[145,146].

Two proliferation patterns have been identified on naïve T-cells following lymphodepletion, the homeostatic proliferation (HP) and the "*spontaneous"/* lymphopenia-induced proliferation (LIP)[147].

HP is a slow homeostatic process, that occurs under steady state conditions, primarily driven by IL-7 and low-affinity biding to spMHC[148]. Through HP naïve T-cells slowly proliferate, thus restoring the naïve pool[141][149].

LIP on the other hand, is a rapid homeostatic process observed in severe lymphopenia, following cytoreductive radio/chemotherapy, T-cell depleting therapy or severe viral infections[150]. It is mostly driven by homeostatic cytokines, such as IL-7 and IL-15, or high affinity spMHC recognition, and results in polyclonal expansion and phenotype switching from naïve to memory and effector T-cells[151–153]. The current understanding on LIP derives from experiments in which naïve T-cells were adoptively transferred into

immune-deficient mice[154]. In this setting, naïve T-cells show high proliferation rate and rapidly acquire characteristics similar to memory cells[146]. Extrapolation of these results to humans may be misleading, considering that in mice, unlike humans, naïve T-cells are primarily sustained by the thymus[126]. To overcome this limitation, humanized mice models have been widely used[155], with the recent example of the Severe Combined Immunodeficiency (SCID) mice transplanted of human fetal thymus, liver, and $CD34^+$ fetal liver cells[156]. This model confirms the existence of two patterns of proliferation within naïve T-cells and reinforce the importance of spMHC for LIP.

Memory T-cells are mostly generated after Ag exposure and are maintained through homeostatic mechanisms for long periods. The longevity of human memory T-cells has been shown in vaccination studies[157]; however, unlike naïve T-cells, the mechanisms for memory T-cell maintenance remain unclear, mostly due to the heterogeneity of this pool and the predominance of memory cells in peripheral tissues[43][158].

Studies in mice show a persistent survival and proliferation of $CD4^+$ and $CD8^+$ memory T-cells in Ag-free/MHC deficient hosts[159,160], implying an important contribution of cytokines, such as IL-7 and IL-15, to the maintenance of memory T-cells. IL-7 regulates both $CD4^+$ and $CD8^+$ naïve and memory T-cell proliferation, while IL-15 primarily regulates memory $CD8^+$ proliferation[161,162], with little effect on memory $CD4^+$ T-cells[163]. This difference seems to be related to the higher expression of IL-15R β-chain (CD122) on $CD8^+$ memory cells[163] and may explain the preferential expansion of this population over $CD4^+$ T-cells in the post-transplant period[164,165]

While mouse studies suggest less stringent requirements for memory T-cell survival and proliferation in comparison to naïve subsets, these results do not truly recapitulate the homeostasis of memory T-cells in humans, where the exposure to pathogens is much more diverse and prolonged[43]. Transcriptome analysis of human memory T-cells shows upregulation of genes encoding TCR-activation markers, including multiple MHC class II molecules, chemokine receptors, CD95 and effector molecules [166]. This data, although scarce, suggests that a constitutive TCR and chemokine signaling is required for human memory T-cell maintenance.

2.3. T-cell apoptosis: regulation of T-cell tolerance and homeostasis

Apoptosis is the most common pathway for cell death in the immune system[167]. Within T-cells, apoptosis plays a fundamental role in the induction and maintenance of T-cell tolerance and peripheral T-cell homeostasis[168]. Indeed, during T-cell development in the thymus, apoptosis regulates the elimination of autoreactive T-cells (negative selection), thereby establishing central tolerance to the self [169]. Likewise, in the peripheral blood, apoptosis eliminates self-reactive and effector T-cells that are overproliferating during immune responses, thus preserving peripheral tolerance and restoring T-cell homeostasis[170].

There are two major apoptotic mechanisms in the immune system[171], the "extrinsic" or *death-receptor pathway*[172], and the "*intrinsic*" or *mitochondrial pathway*[173]. Both mechanisms converge on the activation of caspase-3, that induces proteolysis, with subsequent morphological changes that culminate in the generation of apoptotic bodies, further eliminated by phagocytosis[174].

The *death-receptor pathway* initiates apoptosis after biding of external stimuli to "death-receptors" on the cell surface. CD95 (the pro apoptotic receptor FAS) and its ligand, membrane-bound or soluble CD95L (FasL), is the best representative death receptor/death ligand system in humans, and mediates apoptosis through activation of caspase 8[168].

In the past decade, it became apparent that CD95 has a dual function, since it also has anti-apoptotic effects, depending on the cellular context. Indeed, low levels of CD95L positively costimulate naïve T-cells, supporting its expansion. In contrast, high levels of CD95L during an immune response, result in the induction of apoptosis in activated cells[66].

Repeated cell activation leads to cell death, known as activation-induced cell death (AICD)[170]. AICD eliminates most of the Ag-specific T-cells that expand during immune responses, allowing the termination of immune responses and the restoration of T-cell numbers[175]. Furthermore, AICD contributes to the development of peripheral self-tolerance, by deletion of autoreactive T-cells[176].

The *mitochondrial pathway* initiates apoptosis after an intrinsic stimuli that causes DNA damage, such as radiation, viral infections or chemotherapy[172][177]. This damage acts directly on the mitochondrial outer membrane, disrupting its permeability with the subsequent release of cytochrome C[178]. This pathway is modulated by interactions between a large number of Bcl-2 family proteins[179], that include pro-apoptotic proteins such as Bad, Bax, and Bim, and anti-apoptotic proteins, such as Bcl-2 and Bcl-xl[180,181]. It is the balance between pro-apoptotic and anti-apoptotic Bcl-2 proteins that determines the susceptible to apoptosis.

Studies *in vitro* have shown that activated T-cells, that have an increased susceptibility to apoptosis, express lower intracellular levels of Bcl-2 and increased levels of Bim[182]. In humans, the Bcl-2-regulated apoptosis is mostly involved in the deletion of self-reactive T-cells during T-cell development and in the periphery[183]. During T-cell development in the thymus, the high-affinity interaction between TCR/spMHC leads to the transcription of Bim that deactivates the anti-apoptotic regulators Bcl-2 and Bcl-xL, culminating in cell death/negative selection[169]. A recent study assessed the T-cell susceptibility/ “priming” to Bcl-2-regulated apoptosis, measuring the depolarization of the mitochondrial membrane after challenge with a panel of BH3 peptides (peptides derived from the Bcl-2 homology 3 domains of pro-apoptotic Bcl-2 family proteins). The results show an increase in priming in Treg and $CD8^+$ T-cells compared to Tcon, in both healthy donors and patients[184].

3. $CD4^+CD25^+Foxp3^+$ regulatory T-cells

$CD4^+CD25^+Foxp3^+$ regulatory T-cells (Tregs) are a specialized subset of T-cells with immunosuppressive function that play a critical role in controlling the expansion of self- and alloreactive T-cells, therefore preventing autoimmunity and excessive immune response[185,186].

Treg research began decades ago with experiments that suggested the existence of a T-cell population with suppressive function[187]. However, the identification of this population was hindered by the lack of specific phenotypic markers and the diversity of suppressor immune mechanisms. In the late 1990s, Sakaguchi and colleagues identified in several studies a small subset of $CD4^+$ T-cells that constitutively expressed high levels of the α-chain (CD25) of the IL-2 receptor[188], and had the capacity to transfer tolerance in animals that would otherwise develop autoimmunity after neonatal thymectomy[188]. Furthers studies have shown that these cells specifically co-express the transcription factor Foxp3, a prerequisite for Treg lineage commitment, maintenance and function[105][189]. Foxp3 gene expression is regulated by epigenetic modifications of Conserved Non-coding Sequences (CNS) presented in four elements[190] and remains the most reliable Treg marker in humans[105].

Differently from the mouse, the sole expression of CD25 and Foxp3 are not sufficient to characterize human $CD4^+$ Tregs, since these molecules can be transiently expressed on activated human Tcons upon TCR activation[191,192]. It is now well stablished that human $CD4^+$ Tregs constitutively express high levels of Cytotoxic T-lymphocyte antigen 4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor (GITR); however, similarly to CD25 and Foxp3, the use of these markers to identify Tregs is insufficient due to their concomitant expression on activated Tcons.

The relatively low frequency of $CD4^+$ Tregs in human peripheral blood, combined with the lack of specific surface markers, hamper the exact identification of this T-cell population, which is currently recognized as **$CD4^+CD25^{hi}CD127^{low}Foxp3^+$**[193].

3.1 Treg heterogeneity

Contrary to the initial studies characterizing Tregs as an homogeneous population, it is now well stablished that the pool of human $CD4^+$ Tregs is highly heterogeneous in phenotype, function and epigenetic profile[194].

In the past decade, the combination of CD25 and CD45RA has been used to distinguish three human Treg subsets: $CD25^{low}Foxp3^{low}CD45RA^+$ naïve/resting Tregs, $CD25^{hi}Foxp3^{hi}CD45RA^-/RO^+$ memory/effector Tregs, and $CD4^+CD25^{low}Foxp3^{low}CD45RA^-/RO^+$ Tregs, which correspond to pTregs that transitively express low levels of Foxp3[195]. More recently, CD15s (sialyl Lewis x) was identified as a biomarker of the most suppressive effector Tregs[196]. The co-expression of CD15s and CD45RA allow the identification of naïve $CD45RA^+CD15s^-$ Tregs, highly suppressive $CD45RA^-CD15s^+$ effector Tregs and a non-suppressive $CD45RA^-CD15s^-$ Tregs. Other markers, such as HLA-DR, ICOS and Helios, have been proposed in order to distinguish pTreg and tTregs, since their expression is higher in tTreg[194].

Furthermore, depending on the site of development, Tregs can be classified as natural occurring/ thymus-derived Tregs (tTreg) or peripheral-derived Tregs (pTregs)[197].

Thymus-derived Tregs correspond to the majority of $CD4^+$ Tregs in human peripheral blood, representing approximately 5% of total T lymphocytes[105]. tTregs develop in the thymus medulla and exit the thymus as antigen-primed naïve T-cells, with a high TCR-spMHC affinity and a fully demethylated CNS2/Treg-Specific Demethylated Region (TSDR)[198]. The high affinity TCR increases the sensitivity of Tregs to self-peptides or to microbial antigens cross-reactive with the self[199], enabling rapid activation and suppression even at low Treg numbers. The DNA demethylation at the TSDR warrants a stable expression of the Foxp3 gene, maintaining the functional activity and stability of tTreg[198].

Peripheral-derived Tregs differentiate from naïve Tcons that are present in SLOs and peripheral tissues, such as intestinal mucosa, after recognition of tissue-self-peptides or

foreign antigens not encountered in the thymus (e.g., allergens, food, microbiota, tumor-antigens)[200]. Following antigenic stimulation and under low levels of co-stimulatory molecules, such as TGF-β and IL-2, there is an upregulation of Foxp3 in Tcons, resulting in pTreg induction[201,202]. Despite Foxp3 expression, human pTreg display a TSDR incompletely demethylated, resulting in a less stable Foxp3 expression[198] and the possibility of effector function under inflammatory conditions[191].

Phenotypic markers distinguishing tTregs from pTregs have not been identified yet, making it difficult to measure the relative contributions of each subset to the peripheral Treg pool, which would be particularly important in the context of immunological diseases, cancer and infection. Besides phenotypic characterization, there are Treg-specific molecular markers that contribute to Foxp3 expression, that were proposed for their ability to distinguish tTreg from pTregs[190][203]. As an example, DNA demethylation of both the TSDR and the Foxp3 promoter have been demonstrated to be important for Foxp3 expression and tTreg development, lineage commitment and suppressive phenotype[204].

3.2 Treg Function: immune tolerance and homeostasis

Thymus-derived Tregs play an indispensable role in maintaining immunological peripheral tolerance and homeostasis[185][188][205].

Peripheral tolerance defines the state of unresponsiveness of self-reactive T-cells that escape central tolerance in the thymus. In humans, the role of Tregs in maintaining peripheral tolerance can be illustrated by the loss-of-function mutations in the Foxp3 gene that results in Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome, a fatal autoimmune-inflammatory disease[206]. Decreased Treg activity is also implicated in the development of inflammatory diseases[207], autoimmune liver diseases[208,209], allergy/asthma[210], tumor-growth, and post-transplant cGvHD (described in Section 4)[211,212].

Immune homeostasis defines a state of equilibrium in which the number, diversity and function of lymphocytes are maintained. Tregs have a dominant role in the regulation of immune homeostasis, by recognizing and inhibiting several target cells involved in both innate and adaptive immune responses[213,214].

The mechanisms underlying Treg-mediated T-cell suppression in humans are still not entirely clarified. Conventionally, these can be classified as contact-dependent or contact-independent mechanisms[215–217]:

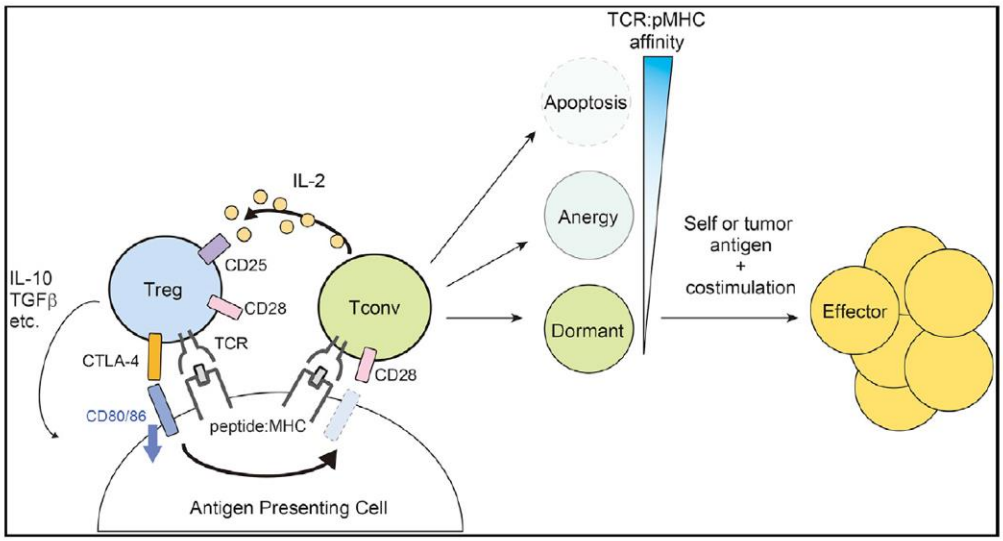
Contact-dependent mechanisms:

Upon antigenic stimulation, antigen-specific Tregs directly suppress the function of effector $CD4^+$ or $CD8^+$ T-cells and/or APCs, which in turn are required for the activation of the former. This direct suppression can be mediated by cytolytic enzymes, granzymes A and B, through a perforin dependent or independent pathway, leading to effector cell apoptosis[218]. Alternatively, activated Tregs downmodulate APC function by interfering with co-stimulation mediated by the CTLA-4/CD28 pathway[219]. The inhibitory protein CTLA-4 on the Treg surface competes with the costimulatory molecule CD28 for the same APC ligands (CD80 and CD86). This leads to reduced CD28-mediated signaling, which is required for the activation of effector T-cells[220]. Under deprivation of CD28 costimulatory signal, T-cells with high-affinity TCR/spMHC die by apoptosis, intermediate-affinity are driven to anergy (functionally inactivated), and low affinity survive and stay dormant for long periods. This mechanism ensure long-term suppression and stable tolerance (Figure 4)[194].

Contact-independent mechanisms:

Human Tregs can also suppress the effector T-cell function or the APC maturation indirectly, through synthesis of inhibitory cytokines that confer suppressive capacity to other immune cells, thus promoting the so-called infectious tolerance[221]. Examples of these molecules are TGF-β[222], IL-10[193] and IL-35[223]. Alternatively, Tregs can modify the microenvironment through an increased uptake of IL-2 by binding the high-affinity IL-2Rα, which is constitutively expressed at high levels on Treg[224]. Such mechanism has been demonstrated *in vitro*, where effector T-cells can be driven to anergy and apoptosis due to IL-2 deprivation, but its role *in vivo* remains controversial[225].

Figure 4. Mechanisms of Treg-mediated T-cell suppression and induction of tolerance



Human CD4+ Tregs can control the cell fate of effector T-cells through several mechanisms conventionally classified as cell-contact or soluble mechanisms. From Wing *et al.*[194].

3.3 Homeostatic maintenance of Tregs

The well established association between dysfunction and/or deficiency of CD4+ Tregs and development of autoimmune and inflammatory diseases has led to a growing interest in exploring the factors and mechanisms that control Treg homeostasis[226] for clinical applications.

The most common factors and mechanisms involved in Treg homeostasis include a continuous and weak TCR-spMHC engagement in SLOs[227]; co-stimulatory signals, namely those mediated by CD28-CD80/86 interactions[228]; and the effects of cytokines, such as IL-2 and IL-7[229].

The relative contribution of each factor for Treg homeostasis varies according to subset maturity and tissue distribution, as validated in preclinical and clinical studies. In mice, the ablation of TCR signal in Tregs results in increased naïve subsets and decreased effector and memory subsets, followed by reduced suppressive function and development of autoimmunity[227]. Similarly, the blockade of CD28-CD80/CD86 interaction and the deletion of IL-2Rα from mature Tregs resulted in decreased Treg proliferation and survival, with subsequent exacerbation of autoimmunity[228–230].

IL-2 has a critical role in T-cell homeostasis. It regulates the development and survival of Tregs in the thymus[106], and the proliferation and maintenance of Tregs in the periphery[231,232]. IL-2 also promotes the proliferation and differentiation of Tcons[233], regulates Th1 and Th2 fate decisions in activated $CD4^+$ T-cells, and interferes with gamma interferon synthesis by $CD8^+$ T-cells[234]. Although IL-2 is mainly produced by activated Tcons, it can act on both Treg and Tcon populations, as both express the IL-2R[235]. The high affinity IL-2R is comprised of three subunits: the α (CD25) chain[236] and the common γ(CD132) and β (CD122) chains, that correspond to the intermediate-affinity receptor. Signaling through the IL-2R upregulates the expression of the α chain (CD25) through STAT5 activation, providing a positive feedback mechanism that favors continued IL-2 secretion by Tcons[233]. In this way, Tregs and Tcons are dynamically linked and reciprocally control each other to maintain immune homeostasis. Recent studies also suggest a critical role for IL-2 in Treg function, as *in vitro* ablation of IL-2Rα or β led to severe autoimmunity and allergy[225], similary to the absence of Foxp3.

The high expression of IL-2Rα in Tregs provides them with increased sensitivity for IL-2. Based on this competitive advantage, clinical studies have tested the administration of low-doses rIL-2 to selectively expand Tregs in several clinical settings. Recently, the group from Dana-Farber Cancer Institute (Koreth *et al*) conducted phase 1 and phase 2 studies using rIL-2 in cGvHD patients. The work aimed to establish whether sub-cutaneous daily low-dose rIL-2 administered for 8 to 12 weeks would have any clinical effect in patients with steroid dependent/refractory (SR) cGvHD, and whether there was any correlation with Treg counts and function. In these trials, approximately 50-60% of the patients showed positive clinical outcome, allowing in some cases, for steroid doses to be tapered. In addition, all patients had a significant increase in Treg counts[237–240]. Predictors of clinical response included earlier rIL-2 initiation and a higher Treg:Tcon ratio at baseline and after week 1 of rIL-2 therapy[240].

The dependency on cytokines, TCR and co-stimulatory signals for Treg homeostasis has been shown for both naïve and memory subsets. The mechanisms controlling effector Treg homeostasis have not been studied in such detail, as this cell subset resides predominantly in non-lymphoid peripheral tissues, which are not as easily accessible as the peripheral blood[241].

4. Allogeneic hematopoietic stem cell transplantation: a disruption in T-cell homeostasis and tolerance

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative form of immunotherapy for patients with hematologic malignancies, immune deficiencies and bone marrow failure syndromes. The number of allo-HSCT/year continues to increase and over a million of HSCT have been reported worldwide in the last six decades[242–244].

The main advantage of allo-HSCT over high-dose chemotherapy is the allo-reactive effect of infused donor T-cells against tumor antigens, that ensure the eradication or long-term control of residual malignant cells, a phenomena known as Graft-*versus*-Tumor effect (GvT)[245]. However, the continued interaction between donor derived T-cells and major or minor histocompatibility antigens (mHA) on recipient tissues requires the prescription of continued immunosuppressive therapy to prevent rejection. Commonly used immunomodulatory drugs include calcineurin inhibitors, such as cyclosporine A (CsA) or tacrolimus (TAC), mammalian Target of Rapamycin (mTOR) inhibitors, such as sirolimus (SIR), mycophenolate mofetil (MMF) and methotrexate (MTX). However, these drugs compromise the immune recovery[246,247]. Besides, the preparative/conditioning regimen used for allo-HSCT can hinder the thymic function and limit the generation of *de novo* thymic naïve T-cells[248,249].This in turn, results in loss of TCR repertoire diversity and poor clinical outcomes[250].

Initial clinical studies on the immune reconstitution after allo-HSCT have provided evidence that T-cell recovery has a significant effect on the success of allo-HSCT[251,252]. Our understanding of post-transplant T-cell immune reconstitution has improved over the past decade and is now well established that T-cell immunity post-transplant is significantly delayed for months or even years, as compared to innate immunity[164][249][253–255]. This delayed recovery leads to uncontrolled immune responses to self and non-self, and the development of further long-term complications such as cGvHD and opportunistic infections[256], with increased morbidity and mortality[257].

T-cell reconstitution post transplant is a long-term process that occurs trough two distinct pathways: the expansion of infused T-cells contained in the stem cell graft and the development of recent thymic emigrants (RTE) from donor hematopoietic stem cells. In patients who receive T-cell replete stem cell grafts, the initial recovery is primarily driven by the homeostatic proliferation of transferred donor T-cells infused in the graft, in response to persistent lymphopenia (LIP) and/or to host allo-antigens[258]. Here, there is a preferential expansion of memory T-cells, which causes the contraction and skewing of the TCR repertoire, resulting in decreased immune response to a broad range of antigens[145][259].The second pathway occurs three to six months post-transplant, and is characterized by *de novo* generation of thymic naïve T-cells from donor hematopoietic stem cells[118]. This latter pathway ensures the generation of a broad and self-tolerant T-cell repertoire, and eventually the complete recovery of the T-cell pool, allowing for the discontinuation of immunosuppressive therapy.

Both, the infused donor T-cells and the recent thymic emigrants, are subject to a variety of influences in the post-HSCT lymphopenic environment that can hamper the favorable T-cell reconstitution. In particular, the excess of IL-7 and IL-15[145][165][260] support the proliferation of naïve and memory Tcon and $CD8^+$ subsets, while the relative deficiency of IL-2 impair Treg reconstitution[258].

The clinical factors affecting T-cell homeostasis after allo-HSCT have been addressed and reviewed in several studies[258][261,262]. Such studies consistently show that advanced patient age[83][262], Human Leukocyte Antigen (HLA) - mismatching[263], conditioning with Anti-Thymocyte-Globulin (ATG)[264], irradiation administered as part of the conditioning regimen[164], and GvHD prophylaxis/development/treatment[246] lead to delayed T-cell recovery. These factors affect T-cell recovery through the dysregulation of thymic-dependent and/or independent pathway, and must always be considered when analyzing and comparing data on immune T-cell reconstitution post allo-HSCT.

4.1 cGvHD: a transplantation major challenge

The success of allo-HSCT is largely limited by the development of cGvHD, the most frequent long-term complication and the main cause of treatment-related mortality after transplant[265].

Chronic GvHD represents a pleomorphic syndrome with autoimmune features, characterized by abnormal immune responses to both auto and allo-antigens resulting in tissue and organ damage[266]. Although cGvHD is associated with a lower relapse rate, presumably because of the GvT effect, the occurrence of cGvHD is mostly associated to a decline in the quality of life due to impaired functional status, and an increased risk of infections, resulting in impaired survival[267].

Despite advances that have improved survival after allo-HSCT, cGvHD remains a leading cause of non-relapse mortality after transplant, due to unsatisfactory treatment. Corticosteroids are the standard of care for initial treatment, but are often not fully effective, and their long-term use leads to multiple complications[268]. Other immunomodulatory drugs, as previously stated, may be used in combination with corticosteroids to minimize the side effects, but no agent has yet demonstrated superiority to steroids alone in a randomized clinical trial[269].

The cumulative incidence of cGvHD at 12 months post-transplant is 40%, with a 37% mortality rate at 5 years[270,271]. Several trends in allogeneic transplantation justify the high incidence of cGvHD, including the use of HSCT in older patients, the predominant use of Peripheral Blood Stem Cells (PBSC) instead of bone marrow stem cells, and the increasing use of unrelated or HLA-mismatched donors.

Despite the advances in clinical practice, preventing and treating cGvHD remains challenging due to the limited understanding of the basic biology and the lack of biomarkers for the diagnosis and assessment of disease activity[266][272]. Insights into the pathophysiology of cGvHD have been gained from numerous preclinical and clinical studies which consistently show that cGvHD results from an imbalance between regulatory mechanisms that maintain immune tolerance and effector mechanisms that cause the disease[273,274].

There is currently a consensus on the main mechanisms involved in cGvHD development, which include[275]: (1) tissue and endothelial cell damage, caused by cytotoxic medications, acute GvHD (aGvHD) and infection[276], (2) thymic damage with emergence of self-reactive T-cells[119], (3) enhanced activation of effector populations and dysregulation of regulatory T-cells[273,274] (4) auto-antibody production by aberrant B-cells[277], and (5) propagation of tissue damage due to aberrant tissue repair and excessive macrophage activation, resulting in fibrotic lesions and irreversible organ dysfunction[278].

The enhanced activation of effector T-cells and the abnormal recovery of regulatory T-cells observed in cGvHD patients led to the development of different approaches to prevent and treat cGvHD[279]. *Ex vivo* graft manipulation with CD34 selection and T-cell depletion[280], and *in vivo* graft manipulation with antithymocyte globulin (ATG)[281,282] or alemtuzumab[283] are strategies that are associated with lower rates of cGvHD[284]. Similarly, the administration of post-transplant cyclophosphamide selectively depletes alloantigen-activated donor T-cells *in vivo* and allows engraftment of hematopoietic stem cells from HLA-mismatched donors[285]. Several preclinical and clinical studies have also suggested that enriching the graft with donor Tregs could decrease the risk of GvHD, while preserving GvT effect[286,287]. The fact that different approaches reduce, but do not completely abrogate cGvHD development is a testimony to the multifactorial nature of this syndrome.

Recent studies have supported a significant role for naïve T-cells in the initiation of cGvHD. This has been shown in mouse models of transplantation, where the infusion of unprimed naïve T-cells induced severe colitis, with a rapid decline in survival and weight loss[288,289]; the same was not observed with the infusion of memory T-cells. Clinical trials are currently being conducted to evaluate naïve-T-cell depletion from stem cell graft. The emerging results are very encouraging, showing a significantly reduced incidence of cGvHD, while allowing transferred memory T-cells to improve immune reconstitution and confer protective immunity[290–292]. These observations, together with the recent identification of T_{SCM} subset, co-expressing naïve molecules, further raise the need to clarify the role of this specific subset in cGvHD development.

All the previous findings show promising results for potential effective therapies for cGvHD based on depletion of allo-reactive T-cells and restoration of regulatory T-cells. Yet, understanding the detailed mechanisms involved in cGvHD biology remains an unmet need.

4.1.1. The role of Tregs in human cGvHD biology

The role of Tregs in the biology of cGvHD in humans has been an area of active investigation. However, the lack of reliable and specific markers to identify human Tregs, together with the use of different gating strategies for phenotypic analysis, and the effect of clinical variables on immune recovery, have hampered the interpretation of the available data[293,294]. Indeed, discrepant results were obtained from various clinical studies. While several reports have found a reduced number/ frequency of Tregs within the graft or in the peripheral blood of patients who develop cGvHD[273][295–297], few studies reported normal[298] or increased Treg counts/ frequency at the onset of cGvHD[299].

Despite conflicting results, prompt recovery of Treg homeostasis seems to prevent cGvHD, while also supporting the recovery of a broad T-cell repertoire[212][303]. These data suggest that a balanced recovery of Treg, Tcon and $CD8^+$ T-cells is needed to control alloimmunity and establish immune tolerance. However, the mechanisms that maintain this balance and regulate the recovery of each T-cell population *in vivo* are not fully understood[265,266].

The evidence suggesting a role for Tregs in cGvHD development has stimulated recent research on targeted therapies, including adoptive Treg transfer and low dose rIL-2 administration. Hence, low dose rIL-2 has led to significant clinical improvement of patients with SR cGvHD[240], while the infusion of donor Tregs has shown to successfully decrease the risk of cGvHD development[286,287]. In the last decade, the safety and feasibility of Treg-based cell therapy for GvHD prevention and treatment has been tested in several clinical trials[301–303]

Although adoptive Treg therapy has been mainly tested in GvHD prevention, ongoing and completed clinical trials investigate the safety and efficacy of polyclonal Treg infusion in the treatment of SR cGvHD[304,305]. In 2015, the international research consortium TREGeneration, coordinated by João Lacerda Lab in Lisbon, was created with the primary aim to explore the safety (phase 1) and preliminary efficacy (phase 2) of different donor-derived Treg products for the treatment of patients with SR cGvHD after allo-HSCT[306]. This research is running in parallel in several institutions, using different protocols for Treg preparation and/or administration. Results from the phase 1 study conducted at the DFCI-Boston were recently published. This group showed that a single infusion of polyclonal Treg-enriched lymphocytes, from the original stem cell donor, with daily low-dose IL-2 was safe and well tolerated, with clinical benefit, including in those with inadequate responses to IL-2 alone[307,308]. Furthermore, there was an increase in Treg repertoire diversity, with expansion and long-term persistence of infused Treg clonotypes.

AIMS AND WORKPLAN

Allogeneic HSCT remains the only curative therapeutic approach for many patients with hematological malignancies. The efficacy of transplantation is hampered by the profound immunosuppression and disturbance to T-cell homeostasis, which requires a timely and balanced reconstitution of both regulatory and effector T-cells. Impaired Treg reconstitution after transplant results in enhanced allo-reactivity and cGvHD development, which is the most relevant cause of late morbidity and mortality post allo-HSCT. Both the adoptive transfer of Tregs, and the infusion of rIL-2 mediate Treg expansion and cGvHD improvement; however, the factors and the endogenous mechanisms that regulate Treg homeostasis *in vivo* remain poorly defined.

The overall aim of this thesis was to **investigate the mechanisms and factors involved in the homeostasis of peripheral blood regulatory T-cells after allo-HSCT**, with the ultimate goal of learning new approaches to prevent and treat cGvHD.

Aim 1. Study *in vivo* the reconstitution of different T-cell subsets after allo-HSCT and identify factors that correlate with Treg inability to establish and maintain immune tolerance in cGvHD patients

Dysfunction of both, the thymus and the peripheral compartment, contribute to an unbalanced and poor recovery of the T-cell pool after allo-HSCT. In patients who develop cGvHD this disruption is more noticeable than in those who do not. We hypothesized that the impaired recovery of Tregs in patients who develop cGvHD results from a homeostatic imbalance that favors survival and expansion of effector $CD4^+$ and $CD8^+$ T-cells over $CD4^+$ regulatory T-cells. Moreover, we speculated that the naïve and memory subpopulations within regulatory and effector T-cells have a different pace of recovery and this may translate into an unbalanced T-cell compartment, leading to cGvHD development.

Workplan:

Considering the primary hypothesis, we prospectively monitored and compared the reconstitution of naïve and memory Treg, Tcon and $CD8^+$ T-cells in the peripheral blood of patients with hematologic malignancies undergoing allo-HSCT after a Reduced Intensity Conditioning regimen (RIC). A detailed phenotypic analysis was performed

using specific immunologic markers to access T-cell differentiation, neogenesis, proliferation and expression of the apoptosis-related proteins Bcl-2 and CD95, at fixed timepoints post transplant. This allowed us to perform the simultaneous characterization of the naïve and memory pool within regulatory and effector T-cells over time. For comparison, we also studied the peripheral blood from Healthy Donors (HD). Finally, we analysed the data separately according to cGvHD status, looking for correlates of disease development.

Chapter I features the results of specific Aim 1.

Aim 2. Elucidate the role of True Naïve and T Memory Stem Cells in the development of cGvHD after allo-HSCT

The contribution of the naïve T-cell pool to the initiation of cGvHD has been suggested and recently validated in preclinical and clinical trials. Likewise, recent studies have found evidence of great phenotypic and functional heterogeneity within the naïve T-cell compartment. Among subsets co-expressing naïve antigens, T Memory Stem Cells (T_{SCM}) have generated great interest in the field of transplantation due to their unique ability to self-renew and differentiate into multiple cell lineages. Although mouse models show that T_{SCM} are able to generate and sustain all memory and effector T-cells in cGvHD reactions, the potential role for T_{SCM} in cGvHD development has not yet been addressed in humans. We hypothesized that cGvHD patients have an early post-transplant expansion of T_{SCM} within Tcon and $CD8^+$ T-cells, accompanied by an impaired recovery of Tregs.

Workplan:

We followed the reconstitution of peripheral $CD4^+$ and $CD8^+$ T-cell subsets after allo-HSCT and made a detailed characterization of T-cells expressing naïve antigens. The phenotypic analysis was performed using the same panel of mAb designed for Aim 1 and a specific gating strategy to distinguish the different subsets and their homeostatic profile, with the main focus on true naïve (pT_N) and T_{SCM}. For comparison, we studied the peripheral blood from HD. Finally, we made a comparative analysis of patients with and without cGvHD.

To take advantage of unique areas of expertise of different centers, this Aim was performed in two institutions, at João Lacerda Lab (Hospital de Santa Maria (HSM)/ Faculdade de Medicina da Universidade de Lisboa (FMUL)/ Instituto de Medicina Molecular João Lobo Antunes (iMM-JLA), and Ritz Lab (Dana Farber Cancer Institute (DFCI)/ Harvard Medical School (HMS), Boston). Dr. Lacerda has performed pioneering work using both haploidentical related[263][309] and unrelated[310] allo-HSCT. Dr. Ritz's Lab is at the forefront of the investigation of human Treg biology and development in the context of transplant. DFCI performs more that 500 transplants per year, including over 250 allo-HSCT. Working together, these teams implemented a prospective immunologic analysis of a large cohort of patients undergoing allo-HSCT in Boston and in Lisbon. The Lisbon cohort included a population of patients undergoing T-cell depleted allo-HSCT after RIC with ATG. The Boston cohort included a population undergoing T-cell repleted allo-HSCT after Myeloablative Conditioning regimen (MAC). Using a similar method to characterize the T-cell compartment after transplant in two different clinical settings presented a unique opportunity to study the effect of clinical variables, such as conditioning regimen and GvHD prophylaxis to the immune T-cell reconstitution post allo-HSCT.

Finally, at João Lacerda Lab, we ran a simultaneous analysis of the thymic function by TREC quantification, and studied the TCR diversity by TCRVβ spectratyping.

The results generated from specific Aim 2 are detailed in Chapter II (II.1 and II.2) of this thesis.

Aim 3. Investigate the T-cell factors that contribute to cGvHD improvement ~~of~~ after low dose rIL-2

Interleukin-2 is the primary homeostatic regulator of Tregs *in vivo*. Previous phase 1/2 trials using daily low-dose rIL-2 in steroid dependent/refractory (SR) cGvHD patients demonstrated an increased numbers of circulating Tregs in all patients, and an effective clinical response in 50-60% [240]. We hypothesized that patients who respond favorably

to rIL-2 infusion can be distinguished by a different pattern of homeostatic T-cell response and repertoire diversity. Moreover, we speculated that non-responders correspond to patients with an exhausted Treg compartment.

Workplan

We examined the effect of low dose rIL-2 on functional T-cell markers, Treg function and T-cell repertoire diversity within Treg, Tcon and $CD8^+$ T-cells in patients with SR cGvHD. To study the impact on T-cell reconstitution and homeostasis, we analysed previously acquired immunophenotypic data[240]. To measure Treg suppressive function we used *in vitro* suppressive assays. Finally, to evaluate T-cell diversity we performed a longitudinal TCR repertoire analysis.

The results generated from specific Aim 3 are detailed in Chapter III of this thesis.

Ana C. Alho contributions to this work:

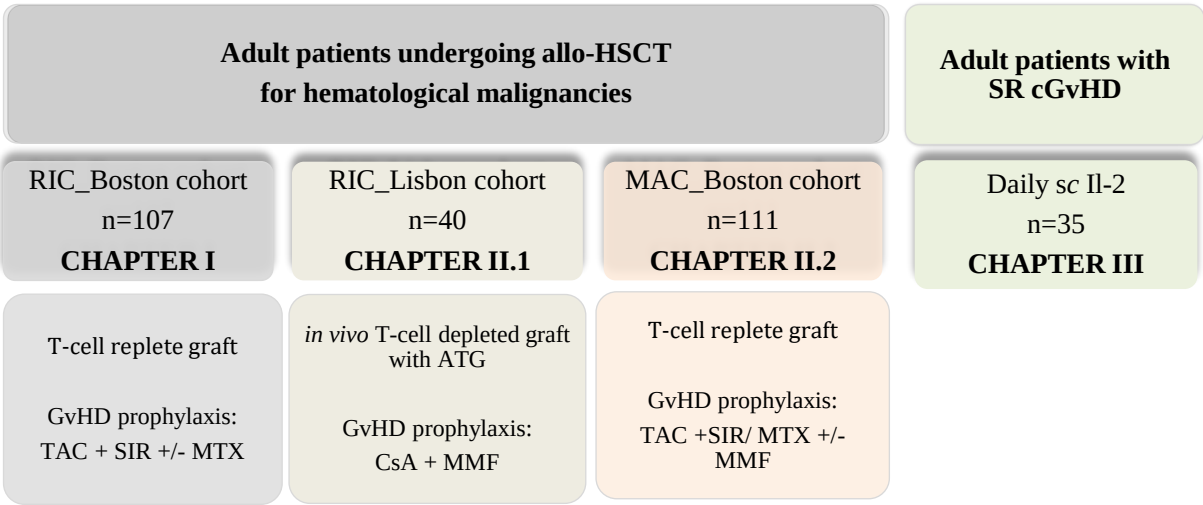
- Chapter I and II.2: planning the research protocol, conducting experiments, analyzing and interpreting the data; writing the manuscript.
- Chapter II.1: acquiring DNA samples for further TCR analysis; interpreting and discussing the flow cytometry data. After a preliminary analysis of the Boston_MAC cohort data, Ana C. Alho alerted the group in Lisbon to the interest of analyzing the T_{SCM} subset in their patients and actively participated in scientific discussions and in the delineation of data analysis strategies.
- Chapter III: conducting flow cytometry staining and analyzing the data.

MATERIALS AND METHODS

1. Patient recruitment and human samples

For this thesis, the population of interest included adult patients undergoing allo-HSCT for hematological malignancies and adult patients with SR cGvHD after allo-HSCT (Figure 5).

Figure 5. Schematic representation of the study population



Allo-HSCT, allogenic Hematopoietic Stem Cell Transplantation; SR, steroid dependent/refractory; cGvHD, chronic Graft *versus* Host Disease; RIC, Reduced Intensity Conditioning; MAC, Myeloablative Intensity Conditioning; CsA, cyclosporine A; TAC, tacrolimus; MMF, mycophenolate mofetil; MTX, methotrexate; ATG, anti-thymocyte globulin; *sc*, subcutaneous.

1.1. Adult patients undergoing allo-HSCT for hematological malignancies

For Specific Aims 1 and 2 we studied prospectively and separately three populations of adult patients who underwent allo-HSCT for hematological malignancies, differing in the intensity of the pre-transplant conditioning regimen and GvHD prophylaxis: RIC_Boston cohort, RIC_Lisbon cohort, and MAC_ Boston cohort.

Peripheral blood samples from patients were collected in EDTA coated tubes at 8 different timepoints post transplant (1, 2, 3, 6, 9, 12, 18, 24 months). Up to 2 weeks of variance in sample collection was allowed. Relapsed patients or death before 9 months post-HSCT were excluded from analysis.

1.1.1. Reduced-Intensity Conditioning (RIC)_Boston cohort

This study included 107 adult patients transplanted at DFCI and Brigham and Women's Hospital (BWH), Boston Massachusetts, between June 2010 and August 2012 (26 months).

All patients received a RIC regimen with fludarabine (120 mg/m^2) plus *iv* low-dose busulfan (3.2mg/kg in 66 patients; 6.4mg/kg in 41 patients) followed by transplantation of unmodified stem cell graft. GvHD prophylaxis consisted of TAC combined with SIR, with or without MTX. In the absence of GvHD, immune prophylaxis was tapered and discontinued between months 6 and 9 post-transplant. Samples were also obtained from 15 Healthy Donors (HD).

1.1.2. Reduced-Intensity Conditioning (RIC) with ATG_Lisbon cohort

This study included 40 adult patients transplanted at Hospital de Santa Maria (Centro Hospitalar Universitário de Lisboa Norte) between February 2013 and December 2016 (46 months).

All patients received a RIC regimen with fludarabine (150 mg/m^2) plus *iv* melphalan (140 mg/m^2), and ATG (4–6 mg/Kg divided in 2–3 days according to HLA compatibility) followed by transplantation of unmodified stem cell graft. Besides ATG, GvHD prophylaxis consisted of CsA plus MMF in all patients. In the absence of GvHD, immune prophylaxis was tapered and discontinued between months 6 and 9 post-transplant. Samples were also obtained from 5 HD.

1.1.3. Myeloablative Intensity Conditioning (MAC)_Boston cohort

This study included 111 adult patients transplanted at DFCI and BWH between July 2010 and December 2015 (66 months).

All patients received a MAC regimen with cyclophosphamide and Total Body Irradiation (TBI), followed by transplantation of unmodified stem cell graft. GvHD prophylaxis consisted of TAC combined with SIR or MTX, with or without MMF. In the absence of GvHD, immune prophylaxis was tapered and discontinued between months 6 and 9 post-transplant. Samples were also obtained from 15 HD.

1.2. Adult patients with SR cGvHD

This study included 35 adult patients with SR cGvHD treated with daily subcutaneous *(sc)* IL-2 (1×10^6 IU/m^2) at DFCI and BWH between July 2011 and January 2014 (30 months). The initial treatment period of 12 weeks was followed by a mandatory 4-week hiatus. Thereafter, improved participants could resume daily IL-2 at the same dose or continue treatment indefinitely.

Fresh peripheral blood samples were obtained at baseline, 1, 2, 4, 6, 8, and 12 weeks during IL-2 infusion; 4 weeks after stopping treatment; every 8 weeks while receiving extended-duration IL-2. Samples were also obtained from 24 HD.

1.3. Peripheral Blood Samples from Healthy Donors

For all the study populations, peripheral blood samples were collected from HD, corresponding to healthy volunteers with no known medical condition and no chronic medication, who volunteered to participate in the studies.

1.4. Ethical Issues

The studies were approved by the Lisbon Academic Medical Center Ethics Committee or the Human Subjects Protection Committee of the DFCI/HMS. They were conducted in strict accordance with universal bioethical principles: the Universal Declaration on Bioethics and Human Rights of UNESCO (19 October 2005); the Charter of Fundamental rights of the EU (2000), and the ethical principles for medical research involving human subjects, Declaration of Helsinki (2008 with 2013 amendments). Moreover, they were conducted in compliance with Good Clinical Practice guidelines, the EU Clinical Trials Directive, and the Portuguese Law No 21/2014 on Clinical Research.

Peripheral blood was collected at the Hematology and Bone Marrow Department at DFCI, BWH and HSM. Written informed consent were obtained prior to sample collection.

2. Clinical Data Collection

Data on patient baseline characteristics was collected from the Clinical Research Information System (CRIS) database, at DFCI, and from patient's medical records, at HSM. All the possible clinical factors that affect immune T-cell reconstitution post-HSCT were assessed (Table 2). Chronic GvHD assessments were made according to the 2014 National Institutes of Health (NIH) consensus criteria[311], except for the IL-2 study in which cGvHD evaluation was made using the 2005 NIH consensus criteria.

Table 2. Clinical factors that affect immune T-cell reconstitution

Host factors	Age	**Source of HSCT**	Peripheral Blood Stem Cells
	Sex		Unmanipulated Bone Marrow
	Conditioning regimen	**Post-HSCT event**	Relapse
	Disease status		Acute GvHD
Recipient/donor matching	CMV matching		Chronic GvHD
	HLA matching		GvHD prophylaxis
			Death

3. Cell Isolation and Immunophenotypic analysis

Immunophenotyping of fresh whole blood and Peripheral Blood Mononuclear Cells (PBMCs) by flow cytometry was used to assess the absolute number of T-cell subsets and the corresponding homeostatic status. According to each study cohort, human whole blood or PBMCs were stained with fluorophore-conjugated monoclonal Antibodies (mAbs) at pre-determined timepoints, using different flow cytometry protocols.

3.1 Flow cytometry protocols

3.1.1. RIC and MAC_Boston cohorts

PBMCs were isolated from freshly collected blood samples using density gradient centrifugation (Ficoll-Hypaque; GE Healthcare). Cells from buffy-coats were re-suspended with PBS containing 1% FBS and 0.1% sodium azide (Sigma-Aldrich), at a concentration of 1.5-2x10^6 PBMCs/ml and further incubated with surface mAbs (Table 3) for 20 minutes at room temperature. Stained cells were then fixed with prepared Fix/Perm buffer (eBioscience) for 30 minutes at 4°C, permeabilized with prepared Perm buffer (eBioscience), and stained with the mAbs directed against intracellular antigens (Table 3) for 30 minutes at 4°C. Cells were then washed with 2%FBS-PBS (BD Bioscience) and immediately acquired on a FACSCanto II cytometer with BD FACS-DIVA software. A minimum of 200 000 events were recorded in the lymphocyte gate. Daily flow cytometer quality control monitoring was performed using Cytometry Setup and Tracking Beads (BD Biosciences).

3.1.2. RIC_ Lisbon cohort

Fresh whole blood (100 microliters (µl)) was incubated with mAbs directed against surface antigens (Table 3) for 20 minutes at room temperature. After red blood cell lysis (BD Pharm Lyse lysing solution) and washing, stained cells were fixed for 30 minutes with Fix/Perm buffer (eBioscience), permeabilized with prepared Perm buffer (eBioscience) and stained with mAbs directed against intracellular antigens (Table 3) for 30 minutes at 4°C. Cells were then washed with 1mL PBS and immediately acquired on LSR Fortessa cell analyser (BD Biosciences), with a minimum of 200 000 events being recorded in the lymphocyte gate. Daily flow cytometer quality control monitoring was performed using Cytometry Setup and Tracking Beads (BD Biosciences). Eight peak calibration Rainbow Beads (BD Biosciences) were used to ensure stable fluorescence.

Table 3. Directly conjugated monoclonal antibodies – Panel I

<table>
<tr><th colspan="2">Boston cohorts</th><th colspan="3">Lisbon cohort</th></tr>
<tr><th colspan="5">SURFACE ANTIGENS</th></tr>
<tr><th>Tube K
(Ki-67/CD31)</th><th>Tube B
(Bcl-2/CD95)</th><th>Treg</th><th>RTE/Porlif</th><th>Apopt</th></tr>
<tr><td colspan="2" rowspan="2">CD3 BV450 (clone UCHT1; BDBioscience)
CD4 Apc-Cy7 (clone RPA-T4; BDBioscience)
CD8 BV510 (clone RPA-T8; BioLegend)
CD45RA PE-Cy7(clone HI100; BDBioscience)
CD62L APC (clone DREG-56; BDBioscience</td><td colspan="3" rowspan="2">CD45RO FITC
CCR7 APCeFlour780
CD3 PerCPCY5.5 (clone OKT3; eBioscience)
CD25 PE-Cy7 (M-A251; eBioscience)
CD4 APC (clone RPA-T4; eBioscience)
CD127 APCeFlour780 (ebioRDR5; eBioscience)</td><td colspan="2">CD3 PerCPCY5.5 (clone OKT3; eBioscience)
CD45RA PE-Cy7 (clone HI100; eBioscience)
CD4 APC (clone RPA-T4; eBioscience)
CD62L APCeFlour780 (clone DREG-56; eBioscience)</td></tr>
<tr><td>CD31 PE
(clone WM59; BDBioscience)</td><td>CD95 PE
(clone DX2; eBioscience)</td><td>CD31 FITC
(clone WM59; eBioscience)</td><td>CD95 PE
(clone DX2; eBioscience)</td></tr>
<tr><th colspan="5">INTRACELLULAR ANTIGENS</th></tr>
<tr><td colspan="2">Foxp3 PerCPCyanine 5.5
(clone PCH101; eBioscience)</td><td colspan="3">Foxp3 e450
(clone PCH101; eBioscience)</td></tr>
<tr><td>Ki-67 FITC
(clone MOPC21; BDBioscience))</td><td>Bcl-2 FITC
(clone Bcl-2/100; eBioscience)</td><td></td><td>Ki-67 PE
(clone20Raj1; eBioscience)</td><td>Bcl-2 FITC
(clone Bcl-2 100; eBioscience</td></tr>
</table>

Panel used to define functionally distinct T-cell subsets and the correspondent homeostatic characteristics. The mAbs directed against surface antigens allowed the identification of T-cell populations (CD3, CD4, CD8), naïve and memory subsets (CD45RA, CD62L, CD31) and T-cell activation/ susceptibility to apoptosis (CD95). The mAbs directed against intracellular antigens allowed the identification of $CD4^+$ Tregs, (Foxp3), quantification of proliferation/activation (ki67) and susceptibility to apoptosis (Bcl-2).

3.1.3. Steroid refractory or dependent cGvHD patients under low dose IL2

This study included data previously acquired from adult patients with SR cGvHD enrolled in a phase 2 study of daily low dose rIL-2 [240]. Data was analysed using two different flow cytometry protocols.

The surface staining protocol was used for the identification and characterization of T-cell populations and subsets. Fresh whole blood (50 µl) was incubated with mAbs directed against surface antigens (Table 4) for 30 minutes at room temperature. After incubation, red blood cells were lysed (BD Pharm Lyse lysing solution), washed and immediately acquired on FACSCanto II flow cytometer (BD Bioscience) with a minimum of 200 000 events being recorded in the lymphocyte gate.

The intracellular staining protocol was used for characterization of T-cell homeostasis. PBMCs were isolated and stained using the flow cytometry protocol previously described (see 3.1.1).

Table 4. Directly conjugated monoclonal antibodies – Panel II

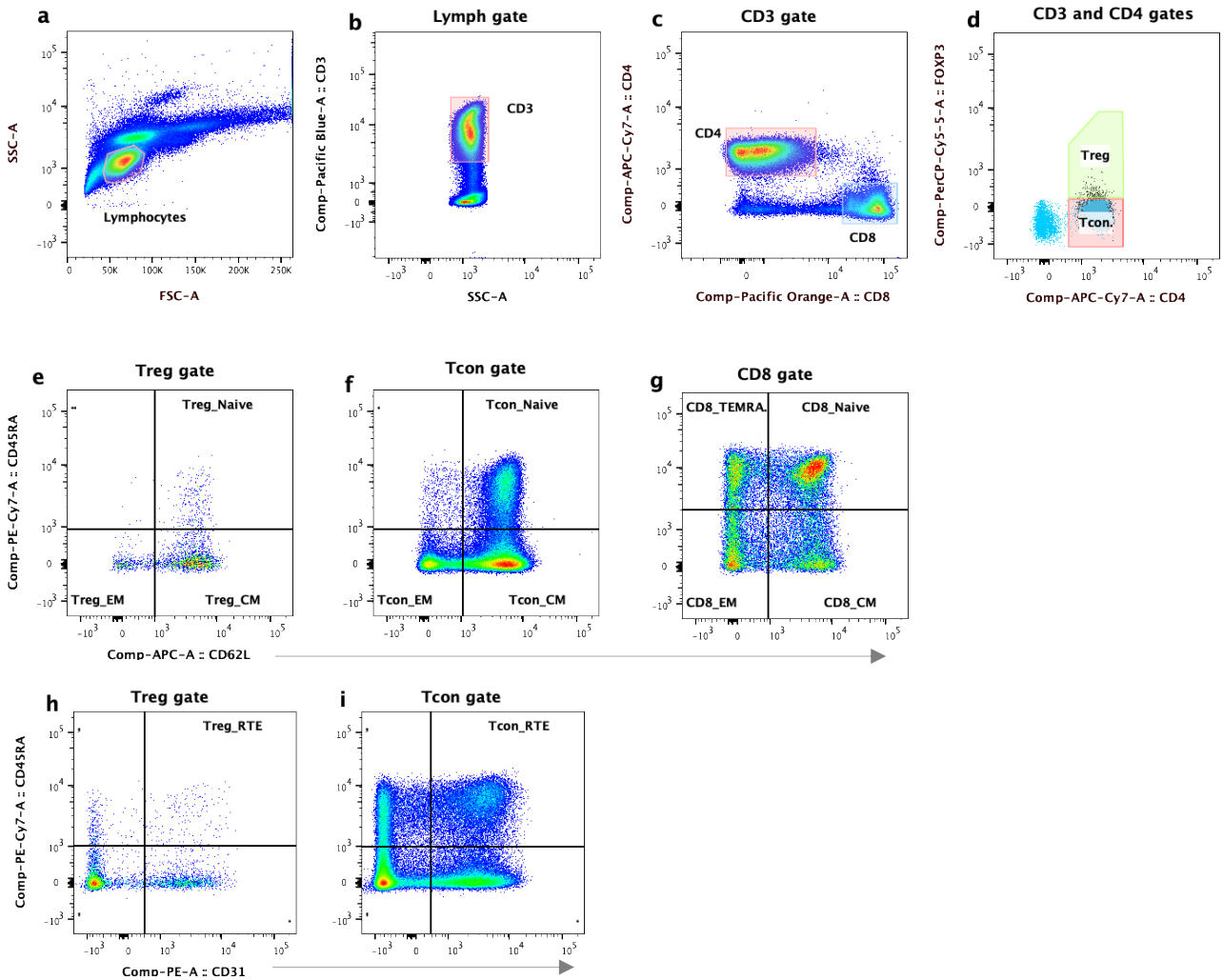
Surface Antigens
CD3 BV450 (clone UCHT1; BDBioscience)
CD4 Apc-Cy7 (clone RPA-T4; BDBioscience)
CD8 BV510 (clone RPA-T8; BioLegend)
CD45RO FITC (clone UCHL1; BD Pharmingen)
CD62L APC (clone DREG-56; BDBioscience)
CD25 PE-Cy7 (clone M-A251; BD Pharmingen),
CD127 PE-Cy5 (clone eBioRDR5; eBioscience),

3.2 Flow cytometry analysis

The data obtained from flow cytometry was analysed using FlowJo software V9 or V10 (Tree Star), with specific gating strategies designed to access different T-cell populations, subpopulations and the corresponding homeostatic patterns, according to each Aim.

To study T-cell reconstitution post allo-HSCT we identified three major T-cell populations, $CD4^+$ Treg, $CD4^+$ Tcon, and $CD8^+$ T-cells, defined as $CD3^+CD4^+Foxp3^+$, $CD3^+CD4^+Foxp3^-$, and $CD3^+CD4^-CD8^+$, respectively. Each population was divided, according to the expression of CD45RA and CD62L (Figure 6), as follows: naïve (T_N; $CD45RA^+CD62L^+$), Central Memory (T_{CM}; $CD45RA^+CD62L^-$) and Effector Memory (T_{EM}, $CD45RA^-CD62L^-$)[45]. Within $CD8^+$ T-cells, expression of CD45RA and lack of CD62L was used to define T_{EMRA}. In order to study the mechanisms involved in T-cell reconstitution, we further measured proliferation, through intracellular Ki-67 expression (%), susceptibility to apoptosis, through Bcl-2 and CD95 expression levels (Mean Fluorescence Intensity, MFI), and thymic production of $CD4^+$ T-cells, through the expression of $CD31^+$ on naïve T-cells [32]. For quantification of Ki-67, Bcl-2, and CD95 a cutoff of 50 events was used as a valid data point for statistical analysis. Healthy donor samples were stained in the same conditions.

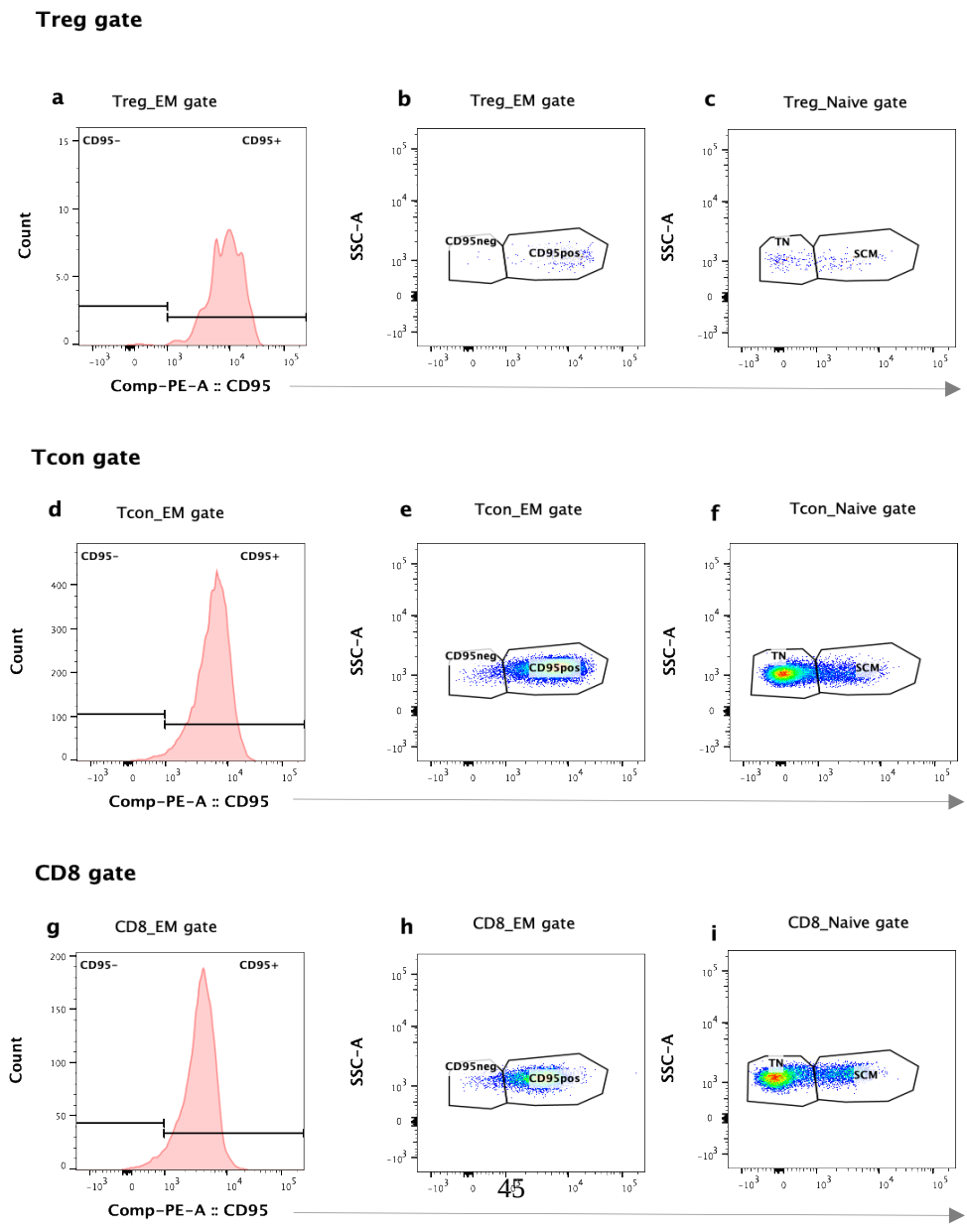
Figure 6. Gating strategy for analysis of naïve and memory T-cell subsets



Lymphocytes were initially gated to determine $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells (a. to c.). Within $CD4^+$ T-cells, Tregs were distinguished from Tcons by Foxp3 expression (d.) Tregs were identified as $CD3^+CD4^+Foxp3^+$ and Tcon as $CD3^+CD4^+Foxp3^-$. Each T-cell population (CD8, Tcon and Treg) was further divided into naïve and memory subsets according to CD45RA and CD62L expression: Naïve ($CD45RA^+CD62L^+$), Central memory (CM, $CD45RA^-$ $CD62L^+$), Effector Memory (EM, $CD45RA^-$ $CD62L^-$), $CD45RA^+$ Effector Memory (TEMRA, $CD45RA^+CD62L^-$) (e. to g.). RTE were identified within Treg and Tcon trough the expression of CD31 and CD45RA (h., i.).

To elucidate the role of peripheral true naïve and T Memory Stem Cell cells in the development of cGvHD after allo-HSCT, we quantified the expression of CD95 within $CD45RA^+CD62L^+$ naïve T-cells. High/dim expression of CD95 distinguished T_{SCM} ($CD45RA^+$ $CD62L^+CD95^{+/dim}$) from pT_N ($CD45RA^+$ $CD62L^+CD95^{-/low}$). Flow cytometry plots illustrating the gating strategy used to identify T_{SCM} are shown in Figure 7.

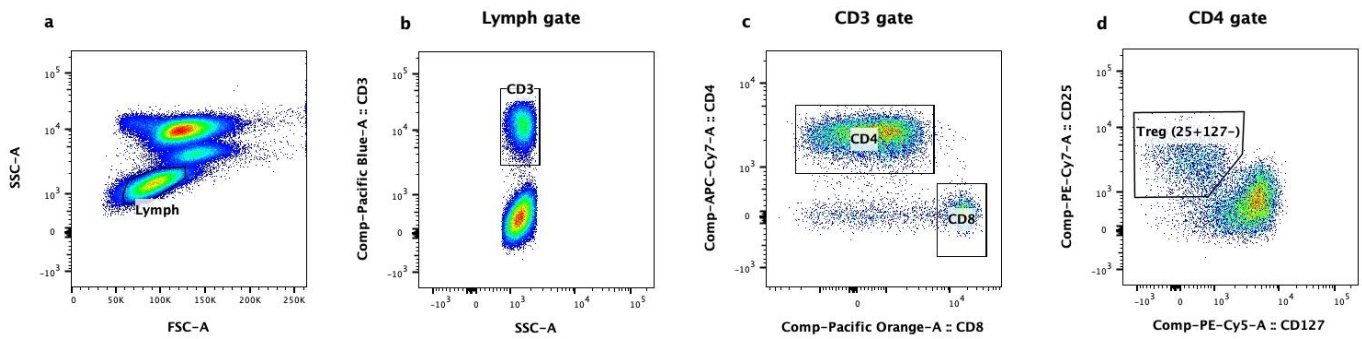
Figure 7. Gating strategy for analysis of peripheral pT_N and T_{SCM} subsets



True Naïve (TN) and Stem Cell Memory (SCM) subsets were identified within naïve Treg, Tcon and CD8^{+} T-cells through the expression of CD95. SCM phenotype: CD45RA^{+}CD62L^{+}CD95$^{+/dim}$; TN phenotype: CD45RA^{+}CD62L^{+}CD95$^{-/low}$. To facilitate the analysis, the threshold for CD95 was initially stablish within the EM subset, which cell number is higher, and then applied into the naïve subset.

To examine the impact of low-dose IL-2 on naïve and memory T-cell compartments and on functional T-cell markers we combined previously acquired data from cell surface and intracellular analysis. Using cell surface staining we defined Tregs, Tcons and CD8^{+} T-cells as CD3^{+}CD4^{+}CD25$^{med\text{-}high}$CD127^{low}, CD3^{+}CD4^{+}CD25$^{neg\text{-}low}$CD127$^{med\text{-}high}$, and CD3^{+}CD8^{+}, respectively. The naïve and memory compartments and the homeostatic profile for each T-cell subset was accessed using intracellular marker analysis, as previously described. Flow cytometry plots illustrating the gating strategy used for surface marker analysis are shown in Figure 8.

Figure 8. Gating strategy for analysis of Treg and Tcon using cell surface markers



Lymphocytes were initially gated to determine CD3^{+}CD4^{+}and CD3^{+}CD8^{+} T-cells (a. to c.). Within CD4^{+} we distinguished Tregs by the expression CD25 and CD127 expression (d)

4. Functional Assays: *in vitro* suppressive assays

To study the suppressive function of Treg and Tcon populations on SR patients under low dose IL2 we used cryopreserved PBMCs. PBMCc were initially thawed, using warm media (RPMI + 10% FBS + 1% Pen/Strep) plus DNase/ $MgCl_2$, then counted, and suspended in 40 uL of MACs buffer (PBS + 0.5% BSA + 2mM EDTA) per 10^7 cells. $CD4^+$ T-cells were isolated by negative selection using biotin magnetic beads (Miltenyi Biotec). Purified $CD4^+$ T-cells were stained with CD4-FITC (clone: OKT4), CD25-PE-Cy7 (clone: MA251) and CD127-APC eF780 (clone: eBioRDR5), and further sorted into Treg and Tcon using BDFacsAriaIII. Populations were confirmed to be 95% pure. Tcons were labeled with CellTrace Violet (Invitrogen), plated with autologous or allogeneic Tregs in a 1:1 ratio and stimulated with 1μg/mL anti-CD3/CD28/CD2–coated beads (Treg Suppression Inspector, human; Miltenyi Biotec). Four days after, cells were harvested and responder cells were analysed for CTV dilution by flow cytometry. For allogeneic assays, Tregs were tested against the same HD Tcon and Tcon were tested against the same HD Treg.

5. Molecular TCR analysis

5.1 TCRβ-chain repertoire analysis

TCRβ-chain repertoire was studied in both RIC_Lisbon cohort and SR cGvHD cohort, using purified $CD4^+$ and $CD8^+$ T-cells isolated from cryopreserved PBMCs.

To study the immune T-cell repertoire diversity we performed a molecular analysis of the TCRβ gene (TRB) repertoire using two different assays: Complementarity-Determining Region 3 (CDR3) spectratyping of each TCRβ chain variable region (Vβ) and Deep-Sequencing analysis.

5.1.1 RNA isolation and TCRβ-chain CDR3 spectratyping (RIC_Lisbon cohort)

Cryopreserved PBMCs from patients and HD were thawed, stained for CD3, CD4 and CD8, and FACSorted (BD FACSAria III) into $CD4^+$ and $CD8^+$ T-cells. Total RNA was extracted and reverse transcribed from each fraction using RNeasy Micro Kit (AllPrep, Qiagen). First-strand complementary DNA was generated after priming with an equivolume mixture of random hexamers and oligo(dT) (Invitrogen Superscript III).

Spectratyping analysis was performed by Dário Ligeiro from Immunogenetics Laboratory, Centro de Histocompatibilidade do Sul, Lisbon. TCRβ transcripts were amplified with TRBV (T-cell receptor ß variable) specific primers and a common TRCB (TCR ß-chain) reverse primer [312,313]. A run-off reaction with a second TRCB FAM-labeled primer was used to extend these products. Each fluorescent TRBV-TRBC fragment was separated using capillary electrophoresis-based DNA automated sequencer.

Data were analysed with GeneMapper (Thermo Fischer Scientific) for size, peak count and fluorescent intensity determination. Profiles of transcript TRB repertoires were classified based on peak count, distribution shape and relative fluorescence intensity (RFI) of each peak (% RFI = 100 x clonal peak area/total peak area) [314].

5.1.2 DNA isolation and TCRβ-chain Deep Sequencing (SR cGvHD patients)

Cryopreserved PBMCs from 12 patients (6 clinical responders, 6 non-responders) and 8 HDs were thawed, stained for CD3, CD4, CD25, CD127 and CD8, and sorted (BD FACSAria III) into Tregs, Tcons and CD8. Genomic DNA was isolated from the sorted cell population using the Qiagen All-Prep DNA/RNA/Protein kit (Qiagen) (supplementary Table 2; p 216). Survey level deep sequencing of the TCRβ was performed using the ImmunoSEQ platform, and sequencing data were analysed using algorithms developed by Adaptive Biotechnologie[315].

Shannon's Entropy was used as a measure of TCRβ diversity/frequency distribution. Entropy values correlate with the amount of sample input DNA. Thus, samples with less

input DNA tend to have lower entropy. To account for differences in input DNA, a weighted correction of the raw entropy measurements was made using a linear regression analysis. Clonality is the reciprocal of Shannon's Entropy normalized for the number of unique productive sequences (clonality = 1 – normalized entropy) with values ranging from 0 (all sequences equally abundant) to 1 (entire sample is comprised of a single sequence)

5.2 Signal-Joint TREC quantification by Real-Time PCR

Signal-Joint TREC sequences in CD4 and $CD8^+$ T-cells were analysed with a multiplex quantitative PCR assay[316], using the same samples purified for the TCRß CDR3 spectratyping. sjTREC sequence copy numbers were extrapolated from standard curves obtained by 10-fold serial dilutions of a triple-insert plasmid containing sjTREC and TRAC fragments in a 1:1:1 ratio (kind gift from L. Imberti, Spedali Civili of Brescia, Italy). Detected sjTREC copies were genome normalized with the mean quantity of TCR α-chain sequences. Results were expressed as sjTREC copies per 10^6 $CD4^+$ or $CD8^+$ T-cells. This analysis was performed by Dário Ligeiro.

6. Statistical analysis

Initially, we made a descriptive analysis of the data, without prior knowledge of cGvHD status. Afterwards, according to cGvHD status, the immunophenotypic data was compared using the exact Wilcoxon-rank-sum test for unpaired group comparison and Wilcoxon-(signed)-rank test for paired group comparison. Correlation studies were made using nonparametric Spearman test.

In each cohort the patient baseline characteristics were compared between cGvHD groups using Fisher's exact test, for categorical variables, and Wilcoxon rank-sum (Mann–Whitney) test, for continuous variables.

All statistical analyses were performed using SAS 9.2 software (SAS Institute), R 3.1.3 (the CRAN project), or GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA).

The data collected at DFCI was discussed with Dr. Haesook Kim, from the Department of Biostatistics and Computational Biology, DFCI, Boston, who provided support for evaluation of complex laboratory data and correlation with clinical outcomes. Statistical tests were two-sided at the significance level of 0.05 and multiple comparisons were not considered. Graphs were made using Prism software (GraphPad, San Diego, CA).

RESULTS

The results presented in this manuscript were published or are currently being prepared for publication in the following scientific journals:

Unbalanced recovery of regulatory and effector T cells after allogeneic stem cell transplantation contributes to chronic GvHD

Ana C. Alho[1,2,3], Haesook T. Kim[4,5,6], Marie J. Chammas[1], Carol G. Reynolds[1], Tiago R. Matos[1,2], Edouard Forcade[1,2], Jennifer Whangbo[1,2], Sarah Nikiforow[1,2], Corey S. Cutler[1,2], John Koreth[1,2], Vincent T. Ho[1,2], Philippe Armand[1,2], Joseph H. Antin[1,2], Edwin P. Alyea[1,2], Joao F. Lacerda[3], Robert J. Soiffer[1,2] and Jerome Ritz[1,2,5]

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Blood Journal, February 14th 2016, Volume 127, Number 5, 646-57. ***Cyted by 78 articles.*** *(facsimile in Annexes)*

Naïve and Stem Cell Memory T cell subset recovery reveals opposing reconstitution patterns in CD4 and CD8 T cells in Chronic Graft versus Host Disease

Maria V. Soares[1‡], Rita I. Azevedo[1‡], Inês A. Ferreira[1,7], Sara Bucar[1,8], Ana C. Ribeiro[1], Ana Vieira[2,9], Paulo N. G. Pereira[1], Ruy M. Ribeiro[3], Dario Ligeiro[4], Ana C. Alho[1,5], António S. Soares[1,10], Nádia Camacho[5], Carlos Martins[5], Fernanda Lourenço[5], Raul Moreno[5], Jerome Ritz[6] and João F. Lacerda[1,5*]

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Frontiers in Immunology. 2019 March 06, Volume 10, Number 334. ***Cyted by 7 articles.*** *(facsimile in Annexes)*

Functional Analysis of Clinical Response to Low-dose IL-2 in Patients with Refractory Chronic Graft-*versus*-Host Disease

Jennifer S. Whangbo[1,2], Haesook T. Kim[3], Sarah Nikiforow[2], John Koreth[2], Ana C. Alho[2,4,] Bryn Falahee[2], Soomin Kim[2], Katharine Dusenbury[2], Marie J. Fields[2], Carol G. Reynolds[2], Edwin P. Alyea III[2], Philippe Armand[2], Corey S. Cutler2, Vincent T. Ho[2], Joseph H. Antin[2], Robert J. Soiffer[2], Jerome Ritz[2]

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Blood Advances. 2019 Apr 9, Volume 3, Number 7. ***Cyted by 9 articles.*** *(facsimile in Annexes)*

Early predominance of effector memory CD8$^+$ T-cells over true naïve and central memory Tregs results in chronic GvHD after myeloablative allo-HSCT

Ana C. Alho[1,2,3], Marie J. Chammas[1], Carol G. Reynolds[1], Sarah Nikiforow[1,2], Corey S. Cutler[1,2], John Koreth[1,2], Vincent T. Ho[1,2], Philippe Armand[1,2], Joseph H. Antin[1,2], Edwin P. Alyea[1,2], Joao F. Lacerda[3], Robert J. Soiffer[1,2] and Jerome Ritz[1,2]

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Manuscript in preparation.

I. CHAPTER I

1.1. Unbalanced recovery of regulatory and effector T cells after allogeneic stem cell transplantation contributes to chronic GvHD

Short Introduction

After allo-HSCT there is a deep disruption of the peripheral T-cell compartment. Defective recovery of the T-cell pool has shown to have significant clinical consequences, including the development of cGvHD, which is the most relevant cause of late morbidity and non-relapse mortality post allo-HSCT. Despite the complex biology of cGvHD, previous studies have suggested that a balanced recovery of $CD4^+$ regulatory T-cells and effetor, Tcons and $CD8^+$ T-cells, is requiered for the establishment of immune tolerance and T-cell homeostasis. In this chapter we studied 107 adult patients who received T-cell replete stem cell grafts after RIC, and characterized in detail the immune recovery of the naïve and memory subpopulations within $CD4^+$ Treg, Tcons, and $CD8^+$ T-cells for a 2-year period.

Results

Patient characteristics

Clinical characteristics of the patients included in the study are summarized in Table 5. The median age was 62 years (range 19-73), 16,8 % of male recipients had a female donor. The most prevalent hematologic malignancy was AML (34,6%). 92.5% of patients received filgrastim-mobilized PBSC and 89.7% underwent transplantation from HLA-matched donors. In 91,6% of patients, GvHD prophylaxis included SIR combined with TAC, with or without MTX. Following transplant, 80% of patients achieved complete donor chimerism in peripheral blood by day +30; 25 patients (23.3%) developed grade II-IV aGvHD; 46 patients (43%) developed cGvHD, within these, 10 (21,7%) had

previously developed aGvHD. Relapse within the 2-year period of follow-up occurred in 46 patients (42.9%), 3 of which had previously developed cGvHD.

Table 5. Patient Characteristics (RIC_Boston cohort)

Total (number)	107	
	N	%
Age, median (range)	62 (19, 73)	
Patient-Donor Gender		
M-M	47	43.9
M-F	18	16.8
F-M	23	21.5
F-F	19	17.8
Diagnosis		
AML	37	34.6
MDS	24	22.4
NHL	12	11.2
MPD	7	6.5
CLL/SLL/PLL	6	5.6
ALL	5	4.7
Mixed MDS/MPD	4	3.7
CML	2	1.9
Hodgkins Disease	3	2.8
MM/PCD	4	3.7
Anemia/ Red Cell Disorder	2	1.9
Immunodeficiency	1	0.9
Disease Risk Index		
Low	17	15.9
Intermediate	46	43
High	37	34.6
Donor HLA type		
Matched unrelated	66	61.7
Matched related	30	28
Mismatch unrelated	11	10.3
Stem Cell Source		
PBSC	99	92.5
Bone marrow	7	6.5
Bone marrow and PBSC	1	0.9
GvHD prophylaxis		

Sir- Tac -MTX	74	69.2
Sir -Tac	24	22.4
Tac-MTX	8	7.5
Sir-MMF-MTX	1	0.9
Acute GvHD (grade ≥ II)	25	23.3
Chronic GvHD		
None	61	57
Extensive	42	39.3
Limited	4	3.7

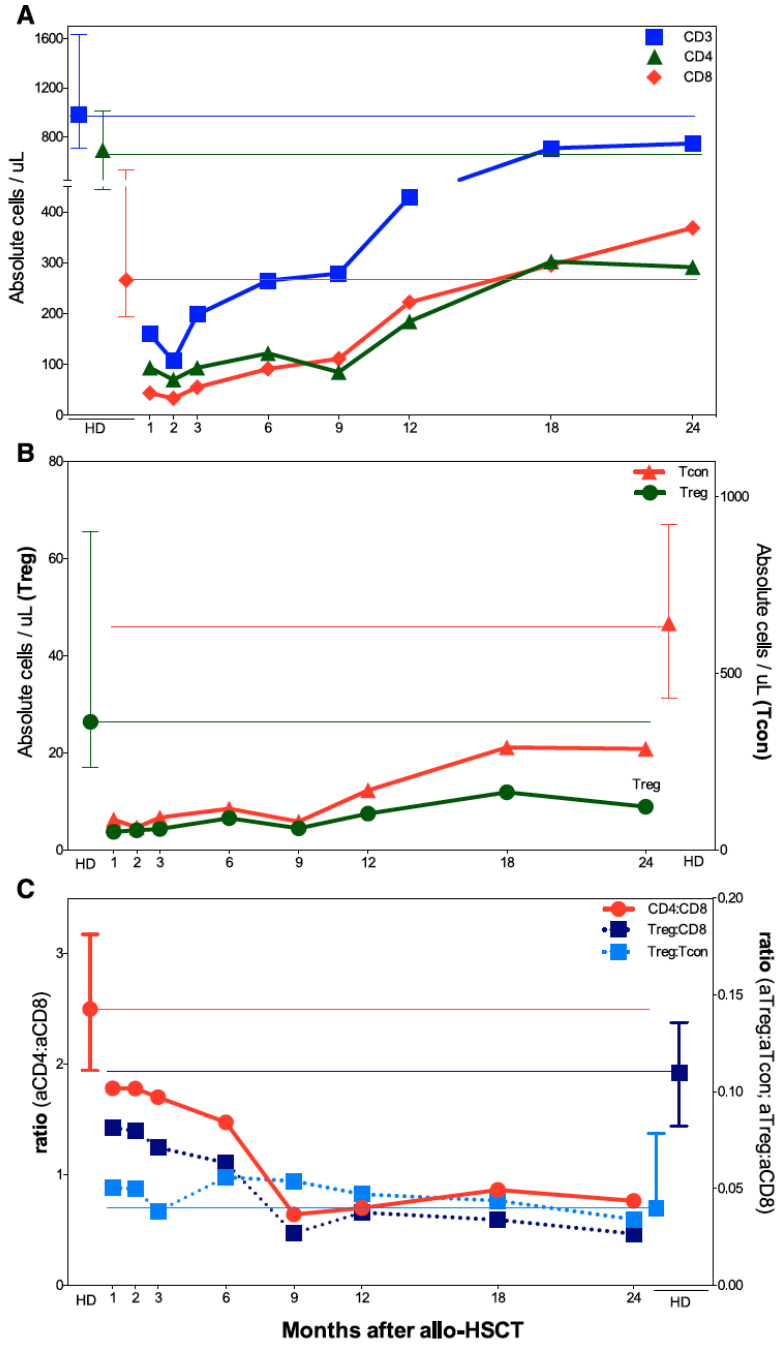
Abbreviations: M: male; F: female; AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; NHL: non-Hodgkin lymphoma; MPD: myeloproliferative disease; CLL: chronic lymphocytic leukemia; SLL: small lymphocytic lymphoma; PLL: pro-lymphocytic Leukemia; ALL: acute lymphoid leukemia; CML: chronic myeloid leukemia; MM: multiple myeloma; PCD: plasma cell dyscrasias; PBSC: peripheral blood stem cell; HLA: human leukocyte antigen; CMV: cytomegalovirus; Tac: tacrolimus; Sir: sirolimus/ rapamycin; MTX: methotrexate; MMF: mycophenolate mofetil.

Kinetics of T-cell reconstitution after HSCT

Recovery of the major T-cell populations is summarized in Figure 9. $CD3^+$ T-cells gradually increased and approached the normal range 18 months after transplant (Figure 9A). Delayed T-cell recovery was primarily due to slow $CD4^+$ T-cell reconstitution. $CD8^+$ T-cells reached normal levels 12 months after transplant but $CD4^+$ T-cells remained below normal throughout the 2-year follow-up period ($p<0.001$).

Within the $CD4^+$ T-cell population, both Treg and Tcon increased gradually, but neither subset reached normal levels ($p<0.001$) 2 years after HSCT (Figure 9B). Despite the more rapid recovery of $CD8^+$ T-cells, $CD4^+$ T-cell numbers were higher than $CD8^+$ T-cells counts for the first 6 months after HSCT. By 9 months, $CD8^+$ T-cell counts were higher than $CD4^+$ T-cell counts resulting in an inverse CD4:CD8 ratio (Figure 9C). Due to the similar pace of reconstitution of Treg and Tcon, the Treg:Tcon ratio remained relatively stable and within normal range throughout the follow-up period. In contrast, CD4:CD8 and Treg:CD8 ratios decreased in the first 9 months and remained below normal ($p<0.001$) throughout the follow-up period (Figure 9C).

Figure 9. Reconstitution of major T-cell populations after RIC allo-HSCT.



The recovery of each T-cell population in peripheral blood was assessed prospectively by flow cytometry. The median cell counts/µl for each population is represented at each timepoint, as well as the corresponding median value in healthy donors (HD), with the inter-quartile (IQ) range (whisker bars). (A) Median CD3$^+$ (blue), CD4$^+$ (green) and CD8$^+$(red) T-cell counts. (B) Median Treg (green) and Tcon (red) counts. (C) Median CD4:CD8, Treg:CD8 and Treg:Tcon ratios. To compare the patterns of recovery of Treg and Tcon in the same graph, different scales were used in panels B and C.

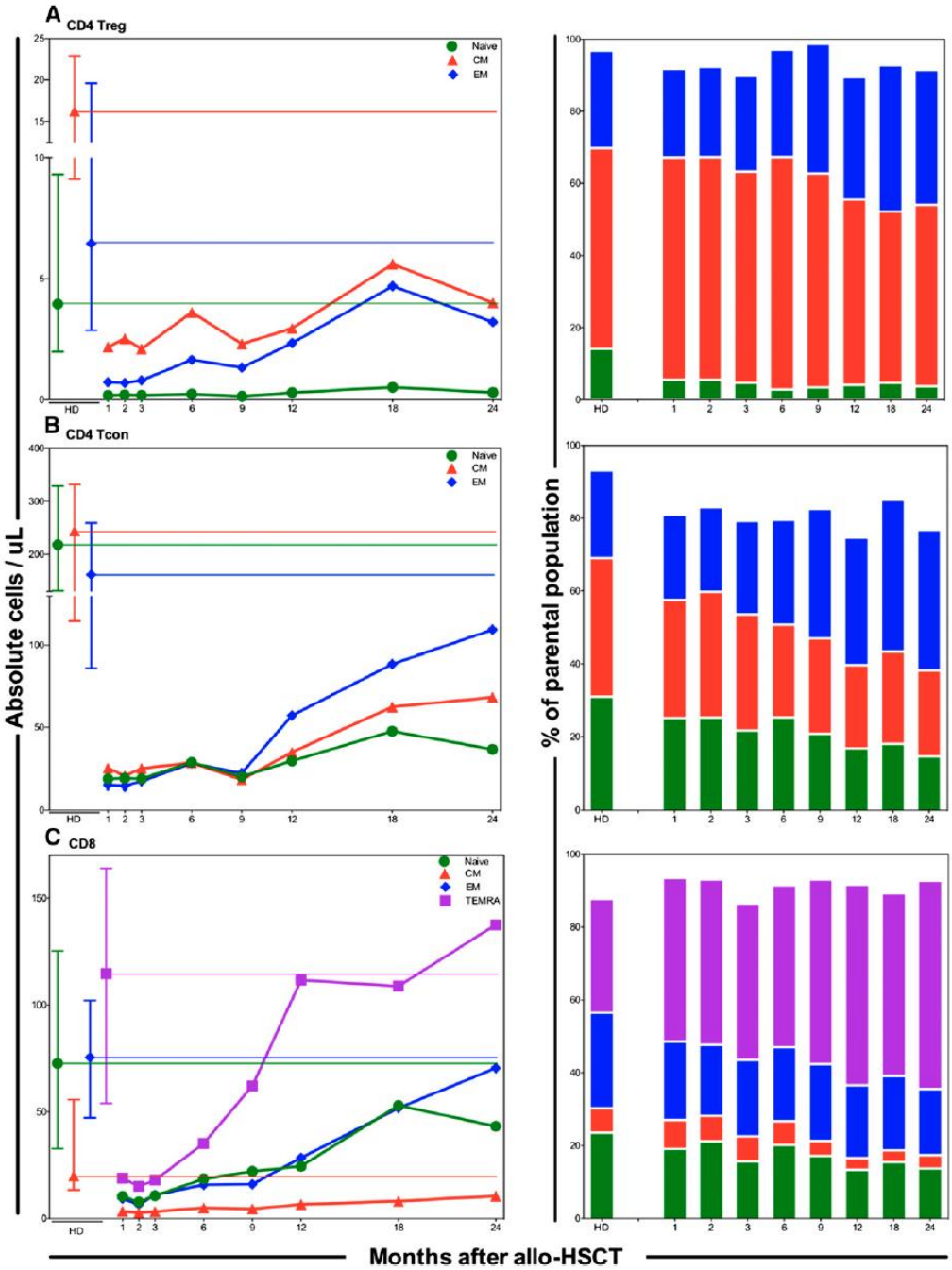
Within Treg, Tcon and $CD8^+$ T-cells populations we determined the fraction of cells that had a naïve, central memory (T_{CM}) or effector memory (T_{EM}) phenotype (detailed gating strategy illustrated in Figure 6). Within $CD8^+$ T-cells we also defined the memory subset that re-express CD45RA. Within each T-cell population, memory cells represented the predominant subsets. This was evident at early as well as late timepoints and the relative distribution at all timepoints was similar to what was found in healthy adults (Figure 10 - right panels).

More detailed analysis of Treg reconstitution (Figure 10A) revealed a gradual increase in both T_{CM} and T_{EM} fractions with very little recovery of naïve Tregs during the entire follow-up period. In HD, naïve cells represent a relatively small fraction (median: 14.1%) of circulating Tregs. Nevertheless, the naïve Treg fraction was significantly decreased throughout the post-transplant period compared to HD ($p<0.001$). In the first 6 months, T_{CM} Treg comprised the major Treg subset. Beginning 9 months after HSCT, there was a gradual increase in T_{EM} Treg subset (Figure 10A right panel).

Analysis of Tcon recovery showed no increase in circulating T-cell numbers in the first 9 months after HSCT (Figure 10B). Beginning 12 months post HSCT, there was a gradual increase in all Tcon subsets, with the greatest increase within T_{EM}. Notably, the frequency of naïve Tcon was relatively normal early post-HSCT and gradually decreased as the T_{EM} increased 9-24 months post-HSCT (Figure 10B right panel).

Analysis of $CD8^+$ T-cells recovery showed that all subsets began to increase in number 6 months post-HSCT (Figure 10C). Rapid recovery of $CD8^+$ T-cells occurred primarily as a result of increasing numbers of T_{EMRA} that achieved normal levels 9 months post-HSCT and comprised >50% of all $CD8^+$ T-cells. Naïve and T_{EM} subsets also showed substantial recovery. In contrast to $CD4^+$ T-cells, the T_{CM} subset represented a small fraction of $CD8^+$ T-cells and showed very little recovery (Figure 10C right panel).

Figure 10. Reconstitution of naïve and memory T-cells after RIC allo-HSCT



Absolute numbers (cells/µl) of each subset in peripheral blood are shown on the left and relative percentages at each timepoint are shown on the right. Graphs on the left show median cell counts/µl for each population and the corresponding median in healthy donors (HD), with the IQ range (whisker bars). Bar graphs on the right show the median percentage of each population and the corresponding median in healthy donors (HD). (A) Treg subsets: naïve (green); CM (red), EM (blue). (B) Tcon subsets: naïve (green), CM (red), EM (blue). (C) CD8^{+} T-cells subsets: naïve (green), CM (red), EM (blue), TEMRA (purple). (CM: central memory; EM: effector memory; TEMRA: terminal effector memory re-expressing CD45RA.

Thymic-dependent T-cell reconstitution

In $CD4^+$ T-cells, recent thymic emigrants (T_{RTE}) can be identified within the naïve T-cell subset by co-expression of CD31 and CD45RA (Figure 11 and 12). In HD the frequency of T_{RTE} is greater within Tcon (median=18%) than within Treg (median=11%; p=0.001). In the first 3 months post-transplant, the percentage of T_{RTE} Treg was within the lower limits of normal but subsequently decreased below normal from 6-24 months post-HSCT (Figure 11A). In contrast, the percentage of T_{RTE} Tcon was within the high normal range 1-6 months post-HSCT and remained at normal levels as Tcon recovery continued (Figure 12A). Taken together, these results indicate that thymic production of $CD4^+$ T-cells strongly favors Tcon over Treg for at least 2 years after transplant.

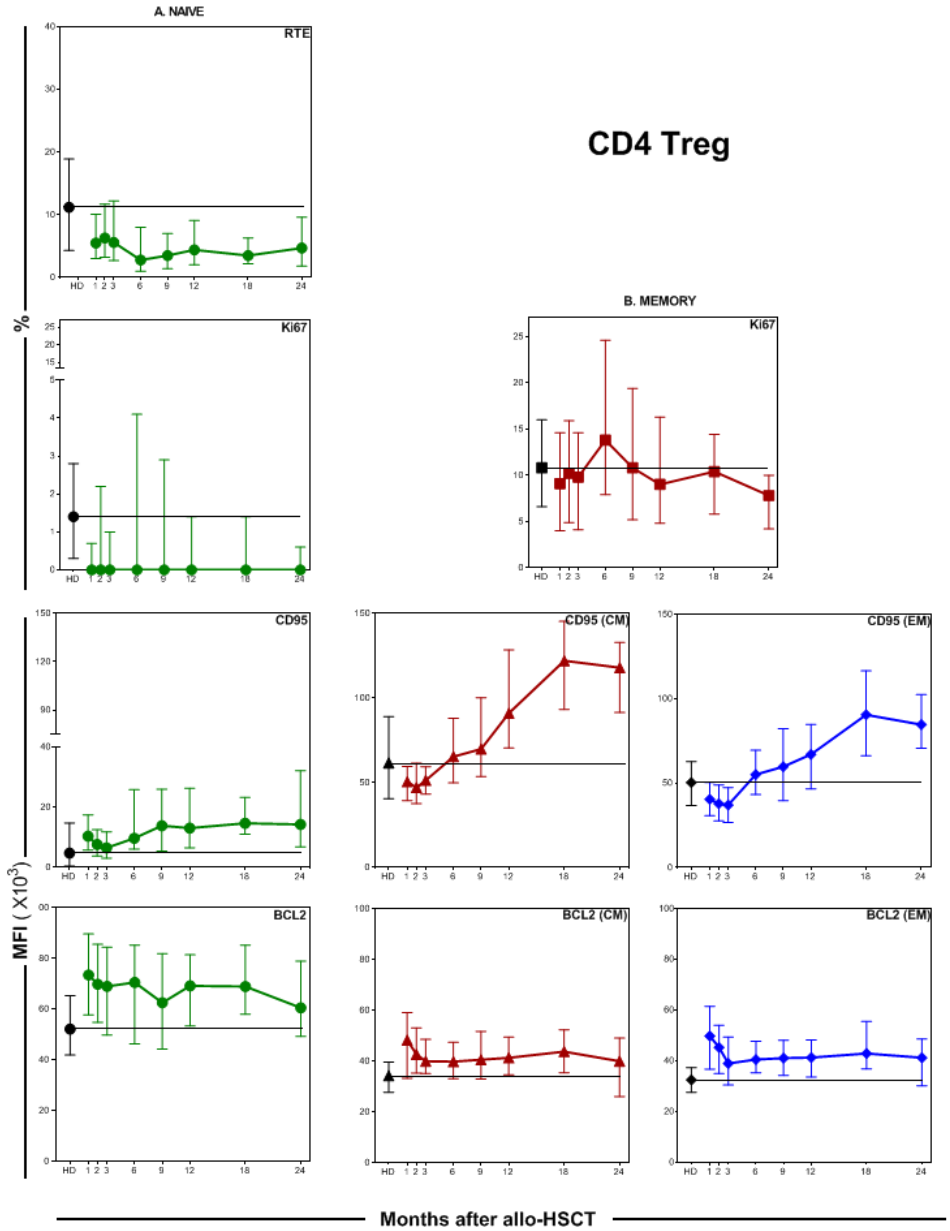
Reconstitution of Treg

T-cell recovery relies on proliferation of mature T-cells as well as generation of naïve cells from lymphoid progenitor cells. Persistence of differentiated cells is also dependent on their susceptibility to apoptosis. In this study, these factors were evaluated by measuring the intracellular expression of Ki-67 (marker of proliferation), the cell surface expression of CD95 (marker of extrinsic pathway of apoptosis and T-cell activation) and intracellular expression of Bcl-2 (marker of intrinsic pathway of apoptosis). Concurrent analysis of each major T-cell population revealed important similarities as well as differences unique to each major T-cell population.

Within the Treg population, naïve Tregs exhibited very low levels of proliferation with no evidence of recovery over a 2-year period (Figure 11A). CD95 expression in naïve Tregs was relatively normal in the first 6 months post-HSCT and then remained stable at levels above normal for the next 18 months. Bcl-2 expression in naïve Tregs was above normal for the entire 2-year period. Although proliferation of naïve Treg was very low, proliferation of memory Treg was consistently normal both early and late post-HSCT (Figure 11B). This is remarkable since steady state proliferation of memory Treg in HD is normally 9-10 fold higher than naïve Treg. In the first 2-3 months post-HSCT, memory Tregs expressed relatively low levels of CD95 and relatively high levels of Bcl-2 compared to HD. Subsequently expression of CD95 gradually increased to very high levels 12-24 months post-HSCT. Bcl-2 expression also remained high 6-24 months post-

HSCT in memory Treg. Expression of CD95 and Bcl-2 was very similar in T_{CM} and T_{EM} Treg subsets.

Figure 11. Dynamic profile of Treg subsets after RIC allo-HSCT

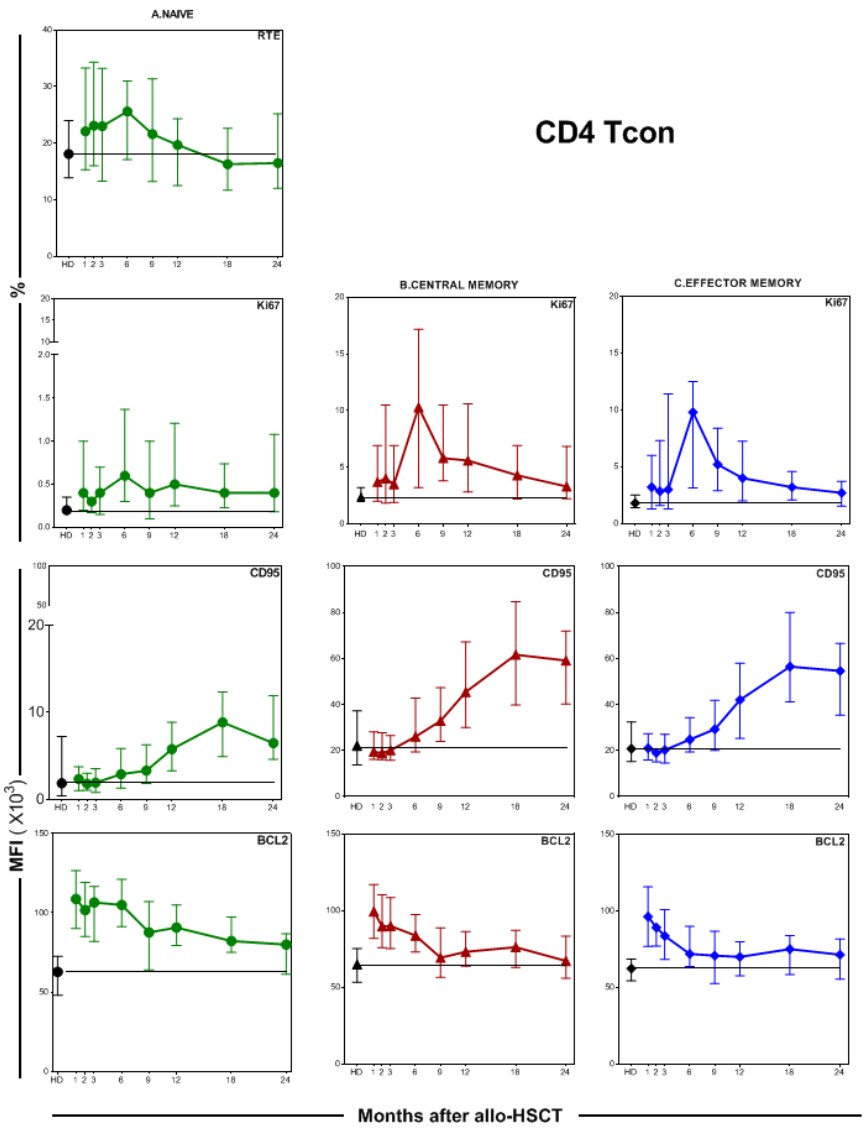


Phenotypic markers of thymic production, proliferation and susceptibility to apoptosis were measured at different timepoints over a 2-year period. Results are shown for (A) naïve and (B) memory subsets of Treg. Individual graphs show % recent thymic emigrants (RTE), % Ki-67 (proliferation), mean fluorescence intensity (MFI) of CD95 and Bcl-2. Median values (dark circle) and the IQ range (whisker bars) are shown for each parameter. Results in healthy donors (HD) are shown in black. CM, central memory; EM, effector memory.

Reconstitution of Tcon

Within the Tcon population, assessment of Ki-67, CD95 and Bcl-2 expression revealed several changes during post-HSCT recovery (Figure 12). Proliferation of naïve, T_{CM} and T_{EM} subsets was consistently at or above normal at all timepoints (Figure 12 top panels). Within the T_{CM} and T_{EM} subsets, proliferation was markedly increased at 6-12 months post-HSCT. Expression of CD95 was relatively normal in all Tcon subsets 1-9 months post-HSCT but then increased to very high levels 12-24 months post-HSCT (Figure 12 middle panels). Expression of Bcl-2 was elevated in all Tcon subsets in the first 6 months post-HSCT (Figure 12 lower panels). Bcl-2 levels gradually decreased to just above normal levels in T_{CM} and T_{EM} Tcon subsets but remained elevated in naïve Tcon.

Figure 12. Dynamic profile of Tcon subsets after RIC allo-HSCT

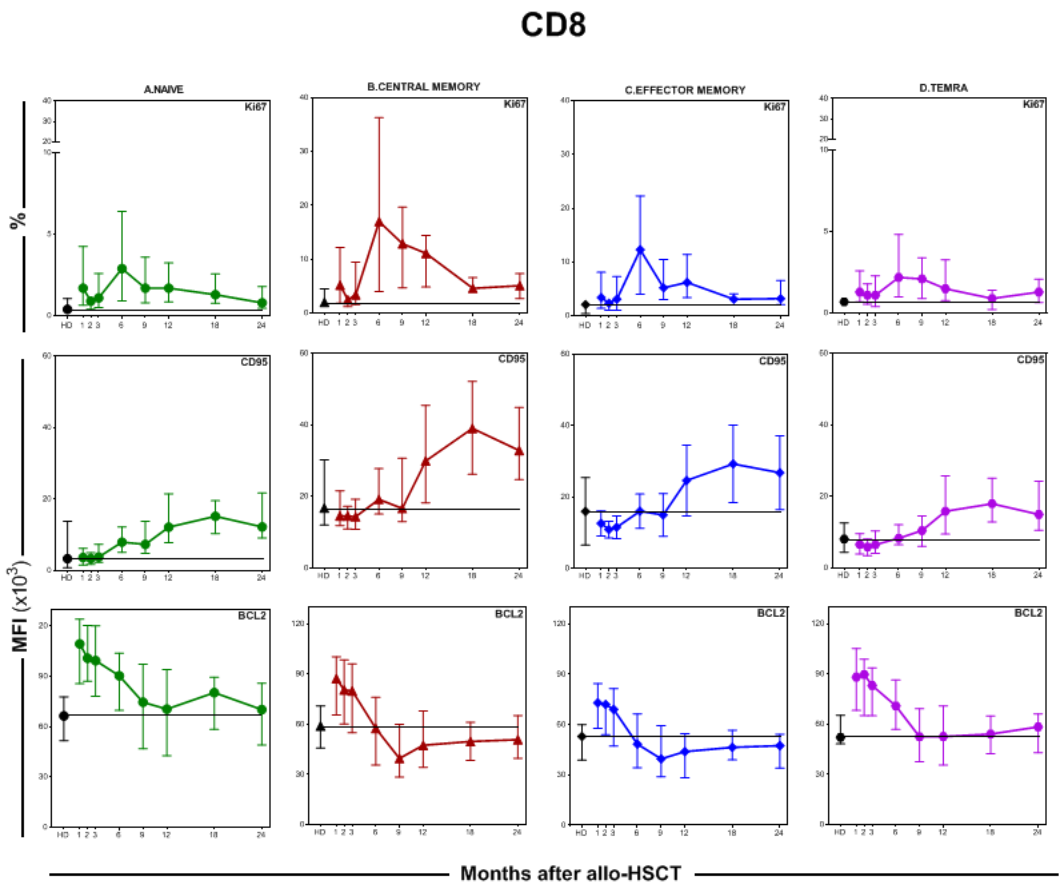


Phenotypic markers of thymic production, proliferation and susceptibility to apoptosis were measured at different timepoints over a 2-year period. Results are shown for (A) naïve, (B) memory and (C) effector memory subsets of Tcon. Individual graphs show % recent thymic emigrants (RTE), % Ki-67 (proliferation), mean fluorescence intensity (MFI) of CD95 and Bcl-2. Median values (dark circle) and the IQ range (whisker bars) are shown for each parameter. Results in healthy donors (HD) are shown in black.

Reconstitution of CD8$^+$ T-cells

Although recovery of CD8$^+$ T-cells was more rapid than Tcon, patterns of proliferation and expression of CD95 and Bcl-2 were remarkably similar in these 2 populations as peripheral counts gradually recovered after HSCT (Figure 13).

Figure 13. Dynamic profile of CD8$^+$ T-cells after RIC allo-HSCT



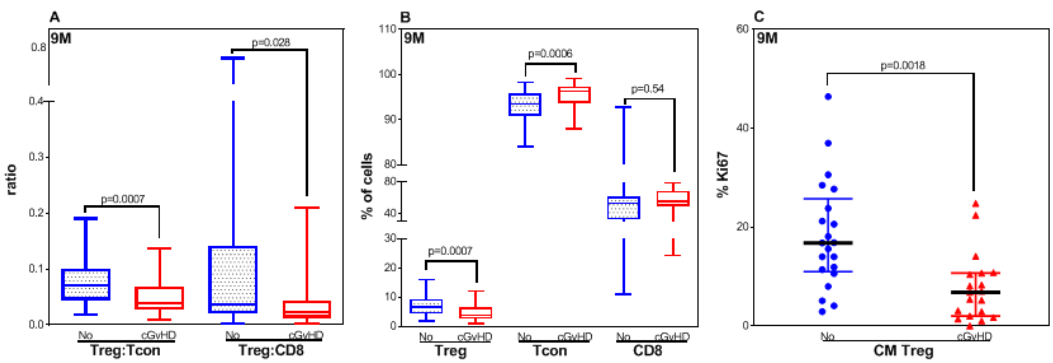
Phenotypic markers of proliferation and susceptibility to apoptosis were measured at different timepoints over a 2-year period. Results are shown for (A) naïve, (B) central memory, (C) effector memory and (D) terminal effector memory re-expressing CD45RA. Individual graphs show % Ki-67 (proliferation), mean fluorescence intensity (MFI) of CD95 and Bcl-2. Median values (dark circle) and the IQ range (whisker bars) are shown for each parameter. Results in healthy donors (HD) are shown in black.

Proliferation of all CD8$^+$ subsets remained normal or above normal throughout the 2-year study period (Figure 13 top panels). High levels of proliferation were observed at 6-12 months post-HSCT in CD8$^+$ T_{CM} and T_{EM} subsets. Expression of CD95 was relatively normal in all subsets 1-9 months post-HSCT and subsequently gradually increased in all subsets as CD8$^+$ counts recovered (Figure 13 middle panels). Bcl-2 expression was relatively high in all subsets in the first 3-6 months post-HSCT and then gradually returned to normal levels 9-24 months post-HSCT (Figure 13 lower panels).

Aberrant - cell dynamics in chronic GvHD

In this cohort, 46 patients (43%) developed cGvHD during the 2-year follow-up period. Immune reconstitution of total Treg, Tcon and CD8$^+$ T-cells were similar between groups, except at 9 months post-transplant where we observed an apparent delayed recovery of CD4$^+$ T-cells in patients who develop cGvHD (data not shown). By this time, 20 patients had developed cGvHD and 20 remained free of any clinical manifestations of cGvHD. As shown in Figure 14A, the ratio of Treg:Tcon and Treg:CD8 were both significantly greater in patients who had not developed cGvHD compared to patients who had developed cGvHD (p=0.0007 and p=0.028, respectively). The significant difference in Treg:Tcon and Treg:CD8 primarily reflects significantly lower %Treg and higher %Tcon in patients with cGvHD, p=0.0007 and p=0.0006 respectively (Figure 14B). %CD8 was not different between patients with and without cGvHD (p=0.54). Central memory Tregs represent the predominant Treg subset at this time and proliferation of this subset was significantly lower in patients with cGvHD, p=0.0018 (Figure 14C).

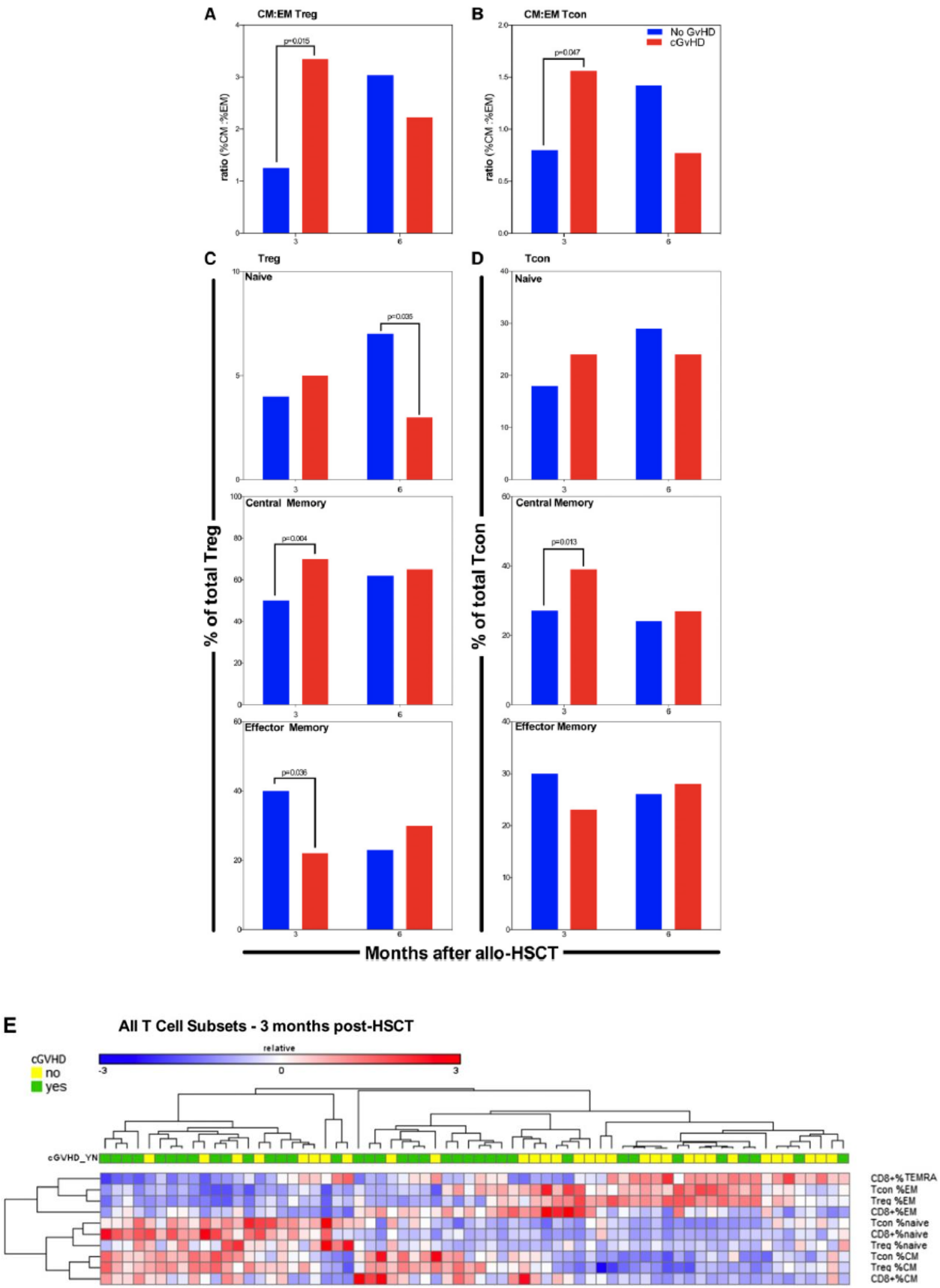
Figure 14. T-cell recovery according to cGvHD at 9 months post RIC allo-HSCT



(A) Treg:Tcon and Treg:CD8 ratios. (B) %Treg, %Tcon and % CD8$^+$ T-cells. (C) Proliferation (%Ki-67) in central memory (CM) Tregs. Each figure compares patients with (red) and without (blue) chronic GvHD (cGvHD). Box plots in plots A and B depict the 75th percentile; median and 25th percentile values and whiskers represent maximum and minimum values. In part C, dots depict the number of patients (n=20 for each group) and the whiskers correspond to the 75th and 25th percentile.

As naïve T-cells encounter antigen and differentiate into memory cells, central memory cells remain relatively immature while effector memory cells acquire functional characteristics of increased tissue homing and increased cytolytic capacity. Examination of the ratio of central memory:effector memory (CM:EM) at 3 and 6 months post-HSCT revealed changes during this period that were significantly different in patients who developed or did not develop cGvHD. As shown in Figure 15A and 15B, the CM:EM ratio was significantly higher 3 months post-HSCT in patients who develop cGvHD. This shift was most evident in Treg but was also present in Tcon. As shown in Figure 15C and 15D, the switch in predominance between T_{CM} and T_{EM} subsets is driven primarily by the increase in T_{CM} in patients who develop cGvHD. The fraction of T_{CM} is significantly increased in both Treg and Tcon at 3 months post-HSCT in patients who subsequently develop cGvHD but there is little change in the T_{CM} fraction at 6 months post-HSCT and the percentage of T_{CM} is similar in patients with and without cGvHD at this timepoint. Also evident at 6 months post-HSCT is the selective increase in naïve Tregs in patients who do not develop cGvHD, with delayed recovery in cGvHD patients.

Figure 15. T-cell differentiation according to cGvHD status after RIC allo-HSCT



Changes in the percentage of the naïve, central memory (CM) and effector memory (EM) subsets were determined at 3 and 6 months after transplant. Results were compared in patients who developed (red) or did not develop (blue) chronic GvHD (cGvHD). (A) CM:EM ratio in Treg. (B) CM:EM ratio in Tcon. (C) Treg - median percentage of the naïve, CM and EM. (D) Tcon - median percentage of the naïve, central memory and effector memory. (E) Frequency profile of the entire T-cell compartment at 3 months after transplant. Data are presented as a heatmap using hierarchical clustering after standardization of each row. Top bar indicates subsequent cGvHD outcome (green – cGvHD; yellow – No cGvHD). Blue, white and red represent low, intermediate, and high subset frequency.

Differences in Treg and Tcon subsets noted 3 months after HSCT led us to undertake a more detailed analysis of all T-cell subsets at this early timepoint comparing patients who would subsequently develop cGvHD with those that remained free of cGvHD. The unsupervised hierarchical clustering of percent naïve, T_{CM} and T_{EM} subsets is shown in the Figure 15E heatmap. Patient clusters on the right are characterized by increased fractions of more differentiated subsets in all 3 major T-cell populations represented by CD8 T_{EMRA} as well as T_{EM} within Treg, Tcon and $CD8^+$ T-cells populations. In contrast, patient clusters on the left are characterized by increased frequency of less differentiated subsets in all 3 major T-cell populations, represented by naïve and T_{CM} subsets. Patients who subsequently develop cGvHD predominate within the latter group of patients that have high percentages of naïve and T_{CM} subsets and low percentages of T_{EMRA} and T_{EM} subsets 3 months after HSCT. In contrast, patients who do not develop cGvHD are characterized by having high percentages of differentiated T-cell subsets and low percentages of naïve and T_{CM} subsets 3 months after HSCT. Further analysis of 3-month data excluding patients who developed acute GvHD did not change these findings (data not shown).

1.1.1. Short Discussion

Full reconstitution of T-cell immunity post allo-HSCT requires a balanced recovery of donor-derived $CD4^+$ regulatory T-cells and effector, $CD4^+$ (Tcon) and $CD8^+$, T-cells. To characterize the peripheral mechanisms involved in T-cell reconstitution post transplant, and elucidate the role of regulatory and effector T-cells in cGvHD development, we performed a detailed quantitative and functional long-term analysis of the naïve and memory T-cell compartments in a cohort of 107 adult patients, who received T-cell

replete stem cell products, after RIC allo-HSCT, using primarily TAC/SIR for GvHD prophylaxis. Results were compared to 15 healthy donors.

Consistent with previous reports, neither Treg nor Tcon achieved normal levels during the 2-year observation period and $CD8^+$ T-cells recovered more rapidly. Within Treg and Tcon, the naïve subset was the slowest to recover. As a result, reconstituting Tregs were predominantly T_{CM} and Tcons were predominantly T_{EM}. Within $CD8^+$ T-cells, T_{EMRA} corresponded to the main subset and naïve T-cells recovered to normal levels 1.5 years after transplant.

To examine the *de novo* production of $CD4^+$ T-cells from lymphoid progenitor cells we monitored the T_{RTE} within Tcon and Treg, measuring CD31 expression on naïve subsets. While T_{RTE} Tcons were maintained within the normal range for all the follow-up period, very few T_{RTE} Tregs were detected. These results must be interpreted carefully, since the expression of CD31 on $CD4^+$ Tcons can be sustained/increased by IL-7 in the post-transplant lymphopenic environment[24]. In this regard, we expected a delayed recovery of both T_{RTE} Tregs and Tcons, resulting from thymic impairment post-transplant[118,119]. The delayed recovery of T_{RTE} Tregs could be exacerbated by insufficient IL-2 levels, produced by Tcons, which numbers were dramatically low[258]. IL-2 has a critical role in Treg development and survival in the thymus[106], and Treg maintenance in the periphery[231,232]. Hence, a relative deficiency of IL-2 may detain the reconstitution of Tregs[258].

Following transplant, mature T-cells undergo homeostatic proliferation to expand the number of cells available for immune surveillance. In this cohort, proliferation as measured by the expression of Ki-67, was maintained at HD levels in the first 3 months post-transplant for both naïve and memory Tcon and $CD8^+$ T-cells. Subsequently, increased proliferation of memory subsets was noted at 6 months post-transplant, while naïve T-cells proliferated to similar levels as HD. Conversely, within Tregs, proliferation of naïve T-cells was markedly reduced for all the follow-up, while proliferation of memory subsets was maintained at HD levels. In a HD, in steady-state conditions, naïve Tregs feature high levels of cytokine-driven proliferation, mostly mediated by IL-2[143] [231,232]. In this cohort, naïve Tregs, contrary to other T-cell subsets and populations,

expressed very low levels of proliferation, which most likely resulted from IL-2 deficiency in the early post-transplant period[258].

Within each T-cell population, proliferation of mature T-cells is balanced by apoptosis of activated cells. In this cohort, the expression of CD95 was similar for all the T-cell subsets, being normal in the first 6-9 months and subsequently increasing between 12-24 months after transplant. Conversely, the expression of Bcl-2 was different according to T-cell subset and population. Within naïve and memory Tcon and $CD8^+$ T-cells, Bcl-2 expression was high in the first 3-6 months post-transplant and then returned to HD levels. Within Tregs, the expression of Bcl-2 was high for all the follow-up period in naïve subsets, while memory subsets only expressed high levels of Bcl-2 in the first 2 months post-transplant, then returned to HD levels. Similar to previous data, in this cohort naïve Tregs displayed a pro-survival phenotype in the first year post-transplant[143]; however, considering the very low rate of proliferation and the lack of thymic generation, the expansion of naïve Tregs was compromised.

Having identified a preferential expansion of Tcon and $CD8^+$ T-cells over Treg, we examined whether this dynamic imbalance was associated with the development of cGvHD. In the first 6 months post-HSCT, recovery of total Treg, Tcon and $CD8^+$ T-cells was similar in patients who subsequently developed or did not develop cGvHD. However, at 9 months post-transplant, some patients had already developed cGvHD and showed lower ratios of Treg:Tcon and Treg:CD8. The lower Treg:Tcon ratio reflects both lower Treg and greater Tcon, but the lower Treg:CD8 ratio is entirely due to a lower percentage of Treg. A major driver of this imbalance appears to be the significantly lower proliferation of T_{CM} Tregs in patients who develop cGvHD. These results must be interpreted carefully, since they may reflect the effect of cGvHD in the T-cell compartment, or rather the immunomodulatory effect of cGvHD treatment.

As we monitored T-cell reconstitution, we also observed altered Treg and Tcon differentiation associated with development of cGvHD as early as 3 months post-transplant. At this early timepoint, the great majority of T-cells were Tcon, but all T-cell populations, including Treg, Tcon and $CD8^+$ T-cells, were skewed towards predominance of naïve and T_{CM} subsets in patients who subsequently developed cGvHD, independently of aGvHD. This skewing was less evident 6 months post transplant and began to reverse

in Treg and Tcon populations as patients were diagnosed with cGvHD. Previous studies in mouse models have shown that T-cells capable of promoting GvHD are primarily contained within the naïve T-cell compartment[288,289]. Moreover, recent clinical trials demonstrated that depletion of naïve T-cells from the stem cell product had little effect on the incidence of acute GvHD but appeared to markedly reduce the incidence of cGvHD[290–292].

In summary, prospective monitoring of T-cell reconstitution in this cohort identified differences in the homeostatic recovery that favors the thymic generation, peripheral expansion and maintenance of Tcon and $CD8^+$ T-cells over Tregs. These homeostatic imbalances contribute to the development of cGvHD, that in this cohort is characterized by a significantly lower percentage of Tregs and higher percentage of Tcon. Moreover, patients who develop cGvHD could be distinguished at three months post-transplant by a predominance of relatively immature T-cell subsets (naïve and T_{CM}), suggesting a role for naïve T-cells in cGvHD biology.

II. CHAPTER II

Short Introduction

The contribution of naïve T-cells to the pathogenesis of cGvHD has been recently validated in clinical trials. However, the role of T memory stem cells (T_{SCM}) in this context has not yet been addressed in human studies. T_{SCM} represent a small subpopulation that phenotypically fall in the traditional naïve T-cell pool, but display functional characteristics of effector and memory subsets. This subpopulation stands out for its unique ability to self-renew and differentiate into multiple cell lineages, and its role in cGvHD has been suggested in preclinical studies.

This chapter illustrates the results from two manuscripts reporting the role of true naïve and T_{SCM} subsets in cGvHD development. The first manuscript describes the immune T-cell recovery, thymic function and TCR repertoire diversity in a cohort of 40 adult patients who received T-cell depleted allo-HSCT for hematologic malignances (RIC_Lisbon cohort). The second manuscript focuses on the characterization of the naïve and memory T-cell compartments early post-transplant and prior to cGvHD development in a cohort of 111 adult patients who received T-cell replete allo-HSCT for hematologic malignances (MAC_Boston cohort).

2.1. Naïve and stem cell memory T cell subset recovery reveals opposing reconstitution patterns in CD4$^+$ and CD8$^+$ T-cells in chronic GvHD

Results

Patient characteristics

Clinical characteristics of the patients included in the study are summarized in Table 6. The median age was 49 years, 25 % of male recipients had a female donor. The most frequent underlying disease was AML (n=17; 42.5%), 24 patients (60%) received PBSC as the source of graft, and 23 (57.5%) were transplanted with a mismatch donor. Besides ATG, GvHD prophylaxis consisted of CsA plus MMF in all patients. After a 24-month follow-up post-HSCT, 19 patients (47,5%) developed grade II-IV aGvHD, 18 patients (45%) developed cGvHD, within these, 17 had previously developed aGvHD. Median cGvHD onset day was 230. Five HD with a median age of 43 (range 36-45), were studied.

Table 6. Patient/donor characteristics according to GvHD (RIC_Lisbon cohort)

	cGVHD	No cGVHD
Patients	18	22
Gender	6 F; 12 M	12 F; 10 M
Age	51 (30–67)	46 (19–69)
DIAGNOSIS		
AML	7	10
ALL	0	4
CLL	1	0
CML	2	2
HL	1	0
NHL	4	3
MDS	2	0
MF	1	0
MM	0	3
CMV Positive	14	18
DONORS		
Gender	11 F; 7 M	9 F; 13 M
Age	35 (21–49)	32 (20–57)
Female Donor to Male Patient	6	4
CMV Pos	12	15

	cGVHD	No cGVHD
HLA MATCHING		
10/10	8	9
9/10	9 (3A, 1B, 4C, 1DQB1)	12 (8A, 1B, 2C, 1DQB1)
8/10	1 (DRB1+DQB1)	1 (A+DQB1)
SOURCE OF GRAFT		
BM	5	11
PBSC	13	11
aGVHD	17/18	9/22
GI	5	2
GII	6	4
GIII	6	3
GIV	–	–
cGVHD	18	–
Mild	7	–
Intermediate	4	–
Severe	7	–
Day of cGVHD Onset	230 (149–455)	–
Follow Up (days)	682 (286–1,051)	483 (90–840)

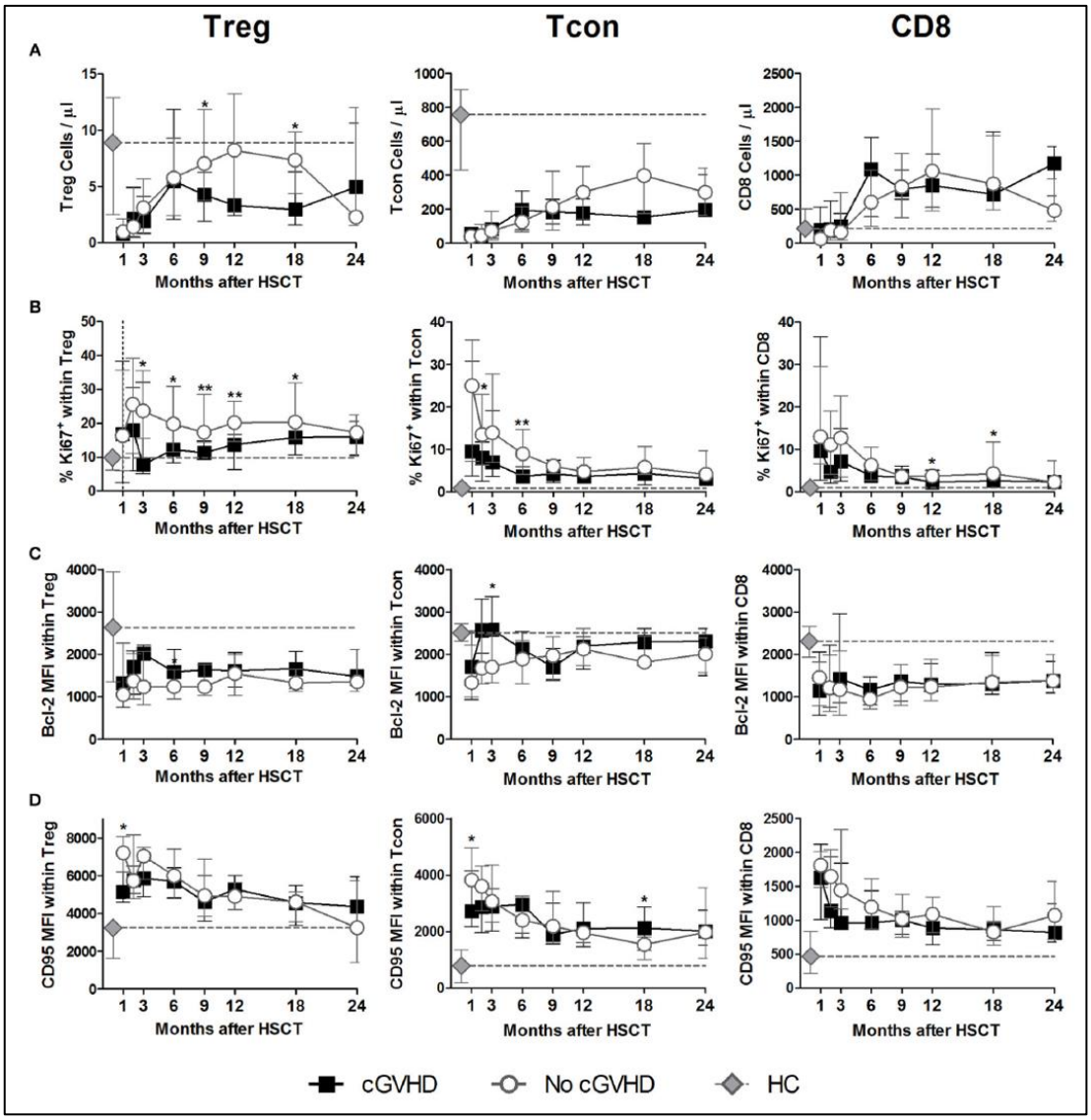
Abbreviations: aGvHD, acute GvHD; ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; cGvHD, chronic GvHD; CMV, cytomegalovirus; F, female; G, grade; GvHD, Graft *vs.* Host Disease; HL, Hodgkin lymphoma; HLA, human leukocyte antigen; M, male; MDS, myelodysplastic syndrome; MF, Myelofibrosis; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; PBSC, peripheral blood stem cell.

Distinct Treg, Tcon and CD8 T-cell reconstitution patterns after HSCT

$CD4^+$ Treg ($CD4^+CD25^+CD127^{low}Foxp3^+$) numbers were significantly low in both patient groups up to month 6 after HSCT (Figure 16A). From months 9 to 18, Tregs were decreased in cGvHD *vs.* No cGvHD patients. Analysis of *ex vivo* proliferation using intracellular Ki-67 staining revealed significantly decreased proliferation from months 3 to 18 in patients developing cGvHD as compared to No cGvHD, suggesting that reduced Treg numbers in cGvHD may be partly due to reduced homeostatic proliferation (Figure 16 B). Lower Treg numbers in cGvHD were not associated with increased susceptibility to apoptosis as assessed by Bcl-2 and CD95 MFI, as these proteins were expressed at similar levels in both patient groups. (Figure 16 C, D). These data suggest that apoptosis mediated by these pathways did not play a major role in the low Treg numbers observed in cGvHD.

We further quantified conventional $CD4^+$ (Tcon; $Foxp3^-CD4^+$) and CD8 T-cells numbers and found no major differences between patients with and without cGvHD (Figure 16A). We did observe significant reductions in Tcon and CD8 proliferation (Ki-67 %), in cGvHD compared to No cGvHD (Figure 16B). On the other hand, no clear trend for altered Bcl-2 or CD95 expression in cGvHD was observed (Figure 16 C-D).

Figure 16. Treg, Tcon and CD8 homeostasis following RIC-ATG allo-HSCT



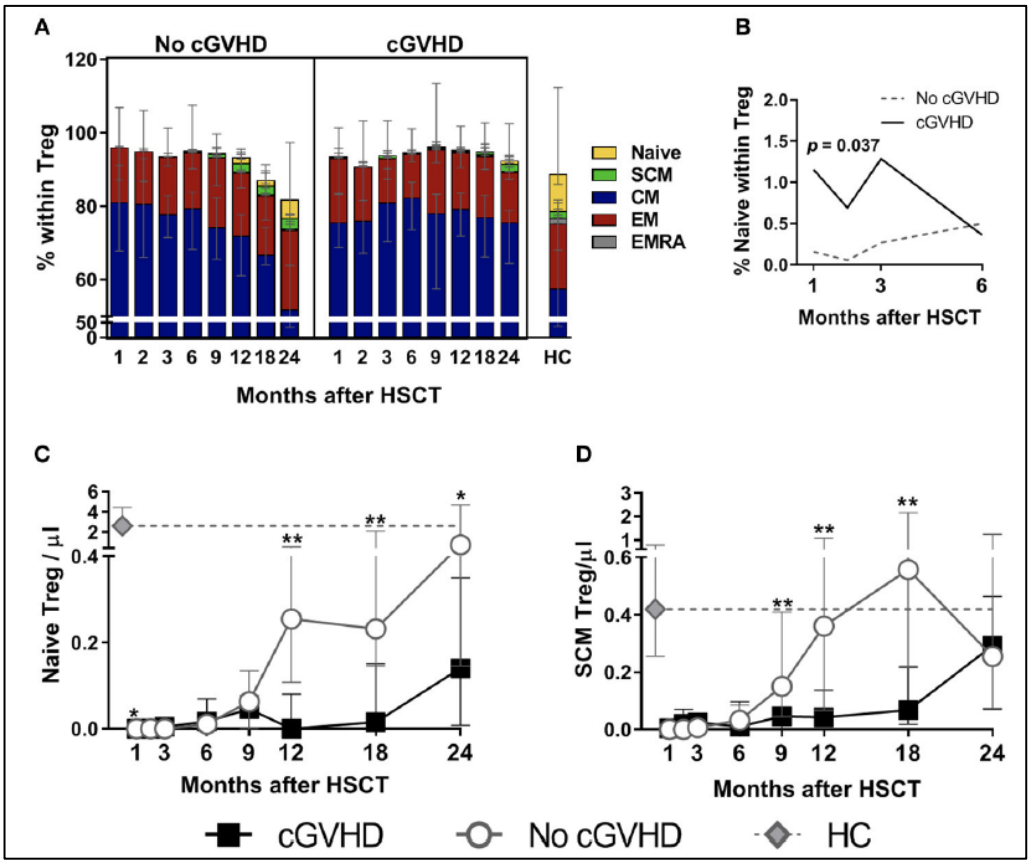
Patients were divided into No cGvHD (grey lines and open circles) and cGvHD (black lines and squares). Healthy controls (HC) are also shown (grey diamonds and dotted grey lines). (A) Absolute counts/µl for Treg ($CD3^+CD4^+CD25^{bright}Foxp3^+CD127^{low}$), Tcon ($CD3^+CD4^+Foxp3^-$) and CD8 ($CD3^+CD4^-$) T-cells. (B) Frequency of Ki-67$^+$ cells within Treg, Tcon and CD8 T-cells. Median fluorescence intensity (MFI) for Bcl-2 (C) and CD95 (D) within Treg, Tcon and CD8 T-cells. Symbols represent median values ± interquartile range (IQR). Asterisks denote statistically significant differences between patient groups (*p=0.01 to 0.05; **p=0.001 to 0.01).

Impaired True Naïve and SCM Treg reconstitution in cGvHD

Quantification of naïve and memory Treg subsets was performed by flow cytometry, using CD45RA and CD62L surface markers, as described in the Methods (Section 3.1.2). We further used CD95 to distinguish the peripheral naïve/true naïve (pT_N) subset, $CD45RA^+CD62L^+CD95^{-/low}$, from the Stem Cell Memory (T_{SCM}) subset, $CD45RA^+$ $CD62L^+CD95^+$[70] (detailed gating strategy is illustrated in Figure 7).

The most prevalent subset within Treg was T_{CM}, followed by T_{EM}, pT_N and T_{SCM}, while T_{EMRA} were an extremely rare subset in this population (Figure 17A). We observed decreased pT_N Treg percentages and absolute counts in cGvHD as compared to No cGvHD throughout the follow-up (Figure 17A, C). The only exception was at month 1 when the frequency and absolute count of pT_N Tregs were increased in cGvHD patients (Figure 17A, C). Despite this, pT_N Tregs remained low in patients developing cGvHD while increasing in the absence of cGvHD. This trend, reflecting an inability of pT_N Treg to recover in patients developing cGvHD, was confirmed in a Linear Mixed-Effects (LME) analysis of the first 6 months post-HSCT. This analysis showed significantly different slopes estimated for the recovery of pT_N percentages, whereby pT_N Treg percentages only increased in the absence of cGvHD (Figure 17B). From month 9 onwards, pT_N Treg percentages and counts were lower in patients developing cGvHD as compared to patients that remained free from cGvHD (Figure 17A, C). Similar to pT_N Treg, the recovery of T_{SCM} Tregs was impaired in patients developing cGvHD. This was the case for percentages and absolute counts, which were significantly reduced in cGvHD from month 9 to 18 (Figure 17A, D). The relative proportion of the remaining memory subsets was largely unaltered by cGvHD (Figure 17A). T_{CM} and T_{EM} Treg absolute counts were either similar or reduced in cGvHD, whereas T_{EMRA} Treg were similar or increased, but no sustained statistical significances were noted. A detailed visualization of the overall data, showing percentage and absolute counts for each subset in all time-points for both patient groups and healthy controls is displayed in supplementary Figure 1B (p.202).

Figure 17.Treg subset recovery in cGvHD patients after RIC-ATG allo-HSCT



(A) The median percentage for each subset within Treg is shown for patients with and without cGvHD, as well as for HC. (B) True naïve Treg percentages in the first 6 months after HSCT in patients with cGvHD (black line) and No cGvHD (dotted grey line), illustrating the results of the LME analysis for this subset. Absolute counts of naïve (C) and SCM (D) Treg in patients without cGvHD (grey lines and open circles), with cGvHD (black lines and squares) and HC (grey diamonds and dotted grey lines). Symbols represent median values ± IQR. Asterisks denote statistically significant differences between patient groups (*p=0.01 to 0.05; **p=0.001 to 0.01).

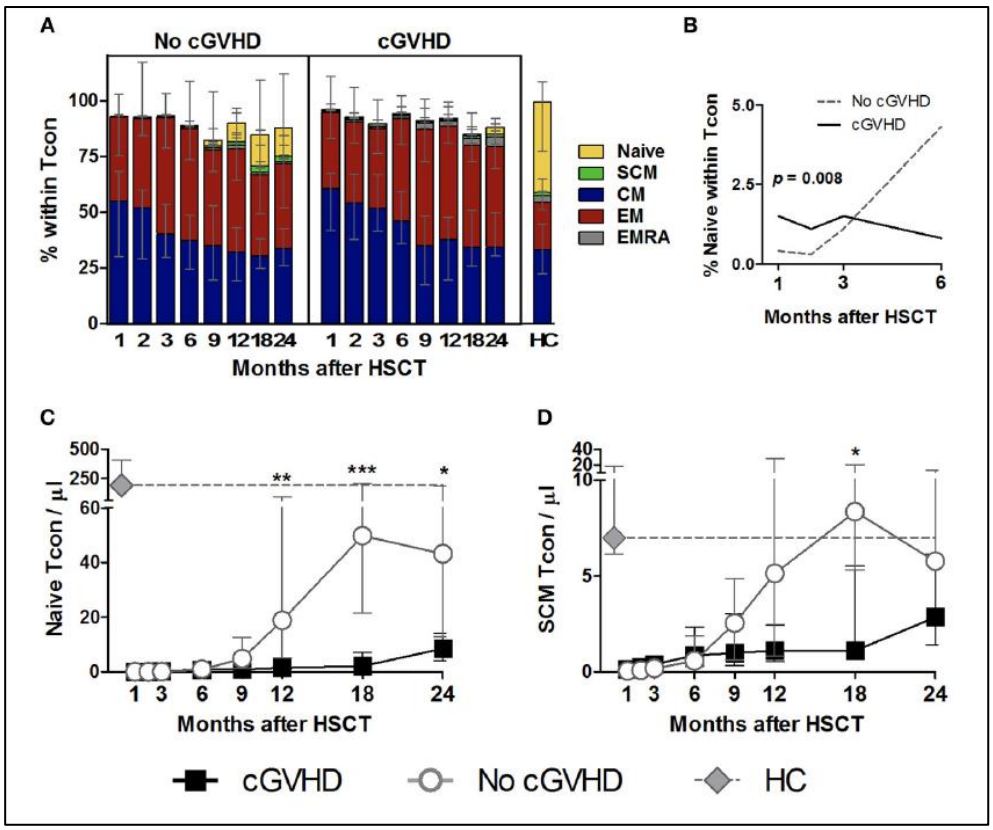
Impaired True Naïve and SCM Tcon reconstitution in cGvHD

Chronic GvHD was associated with a marked impairment in pT_N Tcon recovery as compared to patients who did not develop cGvHD. This became apparent from month 9 onwards in terms of both frequency and absolute counts (Figure 18A, C). Although differences between patient groups only reached significance after month 9, the LME analysis revealed that the percentage of pT_N Tcon up to month 6 was better represented by a model with significantly different slopes for cGvHD and No cGvHD (p=0.008) (Figure 18B). Hence, during the first 6 months after HSCT, pT_N Tcon percentages increased in patients who did not subsequently develop cGvHD, while they remained

stable in patients who develop cGvHD. This translated, at later timepoints, into significant differences in pT_N Tcon frequencies and numbers between patient groups from months 12 to 24 (Figure 18A, C; supplementary Figure 1B and C; p.202). Impaired pT_N Tcon recovery in cGvHD was mimicked by decreased T_{SCM} Tcon levels, reaching significance at month 18 for T_{SCM} Tcon counts (Figure 18A, D). T_{CM} and T_{EM} were the most abundant Tcon subsets post-HSCT and were largely similar between patient groups, while T_{EMRA} showed a non-significant tendency to be increased in cGvHD (Figure 18A). Percentages and absolute counts for all Tcon subsets are detailed in supplementary Figure 1B and C (p.202).

In summary, we did not observe significant disparities in whole Tcon recovery between patients developing cGvHD and those who did not. However, the composition of the Tcon pool after HSCT reveals an impairment in pT_N and T_{SCM} subsets in patients who develop cGvHD, similar to what was observed in Treg.

Figure 18. True Naïve and memory Tcon recovery after RIC-ATG allo-HSCT



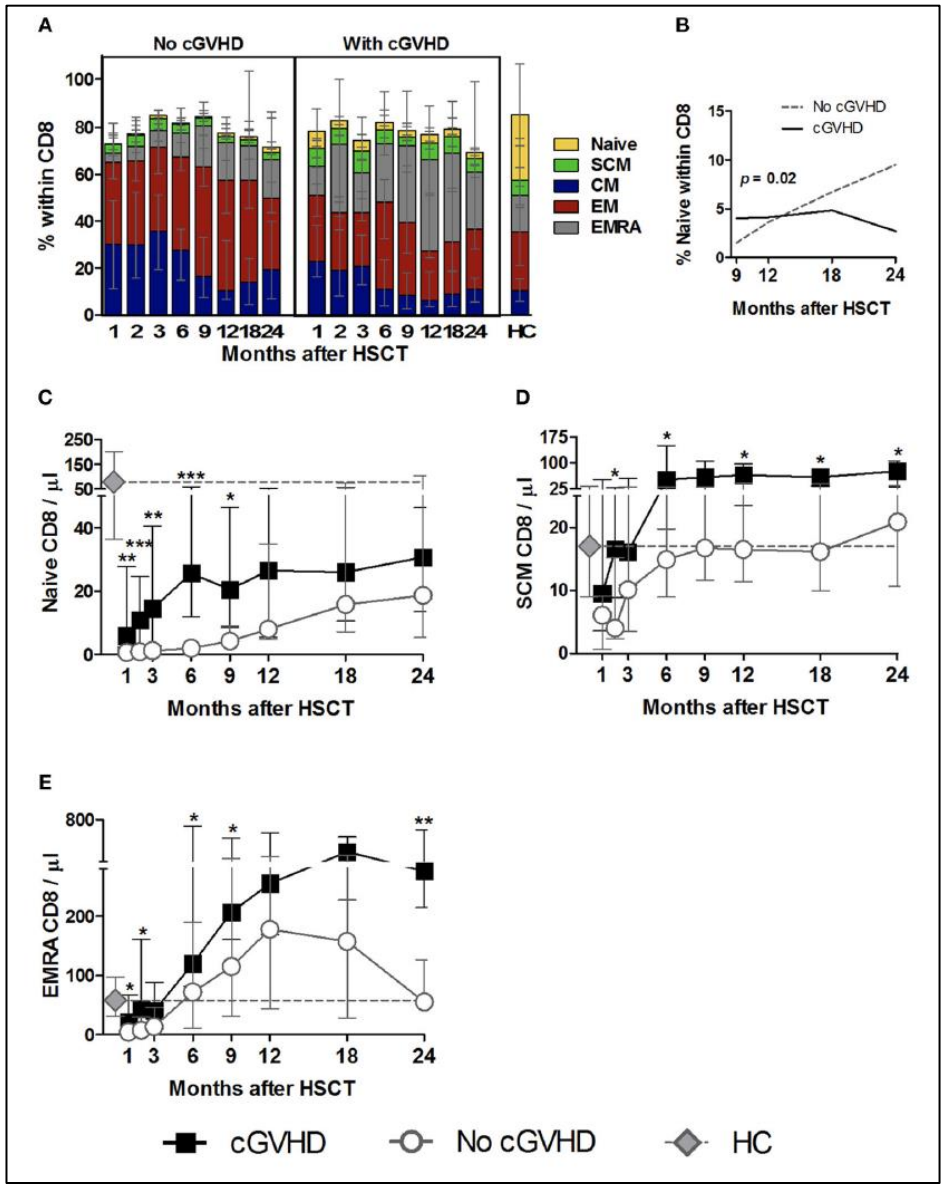
(A) The percentage of each subset within Tcon cells is shown for patients with and without cGvHD, as well as HC. (B) Percentage of true naïve within Tcon in the first 6 months after HSCT in patients with cGvHD (black line) and No cGvHD (dotted grey line), illustrating the results of the LME analysis for this subset. True Naïve (C) and SCM (D) Tcon absolute counts in patients without cGvHD (grey line and circles), patients with cGvHD (black line and squares) and HC (grey diamonds and dotted grey lines). Median values ± IQR are shown. Asterisks denote statistically significant differences between patient groups (*p=0.01 to 0.05; **p=0.001 to 0.01; ***p=0.0001 to 0.001).

Increased CD8 True Naïve, SCM and EMRA in cGvHD patients

The reconstitution of pT_N and memory subsets within the CD8 T-cells differed greatly between patient groups (Figure 19) and had a strikingly different pattern to that observed in Tcons and Tregs. This was particularly prominent not only for the least abundant pT_N and T_{CM} CD8 subsets, but also for the more abundant, T_{EMRA} subset.

Interestingly, we observed significantly increased pT_N CD8 percentages and counts in cGvHD *vs.* No cGvHD during the initial post-transplant period (Figure 19A, C). This statistical significance disappeared from month 12 onwards, as pT_N CD8 increased in patients who did not develop cGvHD. Such accelerated pT_N CD8 T-cells recovery in the absence of cGvHD at later timepoints was also revealed by the LME analysis of months 9 to 24 post-HSCT. This analysis showed a significant difference in the slopes for pT_N CD8 percentages between the two patient groups (Figure 19B). While in patients with cGvHD the percentage of pT_N CD8 stabilized, in the absence of cGvHD the frequency of these cells increased over time. Interestingly, the T_{SCM} CD8 subset was also increased in patients developing cGvHD *vs.* No cGvHD, starting from the early timepoints of the follow-up period, both in percentages and absolute counts (Figure 19A, D).

Figure 19. Ture Naïve and memory CD8 recovery after RIC-ATG allo-HSCT



(A) Percentage of each subset within CD8 T-cells for patients with and without cGvHD, as well as HC. (B) True Naïve CD8 percentages from month 9 to 24 after HSCT in patients with cGvHD (black line) and no cGvHD (dotted grey line), illustrating the results of the LME analysis for this subset. Absolute counts of true naïve (C), SCM (D) and EMRA (E) CD^+ T-cells in patients without cGvHD (grey circles and dotted lines), patients with cGvHD (black squares and lines) and HC (grey diamonds and dotted grey lines). Symbols represent median values ± IQR are shown. Asterisks denote statistically significant differences between patient groups (*p=0.01 to 0.05; **p=0.001 to 0.01).

Despite such prominent differences in pT_N and T_{SCM} subsets, these were always the least abundant populations. Within the most frequent populations the T_{EMRA} showed the most striking differences between patient groups. Hence, T_{EMRA} CD8 T-cells were increased in percentage and counts from the very early post-transplant period in patients developing

cGvHD (Figure 19 A, E), while the remaining memory subsets, T_{CM} and T_{EM}, showed an opposite pattern to that observed in pT_N, T_{SCM} and T_{EMRA}, with significant reductions in percentages being noted in patients developing cGvHD (Figure 19A; supplementary Figure 1B and C; p.202).

The effects of acute GvHD on SCM Tcon and CD8 T-cells

In this patient cohort, all but one of the patients developing cGvHD had a history of previous aGvHD, while within the group of 22 patients that did not develop cGvHD, 9 had developed aGvHD. In order to clarify the contribution of aGvHD and the associated therapies to differences in T-cell reconstitution between patient groups, we tested the effect of adding aGvHD as a covariate in the LME analysis. This analysis showed that during the first 6 months after HSCT, the percentage and absolute number of T_{SCM} within Tcon and CD8 T-cells were significantly affected by aGvHD ($p<0.01$). No impact of aGvHD was observed from month 6 onwards.

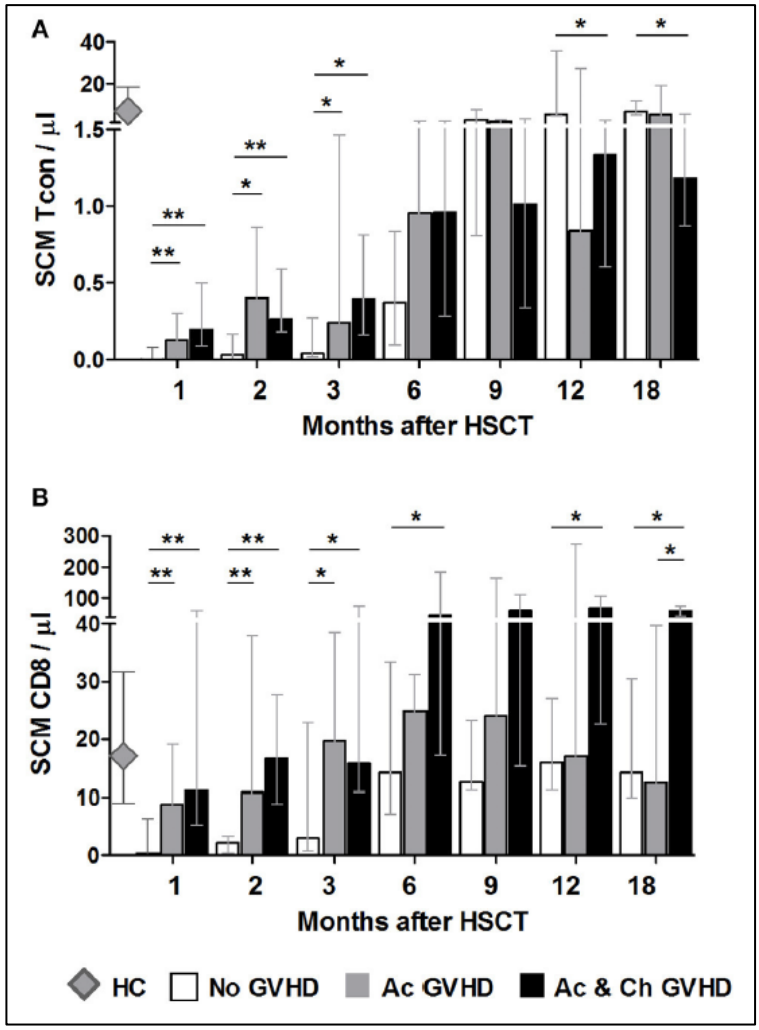
To complement this analysis, we stratified the population into three different groups. The No cGvHD group was divided into acute only (*Ac GvHD*, n=9) and no GvHD at all (*No GvHD*, n=13). The cGvHD group included only one patient who did not have aGvHD prior to cGvHD, who was excluded from this analysis; hence, cGvHD group consisted of patients with acute and cGvHD (*Ac & Ch GvHD*, n=17). Month 24 was excluded from this analysis due to insufficient number of data points.

T_{SCM} Tcon numbers were significantly increased during the first 3 months in patients who developed GvHD (*Ac & Ch GvHD*) *vs.* No GvHD patients (Figure 20A). At later timepoints, this pattern was reversed and while T_{SCM} Tcon reconstituted in the No GvHD and Ac GvHD groups, they became significantly reduced in the Ac & Ch GvHD group. A similar pattern was observed when analyzing T_{SCM} percentages within Tcon (data not shown). Overall, these data suggest that acute GvHD development is associated to early increases in T_{SCM} Tcon.

For $CD8^+$ T-cells, initial timepoints showed a similar reconstitution pattern to Tcon, whereby patients with GvHD (*Ac & Ch GvHD*) showed significantly increased T_{SCM} as compared to No GvHD (Figure 20B). Interestingly, from month 6 onwards patients with

aGvHD showed similar T_{SCM} CD8 counts as patients who did not develop any form of GvHD. On the other hand, patients who subsequently develop cGvHD appear to sustain a significant increase in this population throughout the follow-up period when compared to the other two groups of patients. A similar pattern was observed when analyzing T_{SCM} percentages within CD8 (data not shown).

Figure 20. The effect of aGvHD on T_{SCM} Tcon and CD8 after RIC-ATG allo-HSCT



SCM absolute counts for Tcon (A) and CD8 (B) in patients without any form of GvHD (No GvHD, white bars), patients with acute GvHD only (Ac GvHD, grey bars), patients with acute and cGvHD (Ac & Ch GvHD, black bars) and healthy controls (HC, grey diamonds). Bar graphs shows median values ± IQR. Asterisks denote statistically significant differences between patient groups (*p=0.01 to 0.05; **p=0.001 to 0.01

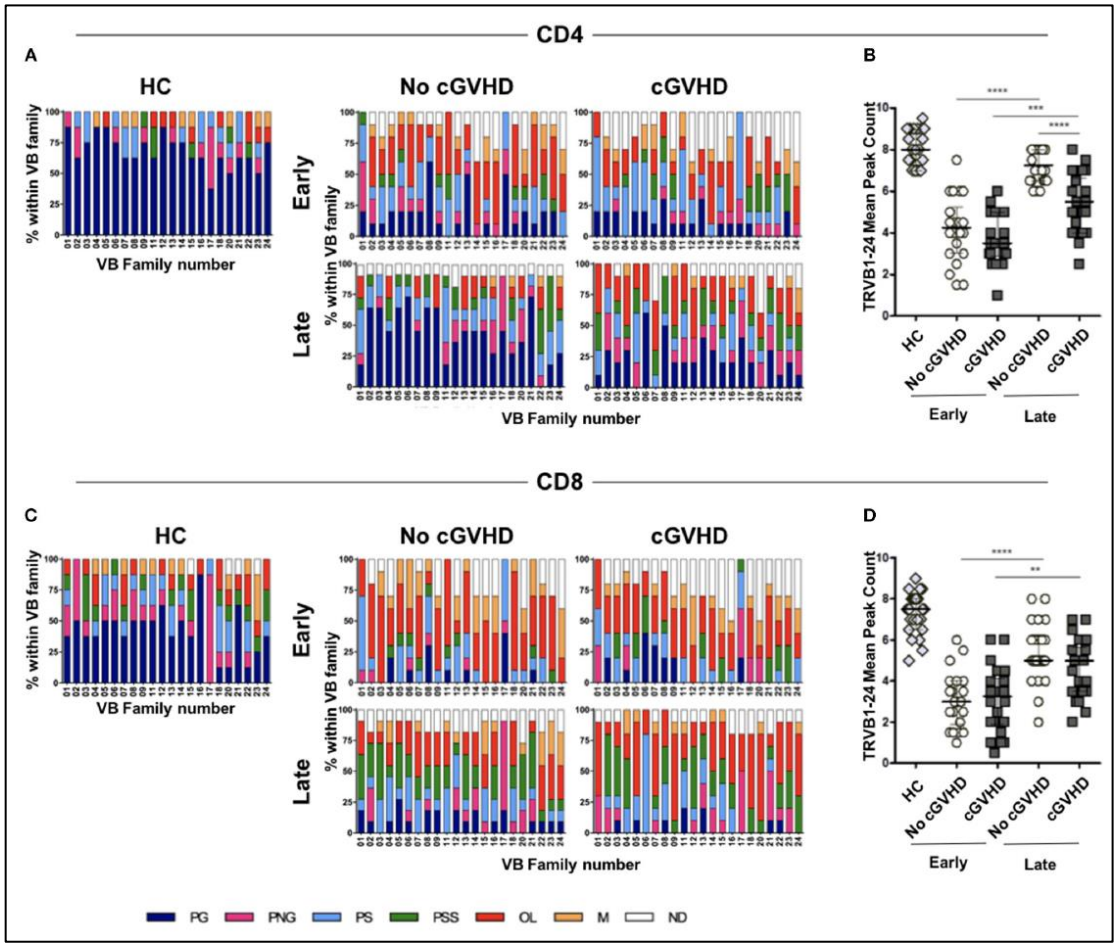
Chronic GvHD is associated with reduced CD4 TCR Vβ Diversity

TCR repertoire diversity was analysed in purified $CD4^+$ and CD8 T-cells from patients at early (month 3) and late timepoints (months 9 or 12) post-HSCT by TCRVβ spectratyping (Figure 21).

Early post-HSCT, we observed no significant differences in TCR diversity between patient groups in either $CD4^+$ or CD8 T-cells (Figure 21B, D). There were few Vβ families displaying polyclonal distributions, while those with skewed, oligoclonal and monoclonal distributions prevailed (Figure 21A, C). When early and late timepoints were compared within each patient group, significant increases in TCR diversity were observed, suggesting *de novo* $CD4^+$ and $CD8^+$ T-cells production overtime. However, when patients developing cGvHD were compared to No cGvHD, a significant reduction in CD4 TCR diversity in cGvHD was observed at later timepoints (Figure 21 B). This was reflected in decreased prevalence of polyclonal Gaussian profiles and the appearance of numerous skewed profiles (Figure 21A), translating into decreased TCR peaks within $CD4^+$ T-cells in cGvHD (Figure 21B).

Similar to CD4, we observed an increase in TCR diversity from early to late timepoints in CD8. However, the median number of CDR3 peak count within the Vβ families did not show statistically significant differences between patient groups (Figure 21 D). This was likely the result of the observed high prevalence of skewed distributions in CD8 T-cells from both patient groups (Figure 21 C).

Figure 21. TCR repertoire analysis in RIC-ATG allo-HSCT patients and controls



TCRVβ diversity analysis was performed on purified CD4⁺ and CD8 T-cells from healthy controls (HC) and patients with (cGvHD) and without cGvHD (No cGvHD). Data shown represent values obtained from patient samples early (month 3) and late (months 9 or 12) after HSCT. Clonality analysis of TRB locus transcripts is displayed as profile frequencies of CDR3 spectratypes for TRVβ1 to TRVβ24 gene families. Profiles were classified on the basis of peak number, distribution shape and relative fluorescence intensity (RFI) as: polyclonal Gaussian (PG, ≥8 peaks with a Gaussian distribution), polyclonal non-Gaussian (PNG, ≥8 peaks with an uneven distribution), polyclonal skewed (PS, 6 peaks or 1 peak with RFI>40%), polyclonal severely skewed (PSS, 1 or 2 peaks with RFI>70%), oligoclonal (O, 2 to 3 peaks), monoclonal (M, 1 peak or a peak with RFI>90%) and not detected (ND). Color stacked columns represent the mean frequency for each of the clonality profiles in CD4 (A) and CD8 (C) in HC, as well as in patients with and without cGvHD early and late after HSCT. TRVβ1-24 CDR3 median peak count in CD4⁺ (B) and CD8 (D) T-cells for HC and patient groups. Asterisks denote statistically significant differences between patient groups (p=0.001 to 0.01; ***p=0.0001 to 0.001; ****p<0.0001).**

Chronic GvHD negatively impacts thymic function

We next analysed sjTREC content as an estimate of thymic activity in purified $CD4^+$ and $CD8^+$ T-cells at early (month 3) and late (months 9 or 12) timepoints post-HSCT. Unfortunately, insufficient cell numbers precluded the analysis at month 3. sjTRECs were significantly reduced in $CD4^+$ (Figure 22A) and $CD8^+$ (Figure 22 C) T-cells in cGvHD and No cGvHD patients at late timepoints.

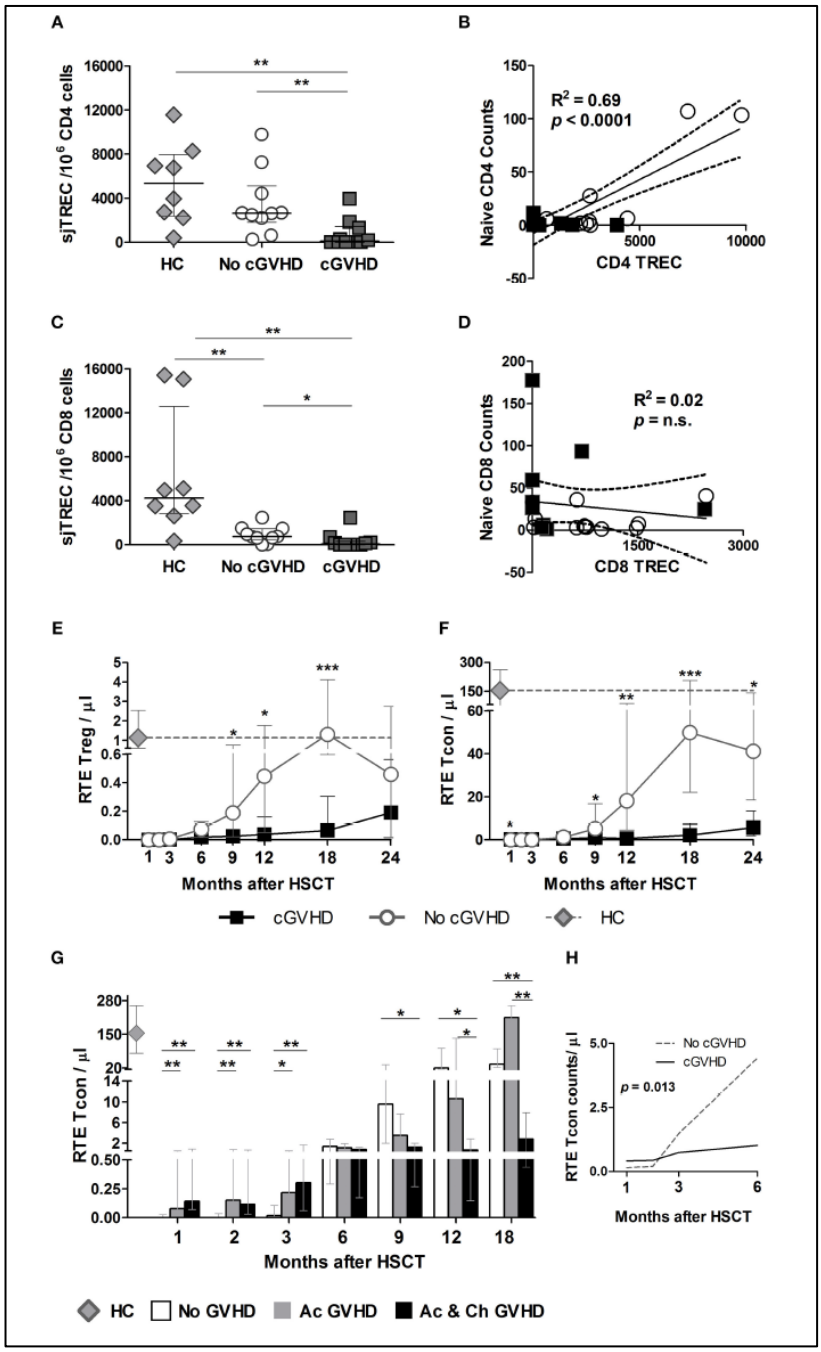
Importantly, pT_N $CD4^+$ counts were positively correlated with sjTRECs in No cGvHD, but not in cGvHD (Figure 22 B). In $CD8^+$ T-cells, no significant correlation was observed between sjTREC content and pT_N $CD8^+$ counts in either patient group (Figure 22 D).

The presence of a significant correlation between $CD4^+$ TREC numbers and pT_N $CD4^+$ counts at month 9 suggests that the pT_N $CD4^+$ T-cell reconstitution observed in patients who do not develop cGvHD is likely the result of *de novo* thymic production. On the other hand, the increase in pT_N $CD8^+$ T-cells observed in patients with cGvHD may depend more on T-cell peripheral expansion. However, TREC data must be interpreted with caution in this context, since it may be affected by events occurring in the periphery, such as T-cell proliferation[29][30][31].

Recent Thymic Emigrants Tcon and Treg in GvHD patients

The expression of CD31 within naïve $CD4^+$ T-cells has been reported to contain a population enriched in T_{RTE}[22]. We therefore quantified T_{RTE} in this patient cohort as an additional measure of thymic function. We found that patients developing cGvHD had significantly reduced T_{RTE} Treg (Figure 22E) and Tcon (Figure 22F) from month 9 onwards. Furthermore, LME analysis of the first 6 months revealed significantly different slopes for T_{RTE} Tcon absolute counts (Figure 22H) and percentages ($p=0.0032$) (data not shown), whereby T_{RTE} Tcon increase in the absence of cGvHD with negligible recovery of this population in patients developing cGvHD.

Figure 22. sjTREC content and T_{RTE} analysis post RIC-ATG allo-HSCT



sjTRECs were quantified in purified CD4⁺ (A) and CD8⁺ (C) T-cells at months 9 and 12 post-HSCT. Median and IQR values for sjTREC content are shown per 10^6 CD8⁺ or CD4⁺ T-cells in healthy controls (HC, grey diamonds), patients with cGvHD (dark grey squares), and patients without cGvHD (No cGvHD, white circles). Linear regression analysis between the absolute counts of true naïve CD4 (B) or CD8 (D) and the sjTREC content are represented; black squares represent cGvHD and while circles No cGvHD patients. Absolute counts of T_{RTE} Treg (E) and T_{RTE} Tcon (F) in patients with (black lines and squares) and without (grey lines and circles) cGvHD, as well as in HC (grey diamonds and grey dotted lines). (G) T_{RTE} Tcon counts in patients without GvHD (No GvHD, white bars), with acute GvHD only (Ac GvHD, grey bars), with acute and cGvHD (Ac & Ch GvHD, black bars), and in HC (grey diamond). (H) T_{RTE} Tcon counts on the first 6 months after

HSCT in patients with (cGvHD, black line) and without (No cGvHD, dotted grey line) cGvHD, illustrating the results of the LME for these subsets. Median and IQR values are shown. Asterisks denote statistically significant differences between patient groups (*p=0.01 to 0.05; **p=0.001 to 0.01; ***p=0.0001 to 0.001).

The addition of aGvHD as a variable to the LME analysis of T_{RTE} Treg and Tcon showed that aGvHD was associated with increased T_{RTE} Tcon counts and percentages during the first 6 months post-HSCT ($p<0.01$). No effect was observed at later timepoints. Accordingly, when the No cGvHD patient group was split into No GvHD and aGvHD, as described earlier, T_{RTE} Tcon were significantly decreased in No GvHD *vs.* both GvHD patient groups at months 1, 2 and 3 (Figure 22G). After month 6, T_{RTE} Tcon counts increased in No GvHD and Ac GvHD, while Ac & Ch GvHD patients showed significantly reduced T_{RTE} Tcon numbers, suggesting that cGvHD may impact on T_{RTE} Tcon reconstitution.

Short Discussion

We have previously found a transient predominance of T-cells with a naïve phenotype in the early post-transplant period in patients who subsequently developed cGvHD. To investigate the role of these cells in cGvHD biology, and the lineage relationship with other T-cell subsets, we extended previous studies looking at T-cell reconstitution post allo-HSCT. The final analysis included a homogeneous population of 40 patients undergoing unrelated donor RIC allo-HSCT with ATG and CsA/MMF for GvHD prophylaxis. Results were compared to 5 healthy donors.

We observed altered Treg homeostasis in patients developing cGvHD. Delayed Treg reconstitution in cGvHD patients was noticeable from month 9 to 18, and was associated with decreased proliferation (from month 3 to 18), without increased expression of apoptosis-related proteins. The low proliferation rate could be related to decreased levels of homeostatic cytokines, particularly IL-2, in this group of patients.

Importantly, we demonstrated a clear association between cGvHD development and severely impaired pT_N and T_{SCM} Treg reconstitution beyond 9 months post-transplant. Decreased pT_N Tregs may result from reduced homeostatic proliferation or reduced

thymic output following thymic damage. To evaluate the contribution of the thymus for Treg recovery post-transplant, we quantified T_{RTE} Tregs and found significantly decreased levels in cGvHD patients. Regarding T_{SCM} Tregs, the delayed recovery may result from decreased pT_N Treg and/or increased differentiation into memory phenotypes, suggesting a lineage relationship between these subsets.

In line with the previously reported negative impact of ATG on $CD4^+$ T-cell reconstitution[317][318–320], the recovery of Tcons in this cohort was impaired in the first year post transplant, irrespective of cGvHD. However, we reported significant distinct Tcon subset composition when comparing both patient groups. Notably, pT_N and T_{SCM} Tcon recovery were impaired in patients developing cGvHD; this was accompanied by a tendency for increased levels of more differentiated subsets, such as T_{EM} and $T_{EMRA.}$ Like Treg, the homeostasis of Tcon was altered in patients developing cGvHD. We observed a tendency for reduced Ki-67 expression within total Tcon, as well as in Tcon subsets (data not shown), in cGvHD patients. Susceptibility to apoptosis, as assessed by the quantification of Bcl-2 and CD95 expression levels, was similar between patient groups, pointing to decreased homeostatic proliferation as a contributing factor to impaired naïve Tcon reconstitution. In order to have a measure for *de novo* thymic production, we quantified T_{RTE} Tcons and showed that from month 9 onwards, this subset increased in the absence of cGvHD, while cGvHD patients had a severely impaired T_{RTE} Tcon reconstitution. To further detail the contribution of the thymus for $CD4^+$ T-cell recovery post-transplant, we quantified sjTREC in total $CD4^+$ T-cells. sjTRECs were significantly reduced in $CD4^+$ T-cells from both cGvHD and No cGvHD patients. Interestingly, $CD4^+$ sjTREC correlated with naïve Tcon numbers in No cGvHD patients, suggesting that, in the absence of cGvHD, the naïve Tcon recovery is likely to result from *de novo* thymic production occurring after HSCT[321]. CD4 TCRVβ diversity was significantly reduced in patients, more so in cGvHD, further pointing to impaired thymic production in patients developing cGvHD. It is therefore likely that a combination of decreased proliferation and impaired thymic output leads to impaired pT_N Tcon and Treg reconstitution in cGvHD patients.

When assessing the effect of aGvHD in T-cell reconstitution, we found increased T_{RTE} Tcon at months 1 to 3, in all patients that developed aGvHD, as compared to No GvHD. We speculate that T_{RTE} Tcon detected early post-transplant may contain self-reactive

clones, while T_{RTE} Tcon present at later timepoints in No cGvHD result from adequate thymic selection processes.

The reconstitution of naïve and memory $CD8^+$ T-cells differed greatly from $CD4^+$ T-cells. Interestingly, cGvHD was associated with a significant increased number and percentage of T_N, T_{SCM} and T_{EMRA} CD8 subsets in the first-year post-transplant, as compared to patients who did not develop cGvHD. When the effects of previous aGvHD were taken into consideration in the statistical analysis, we observed that during the first 6 months post-transplant T_{SCM} Tcon and CD8 were significantly affected by aGvHD. The stratification of patient groups into NoGvHD, aGvHD only and Ac&Ch GvHD confirmed that in the early post-transplant period, patients developing aGvHD had increased numbers of T_{SCM} Tcon and CD8, as compared to NoGvHD patients. A sustained increase in T_{SCM} CD8 was observed in patients developing cGvHD, further suggesting a potential role for this subset in disease development.

Several studies have shown that the thymus is a direct target of aGvHD[322], resulting in impaired negative selection, impaired thymic generation of donor-derived naïve T-cells and emergence of *de novo* self-reactive T-cells, with subsequent development of cGvHD[322,323]. In this cohort, 17/18 cGvHD patients had previously developed aGvHD, and their TCR repertoire at late timepoints, within $CD4^+$ and $CD8^+$ T-cells, was mostly skewed and oligoclonal, suggesting an imbalance between *de novo* thymic generation of naïve T-cells and lymphopenia-induced proliferation. Although the thymus is likely damaged, the reconstitution of T_N CD8 is not compromised, which favors a different mechanism regulating the generation of naïve $CD8^+$ subset. This is supported by the absence of correlation between sjTREC content and pT_N CD8 counts, contrary to what is observed in $CD4^+$ T-cells.

Findings from the present study extended prior observations from Aim one. Previously, we reported an early and transient prevalence of naïve T-cell subsets at 3 months post-transplant in patients further developing cGvHD. Here, we observed a prevalence of both, pT_N and T_{SCM}, Treg, Tcon and CD8 subsets at 1month post-transplant. However, beyond month 1, the recovery of pT_N and T_{SCM} Treg and Tcon was impaired, while CD8 subsets continued to increase. This opposing pattern of reconstitution reflects independent homeostatic mechanisms that regulate $CD4^+$ and $CD8^+$ T-cell subsets. The observed

delayed in pT_N and T_{SCM} Treg and Tcon recovery likely resulted from reduced proliferation. Enhanced pT_N CD8 subset might correspond to infused donor T-cells that expanded after allogeneic recognition, with further differentiation into T_{SCM}[72][73][74]. The increasing number of T_{SCM} CD8 throughout the follow-up translate both the extensive proliferation and self-renewal capacities that characterize this subset[52]. However, the factors behind this extensive proliferation observed in cGvHD patients remain to be clarified.

2.2. Early predominance of effector memory CD8$^+$ T-cells over true naïve and central memory Tregs results in chronic GvHD after myeloablative allo-HSCT

Results

Patient characteristics

Clinical characteristics of the patients included in the study are summarized in Table 7. The median age was 44 years (range 33-52), 18,9 % of male recipients had a female donor. The main indication for transplant was acute leukemia (36% ALL, 33% AML). The vast majority of patients (94,6%) underwent transplantation from an HLA-matched donor and 91% received filgrastim-mobilized PBSC. The GvHD prophylaxis included mostly (92,8%) TAC combined with MTX. Following transplant, with a median follow-up of 398 days (375-777), 46,8% (n=52) of patients developed cGvHD, within these, 24 (21,6%) had previously developed aGvHD. The median onset of cGvHD was 239 days (Q1-Q3: 169-321). Relapse within the 2-year period of follow-up occurred in 28 patients (25%).

According to cGvHD development, we stratified the population into two groups: the *cGvHD* group, that included acute and cGvHD patients (*Ac & Ch GvHD)*, and the *No cGvHD group,* that included no GvHD patients and acute only *(No & Ac).* The patient's characteristics were similar between groups, except for the onset of aGvHD, which occurred earlier in cGvHD patients (24 *vs*. 39 days, p = 0.0158), with a prevalence of 21,6% (n=24).

The overall survival (hazard ratio for death 0.33 [95% CI, 0.15 - 0.71; p= 0.004]) and relapse free survival (hazard ratio for relapse 0.17 [95% CI, 0.065 - 0.46; p<0.001]) was lower for cGvHD patients.

Table 7. Patient Characteristics according to cGvHD (MAC_Boston cohort)

	All patients		*cGvHD (Ch & Ac)*		*No cGvHD (No & Ac)*		*p cGvHD vs. No cGvHD*
	N	%	N	%	N	%	
Total	111	100,0	52	46,8	59	53,2	*ns*
Age (y), median (Q1-Q3)	44(33-51,5)		44(33,5-51)		44(32-53)		
Patient Sex							
M	60	54,1	29	26,1	31	27,9	*ns*
F	51	45,9	23	20,	28	25,2	
Donor Sex							
M	62	55,9	31	27,9	31	27,9	*ns*
F	49	44,1	21	18,9	28	25,2	
Patient-Donor Sex							
MM	39	35,1	22	19,8	17	15,3	*ns*
MF	21	18,9	7	6,3	14	12,6	
FM	23	20,7	10	9,0	13	11,7	
FF	28	25,2	23	20,7	15	13,5	
Diagnosis							
ALL	40	36,0	21	18,9	19	17,1	*ns*
AML	37	33,3	16	14,4	21	18,9	
NHL	15	13,5	8	7,2	7	6,3	
CML	10	9,0	3	2,7	7	6,3	
MDS	6	5,4	3	2,7	3	2,7	
Mixed MDS/MPD	2	1,8	1	0,9	1	0,9	
MPD	1	0,9	0	0,0	1	0,9	
Disease Risk Index							
Low	10	9,0	4	3,6	10	9,0	*ns*
Intermediate	69	62,2	35	31,5	69	62,2	
High	22	19,8	8	7,2	22	19,8	
Very-High	9	8,1	5	4,5	9	8,1	
Unknown	1	0,9	0	0	1	0,9	
Donor type							
MURD	52	46,8	26	23,4	26	23,4	*ns*
MRD	53	47,7	22	19,8	31	27,9	
MMURD	6	5,4	4	3,6	2	1,8	
Graft Source							
PBSC	101	91,0	49	44,1	52	46,8	*ns*
BM	10	9,0	3	2,7	7	6,3	
GVHD prophylaxis							
MTX; Tac	103	92,8	45	40,5	58	52,3	*ns*
Sir; Tac	6	5,4	5	4,5	1	0,9	
MTX; MMF; Tac	2	1,8	2	1,8	0	0,0	
aGvHD grade (grade ≥ II)	39	35,1	24	21,6	15	13,5	*ns*
cGvHD status							
None			59	53,1			*ns*
Limited			7	6,3			
Extensive			45	40,5			
Follow-up , median (Q1-Q3)	388 (359-772)		740 (376-1096)		370 (211-472)		*<0.001*

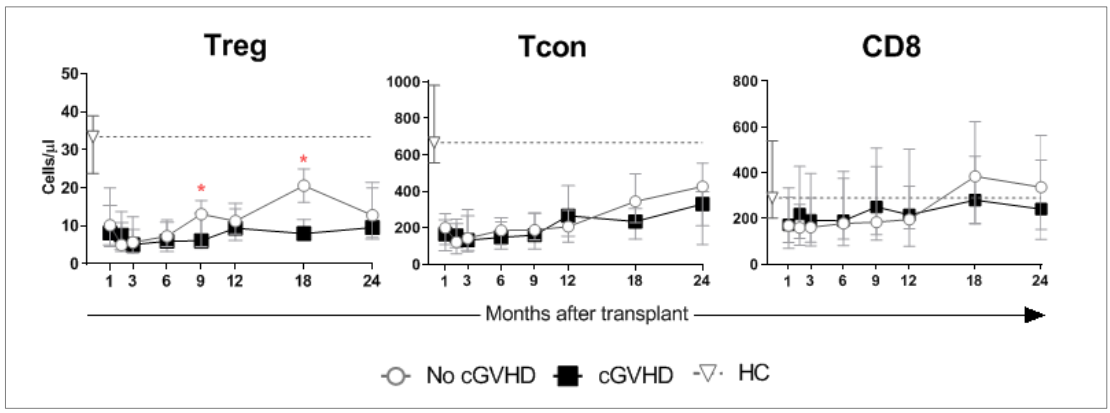
Abbreviations: Ch & Ac, chronic and acute GvHD patients; No & Ac, no and acute GvHD patients; M, male; F, female; ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; NHL, non-Hodgkin lymphoma; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; MPD, myeloproliferative disease; PBSC, peripheral blood stem cell; BM, bone marrow; MURD, matched unrelated donor; MRD, matched related donor; MMURD, mismatched unrelated donor; Tac, tacrolimus; Sir, sirolimus; MTX, methotrexate; MMF, mycophenolate mofetil.

Delayed Treg reconstitution in cGvHD patients post MAC allo-HSCT

Initial analysis included all the population stratified into two groups, according to cGvHD development [(*Ac & Ch GvHD) vs. ((No & Ac)*]. Longitudinal evaluation of T-cell dynamics within Treg, Tcon and CD8 is summarized in Figure 23. $CD4^+$, Treg and Tcon, reconstitution was delayed in both patient groups, and remained below HD numbers throughout the follow-up period ($p<0.001$). In contrast, $CD8^+$ T-cell numbers reached HD values at 2 months post-transplant. Within $CD8^+$ T-cells, T_{EM} corresponded to the main subset (data not shown).

Chronic GvHD patients were distinguished from NoGvHD, by a significantly lower number of Tregs at 9 months post-transplant. By this time, 29/52 (55,7%) patients had been diagnosed with cGvHD and immunomodulatory treatment was initiated/intensified. Recovery of Tcon and $CD8^+$ T-cells was similar between groups.

Figure 23. Treg, Tcon and $CD8^+$ T-cell reconstitution following MAC allo-HSCT

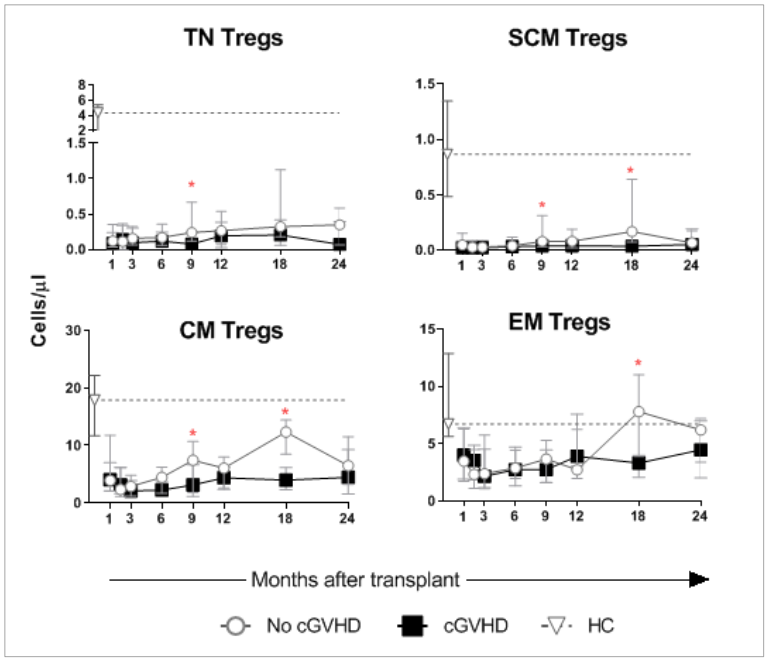


Longitudinal recovery of Treg, Tcon and $CD8^+$ T-cells after MAC allo-HSCT. Median cell counts/µl for each population and the corresponding median in healthy donors/controls (HC) are shown for each timepoint. Patients were divided into No cGvHD ((No & Ac), grey lines and open circles)) and cGvHD ((Ac & Ch GvHD, black lines and squares)). Treg, Tcon and CD8 T-cells were identified as shown in Figure 6a.-c. HC are shown in grey diamonds and dotted grey lines. Interquartile range are represented in whisker bars. Red asterisks denote statistically significant differences between patient groups (*p=0.01 to 0.05).

To investigate the delayed Treg reconstitution we made subsequent analysis of the naïve and memory Treg compartments trough immunophenotyping, as described in Section 3.1.1. The detailed gating strategy used to identify and distinguish pT_N from T_{SCM} subsets is illustrated in Figure 7.

Longitudinal analysis showed a significantly lower number of pT_N, T_{SCM} and T_{CM} Tregs at 9 months post-transplant in cGvHD patients, compared to No cGvHD (Fig 24). The number of T_{EM} Tregs was similar between groups, except at month 18 post-transplant, where cGvHD patients had significantly lower number of memory subsets. We further determined the fraction of cells that had a pT_N, T_{SCM}, T_{CM} and T_{EM} phenotype, and found no differences between groups (data not shown).

Figure 24. Reconstitution of Treg subsets following MAC allo-HSCT

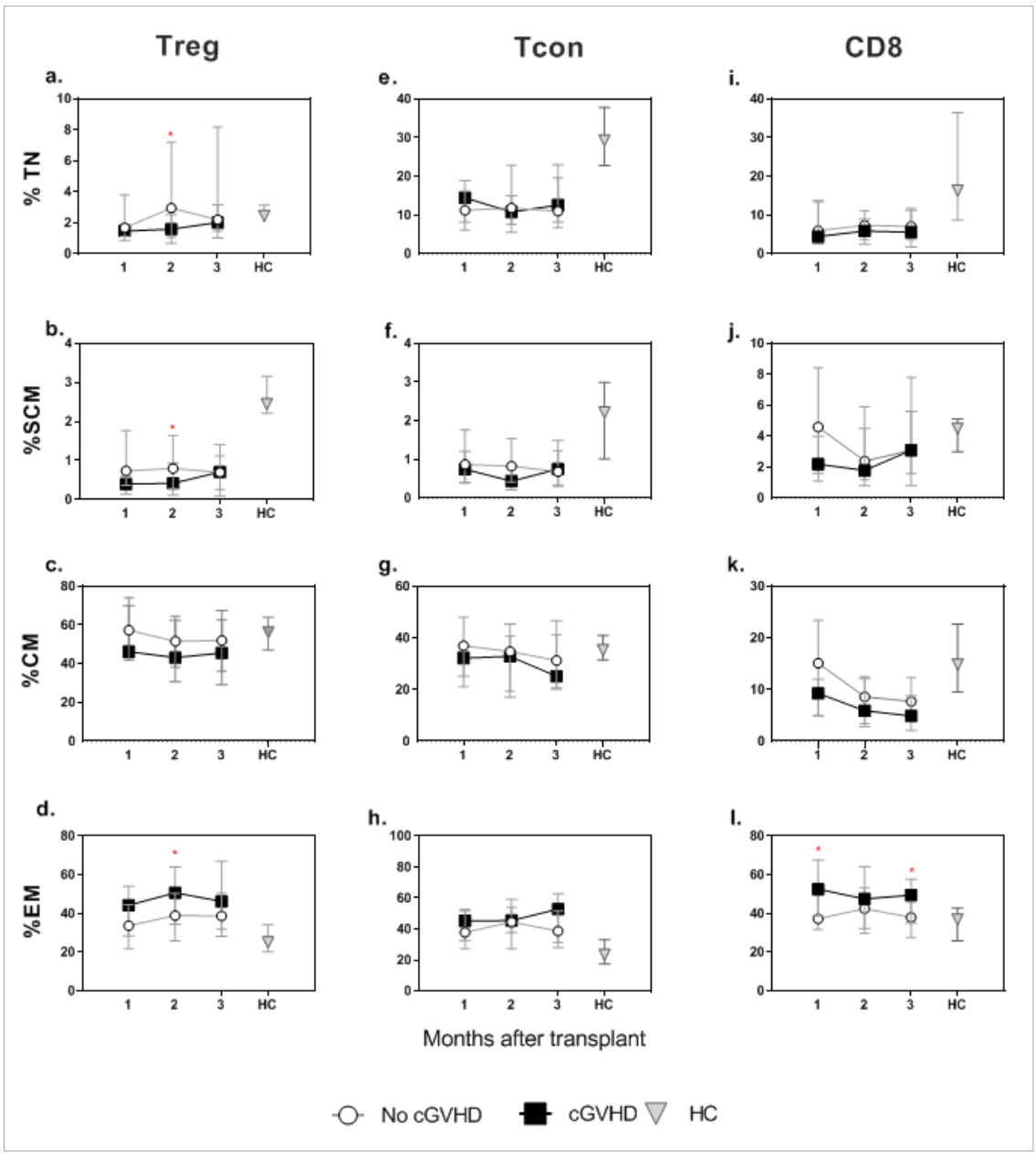


Longitudinal recovery of naïve and memory Treg subsets after MAC allo-HSCT in two groups of patients (No cGvHD vs cGvHD). Median cell counts/µl for each cell subset and the corresponding median in healthy donors/controls (HC) are shown for each timepoint. Patients were divided into No cGvHD ((No & Ac), grey lines and open circles)) and cGvHD ((Ac & Ch GvHD, black lines and squares)). True naïve (TN), Stem cell memory (SCM), Central Memory (CM) and Effector Memory (EM) subsets were identified as shown in Figure 7. HC are shown in grey diamonds and dotted grey lines. Interquartile range are represented in whisker bars. Red asterisks denote statistically significant differences between patient groups (*p=0.01 to 0.05).

T-cell compartment in the early post-transplant period after MAC allo-HSCT

To examine the specific role of pT_N and T_{SCM} in the initiation of cGvHD, further analysis focused on the early post-transplant period (first 3 months) and excluded all the patients who developed aGvHD (Figure 25). In this subanalysis we intended to remove the effect of aGvHD (median onset of 32 days post-transplant) and/or its treatment, on T-cell recovery and function.

Figure 25. Early Naïve and memory T-cell recovery post MAC allo-HSCT

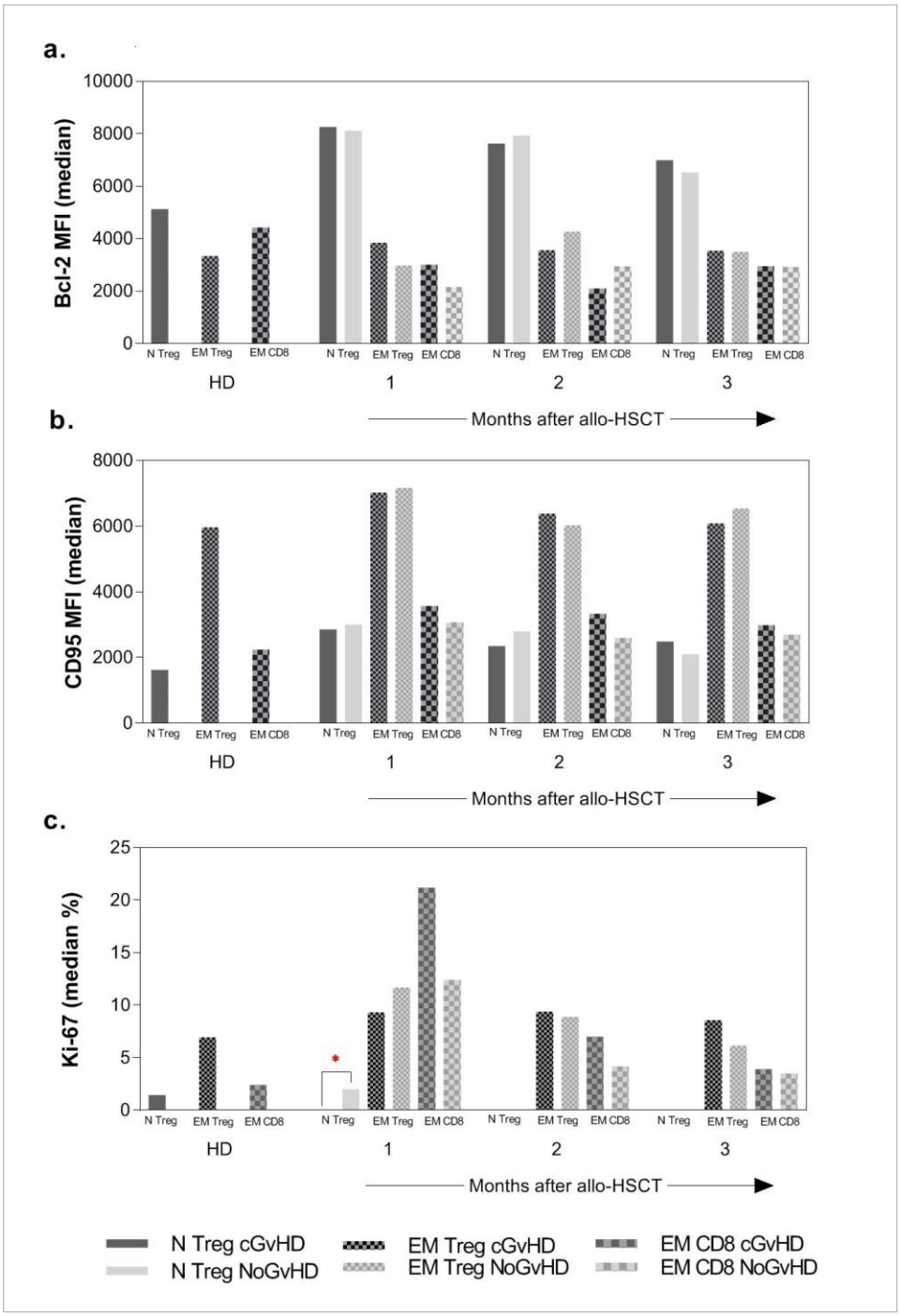


Proportion of T_N, T_{SCM}, T_{CM} and T_{EM} subsets within Treg (a.-d.), Tcon (e.-h.) and CD8 (i.-l.) in the first three months post-transplant, according to cGvHD development. Excluding all patients who developed aGvHD, two groups were stratified into No cGvHD (grey lines and open circles) and cGvHD (black lines and squares). The median percentages and IQR of each subset are shown for patients with and without cGvHD, as well as for donors/controls (HC). True naïve (TN), Stem cell memory (SCM), Central Memory (CM) and Effector Memory (EM) subsets were identified as shown in Figure 7. Symbols represent median values ± IQR. Asterisks denote statistically significant differences between patient groups (*p=0.01 to 0.05).

At 2 months post-transplant, cGvHD patients were distinguished by significantly lower percentages of $pT_{N,}$ T_{SCM} (Figure 25 a.,b.) and T_{RTE} Tregs (data not shown), and significantly higher percentage of T_{EM} Tregs (Figure 25 d.), compared to No cGvHD patients. At 1 and 3 months these patients had also significantly higher percentages of T_{EM} CD8 (Figure 25 l.). Compared to HD, cGvHD patients had significantly higher percentage of T_{EM} Treg and lower percentage of T_{SCM} Treg ($p<0.05$), from 1-3 months post-transplant. The absolute number of Treg, Tcon and CD8 subsets were largely unaltered by cGvHD (data not shown) in the early period post-transplant. $CD8^+$ T_{EMRA} were also analysed, with no differences between groups (data not shown).

To complement this analysis, we accessed the proliferation index through the expression of Ki-67, and the susceptibility to apoptosis through the expression level (MFI) of Bcl-2 and CD95, within naïve Treg, T_{EM} Treg and T_{EM} CD8 in both patient groups (NoGvHD *vs* cGvHD, excluding all the aGvHD patients), in the first 3 months post-transplant. For each subset of interest, we found no differences in the homeostatic profile between groups, except for the expression of Ki-67 within naïve Tregs, that was significantly higher in NoGvHD patients (Figure 26. c). In both groups, naïve Tregs had a pro-survival phenotype, with a significantly higher expression level of Bcl-2 ($p<0,001$) (Fig 26 a.), and significantly lower proliferation rate ($p<0,001$) (Fig 26 c.), compared to HD. In contrast, T_{EM} CD8 had a pro-apoptotic phenotype, with significantly lower MFI of Bcl-2 ($p<0,001$) (Fig 26 a.), and significantly higher Ki-67 percentage ($p<0,001$) (Fig 26 c.).

Figure 26. Homeostatic profile of naïve Treg, T_{EM} Treg and T_{EM} CD8, post MAC allo-HSCT



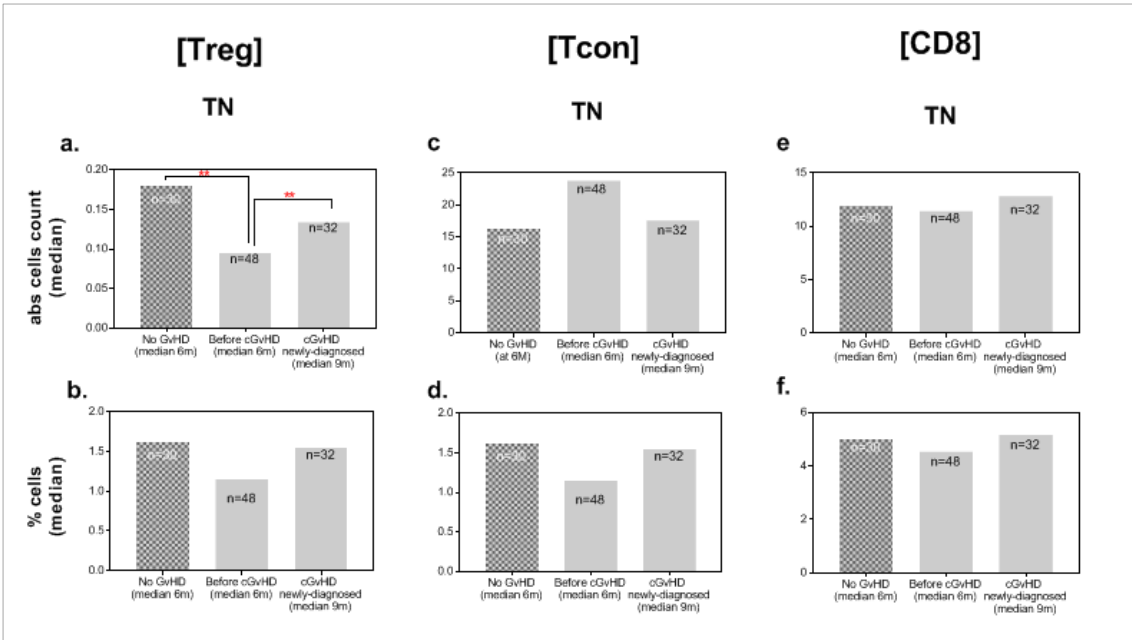
Expression of Bcl-2 (MFI), CD95 (MFI) and Ki67(%) within naïve Treg, T_{EM} Treg, and T_{EM} CD8, in the first three months post-transplant, according to cGvHD development. Excluding all patients who developed aGvHD, two groups were distinguished: cGvHD (purple colour) and No cGvHD (grey colour). The median values of each subset are shown for patients with and without cGvHD, as well as for healthy donors/controls (HC). T-cell subsets were identified as shown in Figure 7. Symbols represent median values ± IQR. Asterisks denote statistically significant differences between patient groups (*p=0.01 to 0.05).

Impaired naïve Treg number immediately prior to cGvHD development

To characterize the naïve and memory T-cell pool immediately before the onset of cGvHD, we performed a cross-sectional analysis of the data, including all the samples. For this approach we accessed the absolute numbers and percentages of T-cell subsets in three different groups of samples: samples collected at *cGvHD* diagnosis (n= 32; median 9 months post allo-HSCT), samples collected at the timepoint *Before cGvHD* (n=48; median 6 months post allo-HSCT), and samples from *No cGvHD*/control group, collected 6 months post allo-HSCT (n=30).

Immediately before the onset of cGvHD, there was a significantly lower number of pT_N Tregs (Figure 27.a.; $p<0.01$), compared to No GvHD patients. With cGvHD development. the numbers of pT_N Tregs increased significantly (Figure 27.a.; $p<0.01$). There were no differences between the groups, in the number and percentage of pT_N Tcon and $CD8^+$ T-cells.

Figure 27. Effect of cGvHD on true naïve T-cells, post MAC allo-HSCT

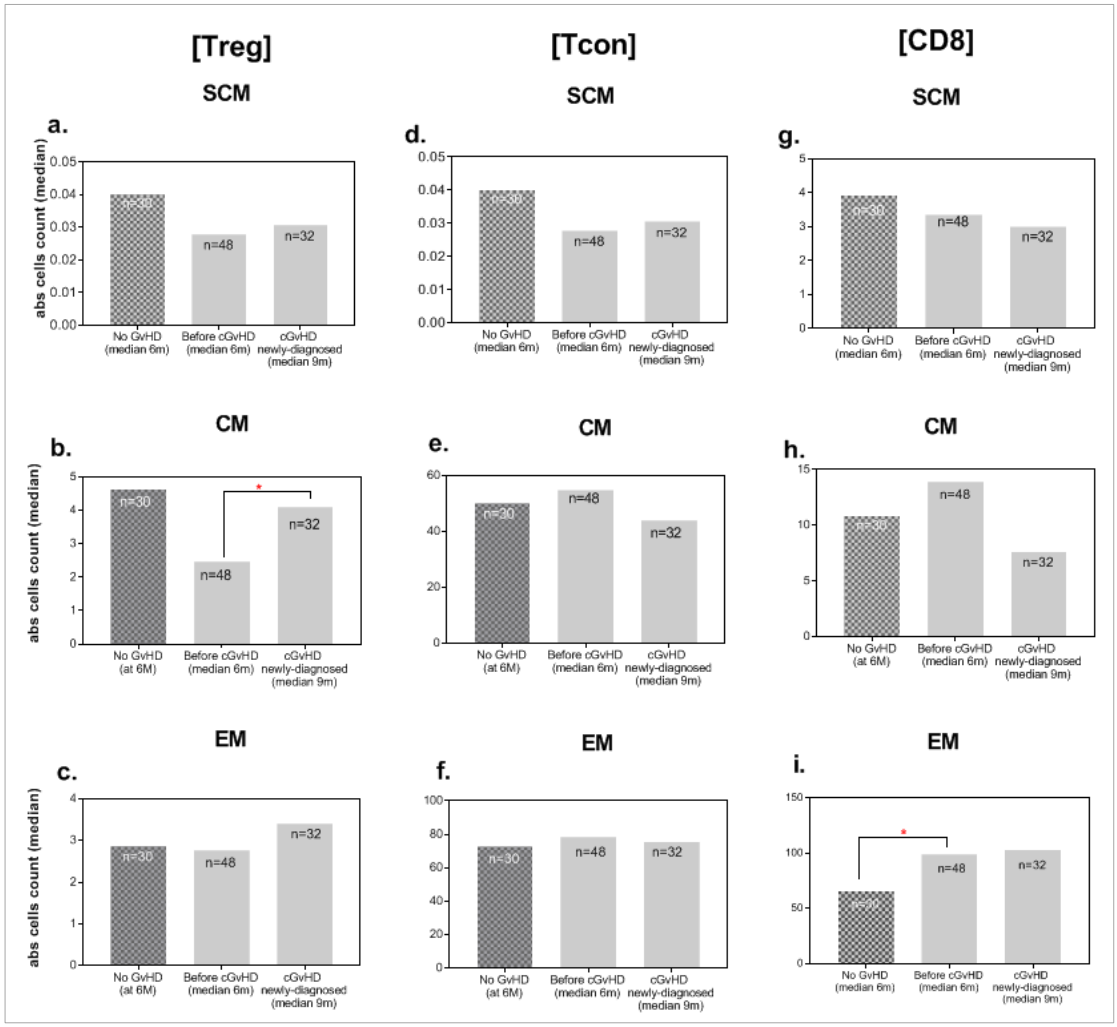


Cross-sectional analysis of the data comparing three different groups: No cGvHD *vs* Before cGvHD *vs* cGvHD newly-diagnosed. The median cell count/µl (top panel) and the median percentage (down panel) are represented for True naïve (TN) within Treg (a., b.), Tcon (c.,d.) and CD8 (e.,f.). The number of patients evaluated in each group is indicated in the corresponding column. Red asterisks denote statistically significant differences between patient groups (p=0.001 to 0.01).**

Reduced T_{CM} Tregs and increased T_{EM} CD8 prior to cGvHD development

Analysis of memory T-cell subsets in the three groups of patients revealed statistically significant lower number of T_{CM} Treg immediately before the onset of cGvHD (Figure 28 b.). This number was lower in relation to No GvHD patients, although not reaching statistical significance. Contrarily, T_{EM} CD8 numbers were statistically significant higher before the onset of cGvHD, when compared to No cGvHD patients (Figure 28 i.). We further determined the fraction of each memory T-cell subset within Treg, Tcon and $CD8^+$ and found no differences between groups (data not shown).

Figure 28. Effect of cGvHD on memory T-cell subsets, post MAC allo-HSCT

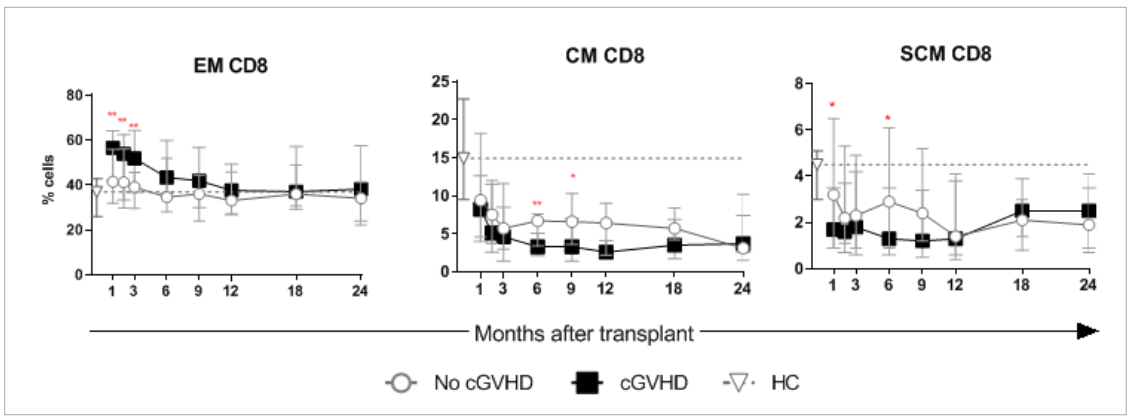


Cross-sectional analysis of the data comparing three different groups: No cGvHD *vs* Before cGvHD *vs* cGvHD newly-diagnosed. The median cell count/µl is represented for Stem Cell Memory (SCM), Central Memory (CM) and Effector Memory (EM) within Treg (a.-c.), Tcon (d.-f.), and CD8 (g.,i). The number of patients evaluated in each group is indicated in the corresponding column. Red asterisks denote statistically significant differences between patient groups (*p=0.01 to 0.05).

Early reconstitution of T_{EM} CD8 in cGvHD patients post MAC allo-HSCT

Analysis of the data in the early post-transplant period (Figure 25 l.) and immediately before the onset of cGvHD (Figure 27 d.) has shown an increased percentage and absolute number of T_{EM} CD8 in cGvHD patients, compared no NoGvHD. This data suggested a role for T_{EM} CD8 in cGvHD development. We further extended this analysis and studied the dynamic recovery of each $CD8^+$ T-cell subset over time. The longitudinal analysis showed that cGvHD patients (Ac & Ch GvHD) had a gradual decline in the percentage of memory subsets (T_{SCM}, T_{CM} and T_{EM}) in the first-year post-transplant (Figure 29), compared to NoGvHD (No & Ac). From month 1-3, the percentage of T_{EM} CD8 was significantly higher in cGvHD patients, compared to HD and NoGvHD; beyond month 3, this percentage reached HD values and was similar between groups. Contrarily, the recovery of T_{CM} CD8 was similar between groups up to 3 months post-transplant; beyond this timepoint the percentage of T_{CM} in cGvHD patients continue to decline until month 12, while NoGvHD group stabilized. The reconstitution of naïve and T_{EMRA} $CD8^+$ subsets was similar between groups (data not shown).

Figure 29. Reconstitution of memory $CD8^+$ T-cell subsets post MAC allo-HSCT



Longitudinal recovery of $CD8^+$ T-cells subsets after MAC allo-HSCT in two groups of patients (No cGvHD *vs* cGvHD). Median percentage of each cell subset and the corresponding median in healthy donors/controls (HC) are shown at different timepoints. Patients were divided into No cGvHD (grey lines and open circles) and cGvHD (black lines and squares). HC are shown in grey diamonds and dotted grey lines. Stem cell memory (SCM), Central Memory (CM) and Effector Memory (EM) subsets were identified as shown in Figure 7. Interquartile range are represented in whisker bars. Red asterisks denote statistically significant differences between patient groups (p=0.001 to 0.01; *p=0.01 to 0.05)**

Short Discussion

To investigate the role of pT_N and T_{SCM} in the initiation of cGvHD and the relationship with other T-cell subsets we made a simultaneous analysis of the different subsets within Treg, Tcon and $CD8^+$ T-cells in 111 adult patients who received T-cell replete stem cell products after MAC allo-HSCT, using primarily TAC/MTX for GvHD prophylaxis. Results were compared to 15 healthy donors.

Similar to previous results, we observed a significant delay on $CD4^+$ T-cell reconstitution during the 2-year observation period. Instead, $CD8^+$ T-cells recovered more rapidly, reaching HD values at 2 months post-transplant. Within $CD8^+$ T-cells, T_{EM} corresponded to the main subset and pT_N recovered to HD levels at 18 months post-transplant. Despite the myeloablative conditioning regimen, there was an early recovery of $CD8^+$ T-cells, which resulted from a markedly increased proliferation, as quantified by the expression of Ki-67%. This preferential expansion might result from lymphopenia-induced proliferation, in the context of elevated levels of cytokines, namely IL-15[260]. Indeed, published data has demonstrated a peak of plasma IL-15 levels in the early period post-transplant, most probably related to tissue damage caused by conditioning regimen[324].

In the early period post-transplant, cGvHD patients could be distinguished by a transient lower percentage of pT_N and T_{SCM} Tregs and a higher percentage of T_{EM} CD8. These differences persisted immediately before cGvHD diagnosis, when the numbers of T_{SCM} CD8 were significantly lower. We speculate that T_{EM} CD8 derived from donor-infused naïve CD8, after allogeneic recognition or lymphopenia-induced proliferation in the presence of high levels of IL-15[260]. In this setting, the early and persistent unbalance between pT_N and T_{SCM} Tregs and T_{EM} CD8 resulted in the subsequent development of cGvHD. Indeed, T_{EM} CD8 remained elevated immediately before cGvHD diagnosis, and this was accompanied by significantly lower numbers of T_{SCM} CD8, which correspond to a reservoir of effector and memory subsets[325].

Following 9 months post-transplant, cGvHD patients (n=52; 46%) were distinguished from NoGvHD by a statistically significant lower number of Tregs. Longitudinal analysis of each subset within Tregs has shown a significant delayed recovery of $pT_{N,}$ T_{SCM} and T_{CM} Tregs also at 9 months post-transplant. By this timepoint, 55,7% (29/52) of cGvHD

patients had already been identified and had initiated/intensified immunomodulatory drugs. Hence, it is difficult to attribute these results to cGvHD alone or to the immunomodulatory treatment. In the cross-sectional analysis, the samples obtained at/immediately after the diagnosis of cGvHD had a higher number of pT_N and T_{CM} Tregs, compared to previous timepoints. We may hypothesize that the immunomodulatory drugs initiated at the diagnosis of cGvHD, namely corticosteroids, favor Treg recovery over effector T-cells[326].

In summary, data from this study suggests that the development of cGvHD is preceded by an early imbalance between Tregs (pT_N and T_{SCM}) and $CD8^+$ (T_{EM}) T-cells. All transplanted patients in this setting had a preferential and sustained expansion of T_{EM} CD8, which may result from severe lymphopenia and/or conditioning-induced tissue damage. However, only those patients with a compromised recovery of pT_N and T_{SCM} Tregs developed cGvHD. Hence, this data confirms the crucial role of Tregs in providing and maintaining peripheral T-cell homeostasis post-transplant and implies an involvement of T_{EM} CD8 in the pathogenesis of cGvHD.

III. CHAPTER III

3.1 Functional analysis of clinical response to low-dose IL-2 in patients with refractory chronic GvHD

Short Introduction

Several studies have demonstrated that patients with cGvHD have a paucity of $CD4^+$ Tregs. These studies suggest that Treg deficiency can enhance alloreactivity and promote the development of cGvHD. Conversely, prompt recovery of Tregs can prevent GvHD while also supporting the recovery of a broad T-cell repertoire. In clinical trials, the daily infusion of low-dose IL-2 for SR cGvHD resulted in peripheral Treg expansion in all patients, although clinical improvement was only observed in 50-60% of patients. In this chapter we studied the impact of low-dose IL-2 for SR cGvHD patients on functional T-cell markers and on the T-cell repertoire of Treg, Tcon and $CD8^+$ T-cells.

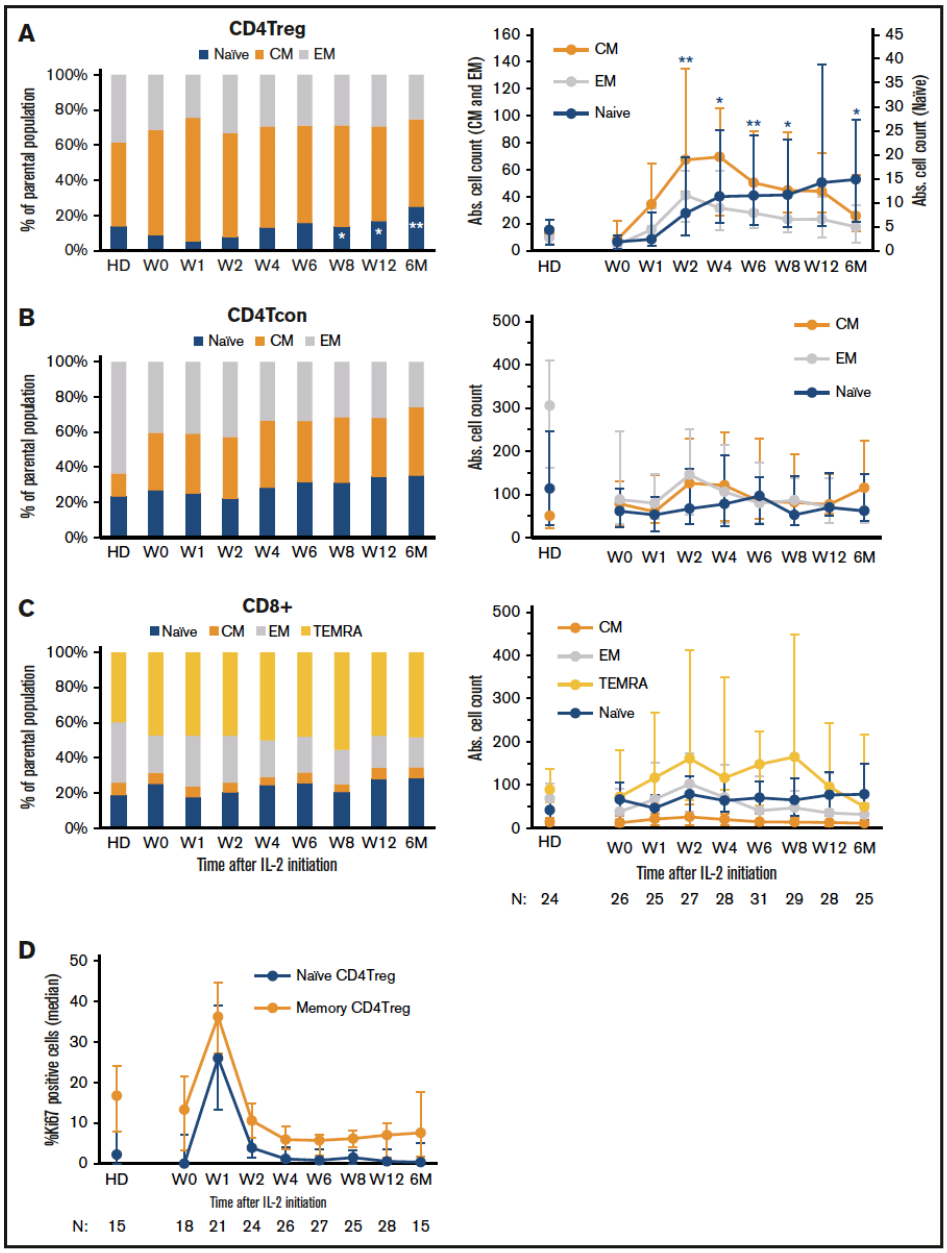
Results

Changes in naïve and memory T-cell subsets in response to low-dose IL-2

All evaluable patients in the previously reported phase 2 study, regardless of clinical response, exhibited preferential expansion of their peripheral blood Treg counts which peaked at 4 weeks and slowly declined. Increased Treg numbers were sustained above baseline throughout the initial 12 weeks of therapy and during extended-duration IL-2 therapy [240]. Here we examined changes in naïve and memory subsets within the Treg, Tcon and $CD8^+$ T cell populations during IL-2 therapy (Figure 30A-C). Prior to starting IL-2, patients with cGvHD had lower absolute numbers of circulating Tregs. Within Tregs, the proportion of naïve and T_{EM} subsets was similar to HD values, but the proportion of T_{CM} was higher (median 61% *vs.* 41.7%, p = 0.0005) (Figure 30). A low-dose IL-2 initially led to relative expansion of the T_{CM} Treg compartment at week 1. Naïve Treg expansion was evident by week 4, leading to a significant increase above baseline after 12 weeks of IL-2 therapy (median 9% ~~to~~*vs*. 16.5%, p = 0.02), and achieved levels above HD by 6 months of extended therapy (median 22.2% *vs.* 12.1%, p = 0.007).

The absolute T_{EM} and T_{CM} Treg cell counts peaked at week 2 and week 4, respectively, then decreased over time, but remained above in the Tcon and $CD8^+$ T-cells, the fraction of naïve cells in patients was similar to HD at baseline and there was no significant change during IL-2 therapy (Figure 30 B and C). Patients had a higher proportion of T_{CM} Tcon than HD (median 29% *vs.* 8%, $p < 0.0001$), and a lower proportion of T_{EM} $CD8^+$ T-cells than HD at baseline (median 20% *vs.* 32%, $p = 0.006$). These differences persisted throughout IL-2 therapy.

Figure 30. Impact of low-dose IL-2 on naïve and memory T-cell compartments



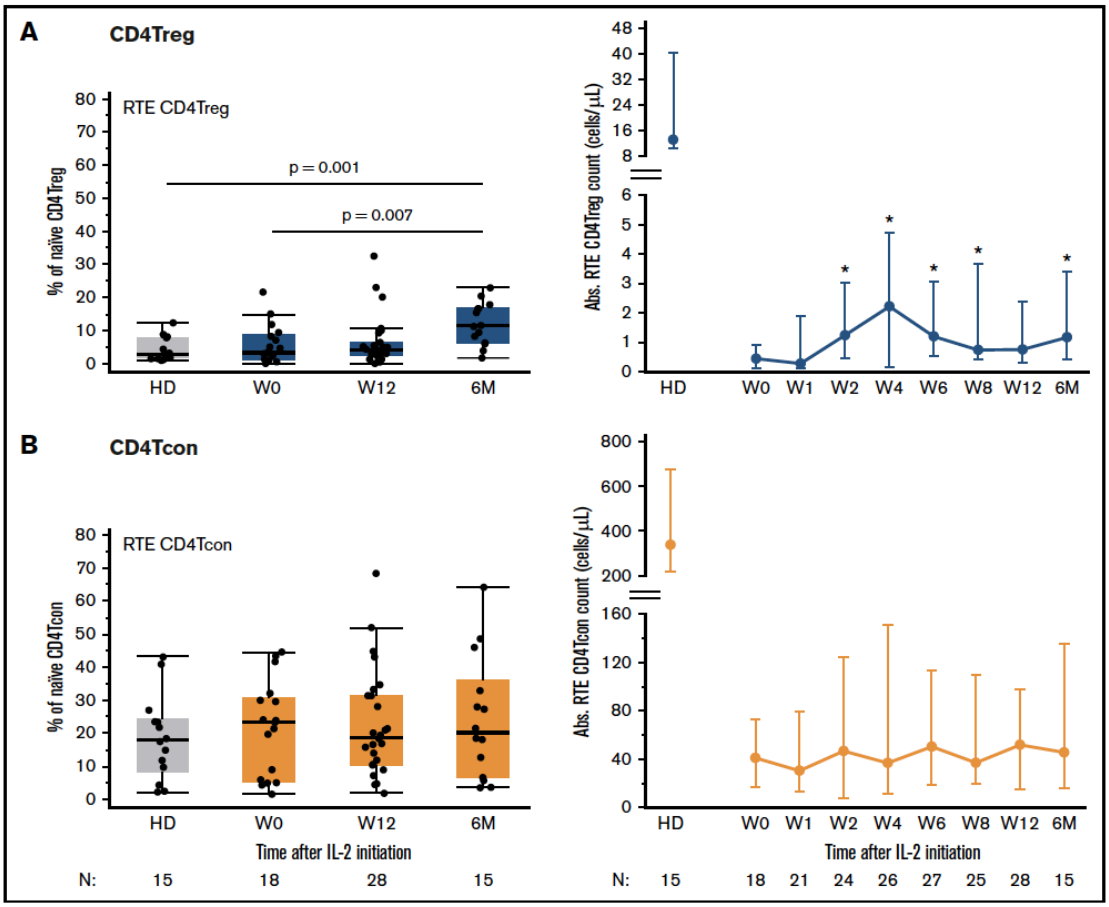
Relative percentages of each subset, at each timepoint, are shown on the left, and absolute cell numbers (cells/ µl) are shown on the right. Bar graphs on the left show the median percentage of each population and the corresponding median in healthy donors (HDs). Graphs on the right show median cell counts/µl for each population and the corresponding median in HDs, with the IQ range (whisker bars). Naïve (blue); CM, central memory (orange); EM, effector memory (gray); TEMRA, terminal effector memory terminal effector memory re-expressing CD45RA (yellow). Timepoints represent weeks (W) and months (M) after IL-2 initiation. Number of patients evaluated at each timepoint is indicated at the bottom of the image. (A) Treg subsets. * $p < 0.05$, ** $p < 0.01$, percentage of naïve cells compared with Week 0 (baseline), 2-sided Wilcoxon signed-rank test. (B) Tcon subsets. (C) $CD8^+$ T-cell subsets. (D) Proliferation marker (Ki-67) expression in Treg. The percentage of Ki-67^+ cells within naïve (orange) and memory (blue) Treg is shown for HDs and for patients at multiple timepoints after IL-2 initiation. Median values and the IQ range (whisker bars) are shown at each timepoint.

To determine whether this expansion was due to increased proliferation, we examined Ki-67 expression in the Treg subsets. Low-dose IL-2 induced rapid proliferation of both memory and naïve Treg subsets with Ki-67 expression peaking after 1 week (22% and 25% increase in Ki-67^+ cells from week 0 to week 1, respectively) (Figure 30D). The expansion of naïve Tregs observed after 4 weeks of IL-2 therapy was not associated with an increase in proliferation.

Naïve Tregs can derive from thymic generation or homeostatic proliferation. To examine the thymic production of naïve Tregs, we used co-expression of CD45RA and CD31 to identify T_{RTE}[33][327]. Low-dose IL-2 preferentially increased thymic output of Tregs, but not Tcons (Figure 31A, B). Although output of T_{RTE} Tregs did not approach the HD range at any time during IL-2 therapy, increased numbers of circulating T_{RTE} Tregs were detected after 2 weeks of therapy and remained elevated above baseline during extended therapy (Figure 31A). At 6 months, the percentage of T_{RTE} Tregs increased to a median of 11.4% ($p = 0.007$, compared to patients at baseline; $p = 0.001$, compared to HD). The percentage of T_{RTE} Tcon remained stable throughout IL-2 therapy (Figure 31B).

During the initial 12-week therapy period, clinical responders and non-responders had similar increases in absolute Treg, naïve Treg, and T_{RTE} Treg counts (supplementary Figure 1; p.217).

Figure 31. Impact of low-dose IL-2 on thymic production



Percentage of recent thymic emigrants (T_{RTE}) are shown on the left and absolute cell numbers (cells per µl) in the peripheral blood at each timepoint are shown on the right. Box plots on the left depict the 75th percentile, median, and 25th percentile values; whiskers represent maximum and minimum values. Dots depict individual HDs or patients. Graphs on the right show median cell counts/µl for each population and the corresponding median in HDs, with the IQ range (whisker bars). Timepoints represent weeks (W) and months (M) after IL-2 initiation. Number of patients evaluated at each timepoint is indicated at the bottom of the image. (A) T_{RTE} Treg (blue). * $p < 0.05$, absolute cell counts compared with Week 0 (baseline), 2-sided Wilcoxon signed-rank test. (B) T_{RTE} Tcon (orange).

Cellular markers of Treg function

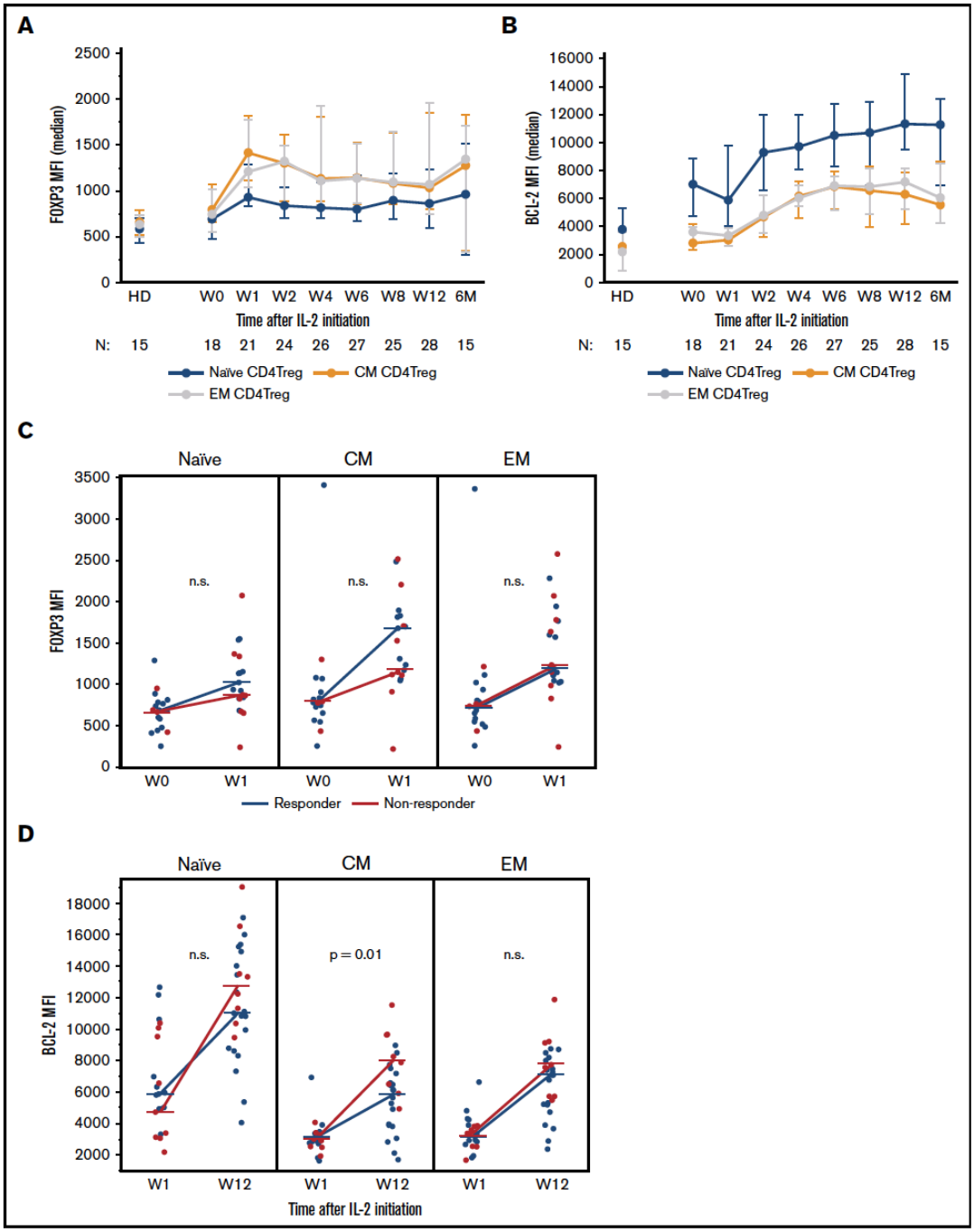
As there were no quantitative differences in Treg expansion or subset composition between response groups, we next examined expression of functional markers.

Stable, high level expression of Foxp3 is required for suppressive function whereas low expression levels are associated with decreased suppressive activity[328][329]. At baseline,

Foxp3 expression in Treg subsets was similar in memory and naïve Tregs (Figure 32A). IL-2 therapy led to a rapid increase in Foxp3 expression, particularly in T_{CM} and T_{EM} Tregs. At week 1, the median Foxp3 median fluorescence intensity (MFI) in T_{CM} Tregs and T_{EM} CD4 Tregs was higher than the median Foxp3 MFI in naïve Tregs ($p = 0.004$ and $p = 0.01$, respectively). Foxp3 levels in Treg subsets peaked between weeks 1 and 2 but remained above baseline during the entire 12-week treatment period (Figure 32A). Clinical responders and non-responders had similar changes in Foxp3 expression from baseline to the peak at week 1 in naïve and memory Treg subsets (Figure 32C).

A previous study showed that IL-2 therapy led to increased levels of the anti-apoptotic Bcl-2 protein in Tregs and enhanced resistance of these cells to apoptosis[238]. Here, we examined Treg subsets and found that Bcl-2 expression levels were highest in naïve Tregs and lower in memory Treg at baseline and during IL-2 therapy (Figure 32B). Bcl-2 expression in all Treg subsets increased 1 week after starting IL-2, peaked at week 12 and remained elevated during extended therapy. Clinical non-responders had a higher increase in Bcl-2 expression from week 1 to week 12 within the T_{CM} Treg subset but no difference between response groups was seen within the naïve or T_{EM} Treg (Figure 32D). There were no significant changes in Bcl-2 expression over time in the Tcon or $CD8^+$ T-cells subsets (data not shown).

Figure 32. Expression of Treg functional markers during IL-2 therapy

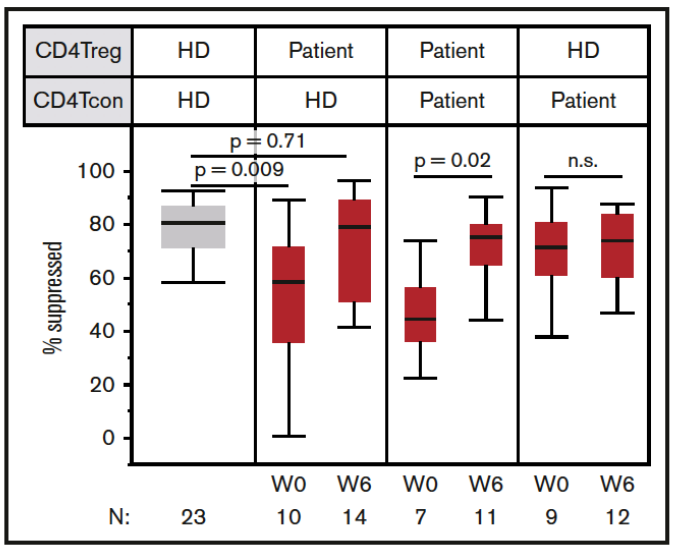


(A) Foxp3 expression in naïve and memory Treg subsets. (B) Increase in Bcl-2 expression from week 1 (W1) to week 12 (W12) in clinical responders and non-responders for naïve and memory Treg subsets. (C) Increase in Foxp3 expression from baseline (W0) to week 1 (W1) in clinical responders and non-responders for naïve and memory Treg subsets. (D) Bcl-2 expression in naïve and memory Treg subsets. (A-B) Median values and the interquartile range (whisker bars) are shown for each parameter. Timepoints represent weeks (W) and months (M) after IL-2 initiation. Number of patients evaluated at each timepoint is indicated at the bottom of the image. (C-D) Dots depict individual patients. Lines connect the median values at each timepoint. The Wilcoxon rank-sum test was used to compare responders *vs* nonresponders. n.s., not significant. HD, healthy donors. Naïve (blue); CM, central memory (orange); EM, effector memory (gray). MFI, mean fluorescence intensity; n.s., not significant.

Impact of low-dose IL-2 on *in vitro* Treg function

To assess Treg function more directly, we tested the ability of Tregs to suppress the proliferation of stimulated Tcons at study baseline and at week 6. Patient Tregs were tested against both autologous Tcons and HD Tcons from the same HD control subject for all *in vitro* assays. At baseline, patient Tregs had a decreased ability to suppress HD Tcons compared with Tregs from HD control subjects (Figure 33). Tregs from patients suppressed HD Tcons to a median of 58% compared with a median of 71% by HD Tregs (p = 0.009). By week 6 of IL-2 therapy, patient Tregs suppressive function improved and was indistinguishable from the HD Tregs (p = 0.71). Significant improvement in Treg function was also observed in the autologous suppression assays using patient Tregs and patient Tcons, with suppression to a median of 44% at baseline and 74% at week 6 (p = 0.02). Tcons from patients and HD were similarly suppressed by HD Tregs at baseline (p = 0.26) and at week 6 (p = 0.09), indicating that Tcon from patients with cGvHD are not more resistant to suppression in this assay.

Figure 33. Low-dose IL-2 improves *in vitro* Treg suppressive activity.



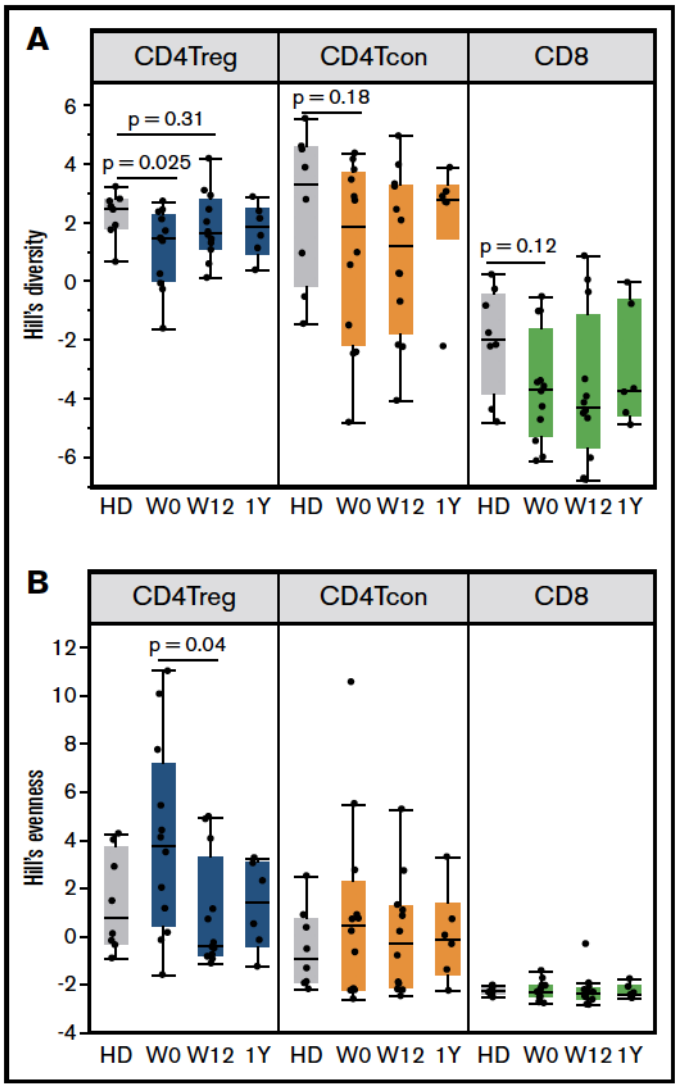
Assessment of suppressive activity of Tregs, at baseline (W0) and at week 6 (W6). From left to right: patient Tregs tested against healthy donor (HD) Tcons *in vitro*; patient Tregs tested against HD Tcons; patient Tregs tested against patient Tcons; HD Tregs tested against patient Tcons. Box plots depict the 75th percentile, median, and 25th percentile values; whiskers represent maximum and minimum values, except for outliers. The box for HD is shaded in gray. *P*-values comparing the suppressive activity of HD Tregs to patient Tregs are shown to W0 and W6; *P*-values are also shown comparing suppression at baseline and W6; two-sided Wilcoxon signed-rank test. n.s., not significant. Number of patients evaluated at each timepoint is indicated at the bottom of the image. For the HD control columns, N represents the number of assays using cells from the same HD

Patient Treg suppression of HD Tcons did not differ between response groups at baseline ($p = 0.55$), and both groups showed improved function at week 6. During extended therapy, Treg-suppressive function remained stable without significant change between week 6 and 6 months of IL-2 therapy (supplementary Figure 2; p. 218). Similarly, low-dose IL-2 did not alter the ability of patient Tcons to be suppressed by HD Tregs at week 6 or after 6 months.

Impact of low-dose IL-2 therapy on T-cell receptor repertoire diversity

Quantitative studies of the TCR repertoire have led to important insights into immune reconstitution and development of GvHD after HSCT[330][331][332][333][334][335]. We examined the effect of low dose IL-2 therapy on TCRß repertoire of purified Treg, Tcon and $CD8^+$ T-cells in serial blood samples from 6 clinical responders and 6 non-responders (supplementary Table 2; p. 216). We first compared TCR diversity between samples using the Hill-based diversity profile[336], in which higher values indicate a more diverse distribution of TCRß sequences. Consistent with previous reports[331][334][337] we found that $CD8^+$ T-cells had lower diversity compared to Treg and Tcon both in HDs ($p = 0.008$) and in patients ($p \leq 0.006$ for baseline and week 12; $p=0.03$ at 1 year) (Figure 34A). In baseline patient samples, diversity was lower in Tregs compared with HDs ($p=0.025$) but not statistically different in Tcons ($p = 0.18$) and $CD8^+$ T-cells ($p = 0.12$). After 12 weeks of IL-2 therapy, Treg diversity increased and was indistinguishable from HDs ($p= 0.31$) whereas diversity did not change within Tcons and $CD8^+$ T-cells. Treg diversity increased to similar extents in both response groups, although this comparison was limited by lower amounts of DNA input for Treg samples in clinical responders (supplementary Table 1; p. 215). We also examined evenness, which represents the extent of clonal expansion of the TCR repertoire by scaling the diversity index to the number of unique TCR clones[336]. High values of evenness indicate that all clones within the population are present at equal frequencies whereas lower values indicate an unequal distribution of clonal frequencies. IL-2 therapy led to significantly decreased evenness in Tregs ($p=0.04$ from baseline to week 12) but did not affect Tcons or $CD8^+$ T-cells (Figure 34B). Together, these observations suggest that low-dose IL-2 therapy both diversifies the clonal composition of Tregs and leads to selective expansion of individual clones.

Figure 34. TCR repertoire diversity in patients receiving low-dose IL-2

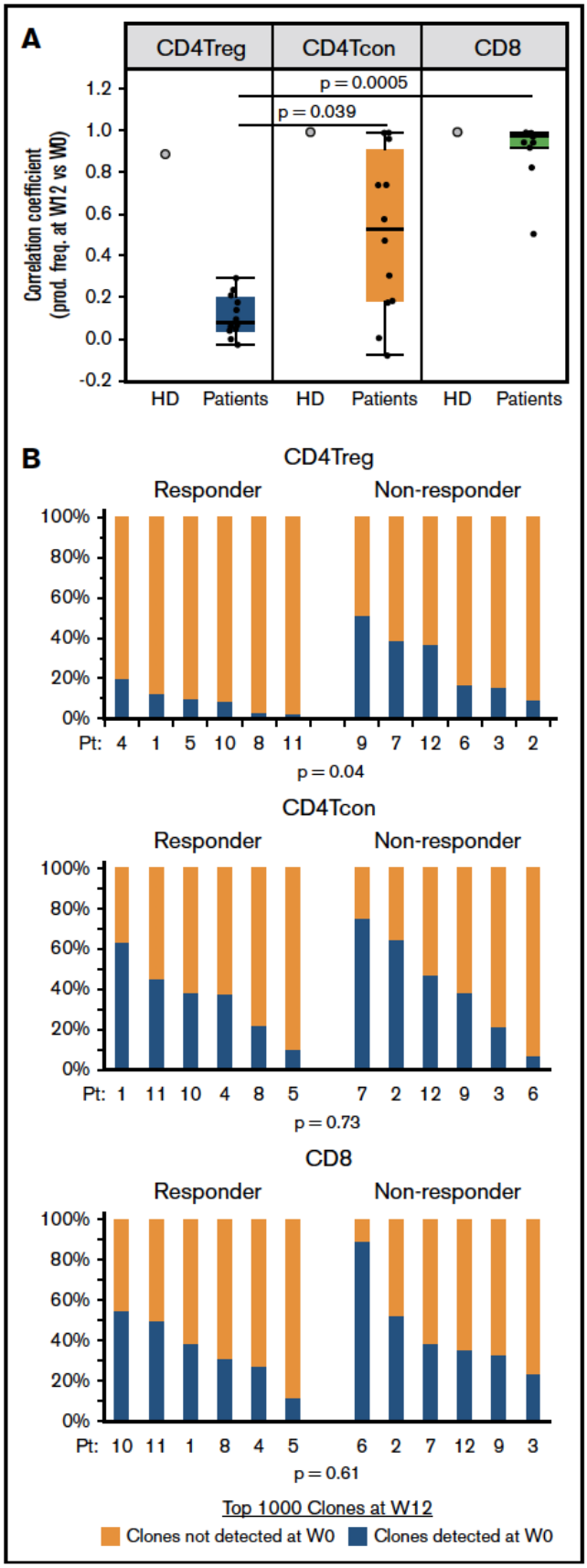


For patients, diversity measurements were assessed at baseline (W0), week 12 (W12) and 1 year (1Y). (A) Hill-based diversity values for Tregs, Tcons, and CD8$^+$ T-cells in patients with cGvHD (n = 12) and healthy donors (HD, n = 8). In HDs, CD8$^+$ T-cells have lower diversity than Tregs (p=0.008) and Tcons (p=0.008). In patients, CD8$^+$ T-cells have lower diversity than Tregs and Tcons at baseline ($p \leq 0.001$), week 12 ($p \leq 0.001$), and 1 year (p=0.03). Diversity in Tcons and CD8$^+$ T-cells is similar in patients compared with HDs and does not change with IL-2 therapy. Diversity in Tregs is lower in patients than in HD at baseline (p= 0.019) but is similar to HDs at week 12 (p=0.2). (B) Hill evenness values for Tregs, Tcons, and CD8$^+$ T-cells in patients with cGvHD and HDs. Evenness is similar between HDs and patients at baseline in Tcons and CD8$^+$ T-cells and does not change with IL-2 therapy. In Tregs, evenness is also similar between HDs and patients at baseline. However, within patients, evenness in Tregs decreases significantly from baseline to week 12 (p=0.04). (A-B) Each dot represents an individual patient. Box plots depict the 75th percentile, median, and 25th percentile values; whiskers represent maximum and minimum values, except for outliers. The boxes for HD are shaded in gray. Two-sided Wilcoxon signed-rank test was used to assess significance.

TCRß clone tracking during IL-2 therapy

To measure turnover of individual T-cell clones, we focused on the 1000 most abundant productive TCRß clones present within each cell type after 12 weeks of IL-2 therapy and asked whether these dominant clones were also detected at baseline. We also examined samples taken 12 weeks apart from an HD (Figure 35A). In the HD, the correlation coefficients between the productive frequencies of the top 1000 clones at the 2 timepoints were high within all 3 cell types, indicating a low level of clonal turnover during this 12-week interval. In patients receiving rIL-2, the correlation coefficients between the productive frequencies of the 1000 most abundant week 12 clones and productive frequencies at baseline (week 0) were significantly lower within Tregs compared with $CD8^+$ T-cells and Tcons (Figure 35 A). This suggests that IL-2 therapy preferentially leads to higher clonal turnover in Tregs. Using clone tracking, a median of 14% (range, 3% to 51%) of the most prevalent Treg clones at week 12 were detected at baseline compared with 38.5% (range, 7% to 75%) of the top 1000 Tcon clones and 36.5% (range, 12% to 90%) of CD8 clones. When examined by response group, clinical responders at week 12 had a significantly higher proportion of clones derived from clones that were not detected at baseline compared with nonresponders in Tregs (p=0.04) (Figure 35B). There were no differences between response groups for Tcons and $CD8^+$ T-cells. These results suggest that greater expansion of novel or low frequency clones within the Treg fraction during IL-2 therapy is associated with a better clinical response. The greatest influx of new clones within Tregs occurred in the first 12 weeks of therapy as TCR overlap between the week 12 and 1-year timepoints was higher than the overlap between baseline and week 12 (supplementary Figure 3A; p.219). Among patients who received extended-duration therapy, a median of 9.5% (range, 3% to 20%) of the dominant Treg clones at week 12 was shared with the baseline population, whereas a median of 34% (range, 12% to 57%) of the dominant Treg clones after 1 year of IL-2 therapy was shared with the week 12 population (p=0.03) (supplementary Figure 3B; p.219).

Figure 35. Origin of dominant clones in each T-cell population at week 12 of IL-2.

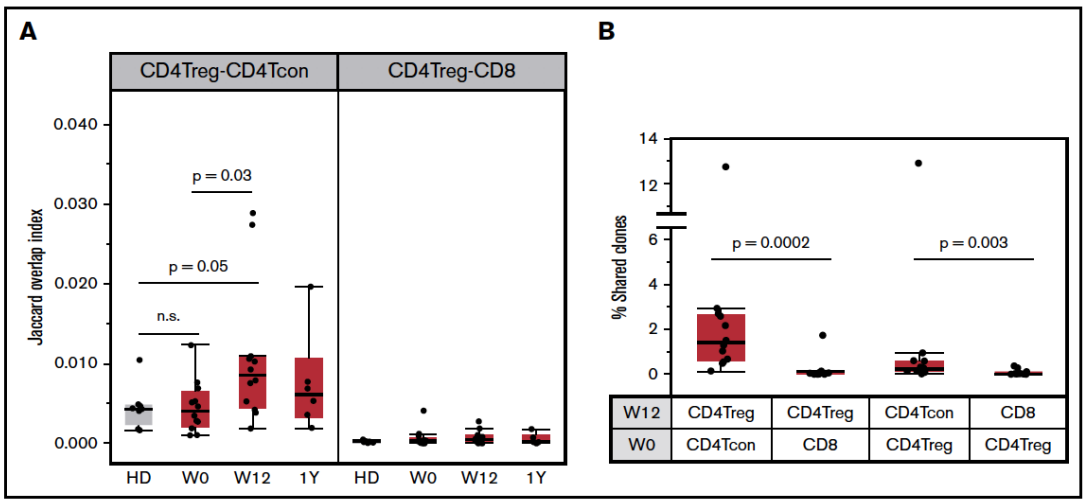


(A) Correlation coefficients between the productive frequencies of the top 1000 clones at week 12 (W12) and the productive frequencies of the same clones at baseline (W0) for each cell type are shown for an HD (gray filed circles) and patients (black dots). Each dot represents the correlation coefficient for an individual patient. Box plots depict the 75th percentile, median, and 25th percentile values; whiskers represent maximum and minimum values, except for outliers. The correlation coefficients for Tregs were significantly lower than for Tcons (p=0.039) and CD8^{+} T-cells (p=0.0005). (B) Origin of the dominant 1000 clones at W12 in clinical responders (n=6; patients 1,4,5,8,10,11) *vs* non-responders (n=6, patients 2,3,6,7,9,11). For each cell type, bar graphs show the percentage of dominant week 12 clones that were detected (blue) at W0 and the percentage of dominant W12 clones that were not detected (orange) at baseline. Each column represents an individual patient. Two-sided Wilcoxon signed-rank test was used to assess significance.

Peripheral conversion of Tcon to Treg during IL-2 therapy

Apart from thymic production, a potential source of new Treg clonotypes is through peripheral conversion of Tcon into Treg[197]. We compared the TCR overlap between productive Treg and Tcon sequences. At baseline, the TCR overlap (Jaccard index) between Tregs and Tcons was low in both patients and HDs (Figure 36A). After 12 weeks of IL-2 therapy, the Treg-Tcon overlap index increased above the baseline level (p=0 .03) whereas there was no change in the Treg-CD8 overlap. We performed a similar analysis using samples taken 12 weeks apart from an HD and found that Treg-Tcon TCR overlap did not increase (supplementary Figure 4; p.220). In all patients, we identified individual Treg clones at week 12 that were detected exclusively in the baseline (week 0) Tcon population (Figure 36B), further supporting the conversion of Tcons into Tregs during IL-2 therapy. However, the calculated TCR overlap index between week 0 Tcon and week 12 Treg samples increased in only some patients (supplementary Figure 5A p.221). Thus, although IL-2 treatment enhances peripheral conversion of Tcons into Tregs, it likely remains a low frequency event. Conversion of Tregs to Tcons occurs to a lesser extent as we could also detect clones within the week 12 Tcon population that were shared exclusively with baseline Tregs (Figure 36B). As expected, there was almost no clone sharing between the Treg and CD8^{+} T-cell populations. There were no differences in Treg-Tcon clone sharing or TCR overlap between response groups (supplementary Figure 5B-C; p.221).

Figure 36. Clone sharing between different T cell populations



(A) TCR overlap (Jaccard overlap index) between Treg and Tcon repertoires at baseline (W0), week 12 (W12), and 1 year (1Y). Overlap in HD (gray) is shown for comparison. (B) Percentage of the total unique clones at W12 that were detected within different baseline populations. Each column shown the percentage of W12 clones within the various cell types that are exclusively shared with a particular baseline population. For example, the first column on the left shown the percentage at W12 of Treg clones shared exclusively with the baseline Tcon population. There is significantly higher clone sharing between Tregs and Tcons than between Tcons and CD8$^+$ T-cells T-cells. In both panels, each dot represents a patient (n=12) or an HD (n=8). Box plots depict the 75th percentile, median, and 25th percentile values; whiskers represent maximum and minimum values, except for outliers. Two-sided Wilcoxon signed-rank test was used to assess significance between the different combinations.

Short Discussion

Previous studies have demonstrated that administration of low-dose rIL-2 leads to a rapid increase in peripheral Tregs with little, if any, effect on Tcon or CD8$^+$ T-cell[237][338,339]. In SR cGvHD patients, clinical responses were documented in 50-60%, even though all patients experienced an increase in the absolute numbers of circulating Tregs[240]. To investigate the T-cell factors that contribute to amelioration of cGvHD in this setting, we studied functional T-cell markers, Treg function and T-cell repertoire diversity within Treg, Tcon and CD8$^+$ T-cells.

We found that low-dose IL-2 selectively induced several changes in Treg homeostasis and function, as previously demonstrated in mouse models[225][340,341]. The earliest effect observed was a rapid proliferation of T_{CM} Tregs, which was followed by a gradual

increase on naïve Tregs. Initially, naïve Tregs arised from homeostatic proliferation; following 4 weeks of therapy, naïve Tregs arised primarily from thymic generation. IL-2 also induced significant functional effects in Tregs, including increased expression of Bcl-2 and Foxp3, and enhanced *in vitro* suppressive activity. Importantly, these changes occurred in both clinical non-responders and responders, suggesting that the lack of clinical response was not due to resistance of Tregs to the functional and homeostatic effects of IL-2. This analysis was limited by the number of assays available to measure Treg function *in vitro*. The standard assay used to examine Treg function measured the ability of Tregs to inhibit proliferation of stimulated Tcon. This output reflects the ability of Tregs to sequester IL-2, but does not quantify other mechanisms of Treg suppression, as described in Section 3.2 [215][216][217].

Quantitative assessment of the TCR repertoire provides another tool for investigating the impact of low-dose IL-2 therapy[91]. Using high throughput TCRß sequencing, we examined the TCR repertoire of highly purified Treg, Tcon and $CD8^+$ T-cells. At baseline, patients with SR cGvHD, had significantly lower TCR diversity within Tregs compared with HD. Low dose IL-2 increased diversity within Tregs after 12 weeks of therapy, regardless of clinical response. TCR diversity within Tcons and $CD8^+$ T-cells was similar between patients and HD at baseline and did not change with IL-2. This analysis is limited by the semiquantitative nature of the ImmunoSEQ platform[315][342] and by the low DNA input from $CD4^+$ Treg samples.

IL-2 therapy increased TCR diversity within Tregs and decreased TCR evenness, which suggests an expansion of specific Treg clones. We further examined the expansion and contraction of clonotypes during IL-2 therapy by tracking the most abundant TCRß sequences in serial samples. In contrast to Tcon and $CD8^+$ T-cells, where there was very little change in clonotypes over time, low-dose IL-2 induced rapid and extensive turnover of individual Treg clones. Notably, expansion of Treg clones that were not detectable at baseline occurred more frequently in clinical responders compared to non-responders after 12 weeks of therapy. By tracking individual T-cell clones from baseline to week 12, we also demonstrated that at least some Treg clones that expanded during IL-2 therapy were recruited from the baseline Tcon population.

Overall, this data suggests profound effects of IL-2 on Treg homeostasis and function, regardless of clinical response. Clinical improvement was associated with a rapid expansion of low frequency or novel Treg clones, which suggest an important role of specific Treg clones for achievement of peripheral tolerance post allo-HSCT.

General Discussion

Despite the recent advances in the field of allogeneic transplantation, around 40% of patients fail to benefit from this immune therapy due cGvHD development, which remains the leading cause of morbidity and non-relapse mortality post-transplant[270,271].

Although multifactorial in nature, cGvHD has been associated with imbalances between effector T-cells (Tcon and CD8), and Tregs, resulting in loss of immune tolerance post-transplant[273,274]. The impaired recovery of Tregs seems to have an important role in the pathophysiology of cGvHD[273,274]. However, there are multiple factors involved in T-cell reconstitution and the mechanisms that can lead to Treg deficiency or disfunction are poorly understood.

The primary aim of this thesis was to investigate the endogenous mechanisms and factors involved in the homeostasis of peripheral blood $CD4^+$ regulatory T-cells after allo-HSCT, with the ultimate goal of developing new approaches to prevent and treat cGvHD. In order to accomplish this goal, we conducted, for the first time, a simultaneous characterization of the naïve and memory compartment within Treg, Tcon and $CD8^+$ T-cells, and its homeostatic profile in terms of thymic generation, proliferation and susceptibility to apoptosis. This study was performed in three allotransplant cohorts, differing primarily in the conditioning regimen, level of HLA matching and GvHD prophylaxis.

First, we addressed the dynamics of T-cell reconstitution and its homeostatic regulation over a period of 2 years after unmodified RIC allo-HSCT in a large cohort of patients with hematologic malignances (RIC_Boston cohort). Matched donors were the most frequent source of PBSC (89,7%) and GvHD prophylaxis consisted primarily of TAC/SIR. In this setting, the recovery of $CD4^+$ T-cells was delayed throughout the follow-up, while $CD8^+$ T-cells reached normal levels at 12 months post-transplant. Moreover, T-cell homeostasis favored the proliferation of memory Tcon and $CD8^+$ T-cells, while the thymic generation and proliferation of naïve Tregs were selectively compromised in all patients for the entire follow-up period. This divergent homeostatic pattern reflects a different homeostatic regulation of Treg, Tcon and $CD8^+$ T-cells[258]. Hence, reduced numbers of Tregs most likely resulted from insufficient IL-2, that regulates Treg development and survival[231], while increased numbers of memory subsets within Tcon and $CD8^+$ T-cells resulted from expected high levels of IL-7 and IL-

15[145][151][161,162], induced by profound lymphopenia. The 46 patients (43%) that developed cGvHD were distinguished by an early and transient prevalence of immature subsets (naïve and T_{CM}). This early expansion occurred irrespectively of aGvHD and most likely resulted from donor infused naïve T-cells that expanded in response to allo-antigens. We speculate that the concurrent immunomodulatory treatment avoided an acute immune response. Nevertheless, with the reduction of immunomodulatory drugs, the insufficient number of Tregs enabled the expansion of alloreactive naïve T-cells, with subsequent development of cGvHD. Chronic GvHD patients were also distinguished by significantly lower Treg:Tcon and Treg:CD8 ratios at 9 months post-transplant, as a consequence of delayed Treg recovery. Results from this study confirmed a different homeostatic regulation of each T-cell population and subset, and raised the need to clarify the role of naïve T-cells in the induction of cGvHD.

Secondly, we investigated the contribution of true naïve (pT_N) and T memory stem cells (T_{SCM}), within Treg, Tcon and CD8, in the development of cGvHD. Despite the suggested involvement of naïve T-cells in the initiation of cGvHD, increasing evidence shows that this subset may not truly correspond to functionally naïve T-cells. Indeed, a proportion of cell expressing naïve T-cell markers display phenotypical and functional characteristics reminiscent of effector and memory T-cells, the so-called Memory Stem Cells[11][20]. To elucidate the role of each specific subset, pT_N and T_{SCM}, on cGvHD development, we studied separately two distinct cohorts of transplanted patients.

Following *in vivo* T-cell depleted RIC allo-HSCT with ATG and CsA plus MMF for GvHD prophylaxis (RIC_Lisbon cohort), the recovery of pT_N and T_{SCM} subsets in cGvHD patients revealed opposing reconstitution patterns in $CD4^+$ and $CD8^+$ T-cells. The 18 patients (45%) that developed cGvHD were distinguished at month 1 post-transplant by significant high numbers and percentages of pT_N and T_{SCM} Treg, Tcon and CD8 subsets. These subsets most likely corresponded to donor-infused naïve T-cells that escaped T-cell depletion and differentiated into T_{SCM} after allogeneic recognition or through lymphopenia-induced proliferation [72–74]. Following the first month, however, cGvHD patients had a divergent pattern of recovery, with delayed reconstitution of pT_N and T_{SCM} $CD4^+$ subsets and increased pT_N, T_{SCM} and T_{EMRA} $CD8^+$ subsets. The significant delayed recovery of pT_N and T_{SCM} $CD4^+$ T-cells was observed beyond month 9, and coincided with significantly reduced proliferation and thymic generation of total Treg

and Tcons, and decreased CD4 TCR repertoire diversity. Instead, the early increase on pT_N, T_{SCM} and T_{EMRA} $CD8^+$ subsets coincided with increased proliferation and reduced sjTRECs levels. These results confirm previous data suggesting a distinct homeostatic regulation of $CD4^+$ and $CD8^+$ T-cell subsets[121]. In this cohort, despite *in vivo* T-cell depletion with ATG, there was a high incidence of aGvHD, likely associated to the high number of HLA mismatches between donor and host (55,5%; 10/18). Acute GvHD and its treatment have previously been shown to modify the immune T-cell compartment early post-transplant[276]. In this cohort, aGvHD affected particularly T_{SCM} numbers and percentages within Tcon and CD8 in the first 6 months post-transplant. Following this timepoint, a sustained increase in T_{SCM} CD8 was observed in cGvHD patients, regardless of aGvHD, pointing to a possible role for this subset in disease development. T_{SCM} have been reported to differentiate from naïve T-cells[72–74], to be increased in autoimmune diseases[75,76], and to induce lethal GvHD in mice[68]. In this analysis, findings of early increase on pT_N CD8 and sustained expansion of T_{SCM} CD8 in cGvHD patients suggests and involvement of this latter subset in the pathogenesis of cGvHD, by yet undescribed mechanisms.

Following unmodified MAC allo-HSCT, using primarily TAC/MTX for GvHD prophylaxis (MAC_Boston cohort), cGvHD developed in 46,8% (n=52) of patients, and was preceded by reduced pT_N and T_{SCM} Tregs and increased T_{EM} $CD8^+$ T-cells. This imbalance was observed in the early post-transplantation period and persisted immediately before cGvHD diagnosis. The early preferential recovery of T_{EM} CD8 could result from lymphopenia-induced proliferation of transferred donor naïve $CD8^+$ T-cells, in the presence of high levels of homeostatic cytokines, namely IL-15, which selectively activates $CD8^+$ memory T-cells[260]. Previous data support this hypothesis, by showing increased IL-15 levels in the early post-transplant period, likely resulting from conditioning-induced tissue damage and severe lymphopenia[324]. In line with other reports, we observed a significant delay in Treg reconstitution beyond 9 months post-transplant. Analysis of the samples obtained at or immediately after the diagnosis of cGvHD showed an increased number of pT_N and T_{CM} Tregs, compared to samples obtained before cGvHD. We speculate that this increase in the number of Treg subsets, that coincide with the diagnosis of cGvHD, translates the acute effect of immunomodulatory drugs[326]. Results from this study emphasize the role of a balanced

recovery between effector and regulatory T-cells for immune tolerance post transplant, and imply an involvement of T_{EM} CD8 in the pathogenesis of cGvHD.

Finally, to clarify the mechanisms likely involved in the recovery of T-cell tolerance, we performed a simultaneous analysis of the T-cell subsets, Treg function and T-cell repertoire in a cohort of SR cGvHD patients receiving daily low-dose rIL-2. Interleukin-2 is the primary homeostatic regulator of Tregs *in vivo*[232], and the high expression of IL-2Rα in Tregs enhances its sensitivity for this cytokine[188]. Prior research demonstrated that administration of low-dose rIL-2 increased the total Treg numbers in all patients, although only 50-60% had clinical improvement of cGvHD[240]. This finding raised the need to clarify the T-cell factors associated with amelioration of cGvHD. In this analysis we observed profound effects on Treg homeostasis with rIL-2, including increased proliferation, improved thymic generation of naïve Tregs, and increased and sustained expression of Foxp3 and Bcl-2 within all Treg subsets. Furthermore, rIL-2 enhanced *in vitro* Treg suppressive activity. These findings were in agreement with previous data regarding the effects of IL-2 on Tregs[106][232][231]. Unlike Treg, Tcon and $CD8^+$ T-cells were unaffected by rIL-2 in this study, likely due to the low dose administered. Despite the relevance of these results, they were observed in both clinical responders and nonresponses. Hence, we further examined the impact of rIL-2 on TCR repertoire within Treg, Tcon and $CD8^+$ T-cells. Chronic GvHD improvement correlated with the expansion of low frequency Treg clones and the emergence of novel clones, which suggests a role for Treg TCR specificity to an optimal Treg function, and subsequent cGvHD regression/ prevention.

CONCLUSION AND FUTURE PERSPECTIVES

The mechanisms and factors implicated in Treg impairment post allo-HSCT remain elusive. The findings from human studies that addressed T-cell reconstitution post allo-HSCT and the association with cGvHD development are heterogeneous, which reflect the complexity of factors and mechanisms regulating T-cell homeostasis[164][251–255]. In this work we performed a comprehensive analysis of the T-cell compartment in different transplant settings in order to clarify the factors and the endogenous mechanisms that regulate Treg homeostasis *in vivo*.

Altogether, the dynamics of T-cell reconstitution varied according to the transplant setting, as did the contribution of each T-cell subset to the pathogenesis of cGvHD. This heterogeneity confirms the combined effect of clinical variables that distinguish the three cohorts, such as the conditioning regimen[264], the immunosuppressive therapy[246], the level of HLA matching and the development of aGvHD[276], on T-cell immunity post-transplant.

Our main findings and conclusions were:

- **$CD4^+$ T-cell reconstitution post allo-HSCT was delayed throughout the follow-up period, while $CD8^+$ T-cell numbers recovered within the first-year post-transplant**. This was observed in all cohorts and occurred irrespective of cGvHD development. The late recovery of $CD4^+$ T-cells coincided with a sustained low proliferation of naïve Tregs and decreased thymic generation of both Treg and Tcon, without significant changes in the pathways of apoptosis. Conversely, the early recovery of $CD8^+$ T-cells coincided with increased proliferation of memory and naïve subsets, despite decreased thymic generation. The preferential recovery of $CD8^+$ over $CD4^+$ T-cells is in agreement with published data showing that homeostatic proliferation is insufficient to restore $CD4^+$ T-cell numbers that, unlike $CD8^+$ T-cell homeostasis, relies largely on thymic function[121]. Hence, the high numbers of memory Tcon and $CD8^+$ T-cells likely resulted from increased levels of IL-7 and IL-15 reported in lymphopenia[145][151][161,162]. On the other hand, the selective decrease in the proliferation of naïve Tregs most likely resulted from deregulated cytokine secretion, namely IL-2, which is a critical regulator for Tregs *in vivo*[106][231,232].

- **Patients who developed cGvHD could be distinguished, at 9 months post allo-HSCT, by a significantly delayed recovery of Tregs, regardless of the patient cohort.** We consistently found a significantly lower number of Tregs at 9 months post-transplant in patients developing cGvHD, that coincided with decreased Treg:Tcon and Treg:CD8 ratios. This consistent observation emphasizes the contribution of T-cell homeostatic imbalance in cGvHD pathology. However, considering the median time of cGvHD onset of 230 days (~7,5 months), this result is difficult to interpret, as it may reflect the effect of cGvHD or its treatment.

- **The thymic generation of $CD4^+$ T-cells was compromised in all cohorts, and became significantly delayed after cGvHD development.** The generation of *de novo* donor-derived naïve T-cells occurs three to six months post-transplant[118], and relies on the preservation of thymic function to generate a diverse and self-tolerant T-cell repertoire. However, the thymus is easily damaged in the post-transplant setting, due to the conditioning regimen[248,249], severe infection[117], and GvHD[118,119]. In our studies, there was a delayed recovery of RTE Treg and Tcon throughout the follow-up, that became more prominent after cGvHD development. Beyond month 9, $CD4^+$ T-cell sjTREC content was significantly reduced in cGvHD patients. Likewise, CD4 TCR diversity at such late timepoints was significantly decreased, compared to patients with No GvHD. Collectively, these results suggest that thymopoiesis is always compromised after allo-HSCT, independently of the conditioning regimen. In addition, the data further suggests that cGvHD development enhances thymic dysfunction. The lack of a functional thymus, together with the preferential expansion of memory subsets likely involved in cGvHD pathogenesis, results in a skewed $CD4^+$ TCR repertoire in cGvHD patients.

- **The contribution of pT_N and T_{SCM} subsets to the development of cGvHD varied between patient cohorts**. Previous studies suggested a role for donor-infused naïve T-cells[290–292] and T_{SCM} CD8[68] in cGvHD development, in humans and mice, respectively. In this work we studied, for the first time, the association between pT_N and T_{SCM} T-cells and cGvHD development, in humans. The dynamic recovery of pT_N and T_{SCM} subsets was unique to each cohort. Hence, with regards to pT_N subsets, we found an early and transient increase in patients

developing cGvHD, in two cohorts. Regarding T_{SCM}, the association with cGvHD was only found in one cohort. We speculate that the observed differences result from disparities in clinical variables, such as HLA matching, intensity of conditioning regimen, T-cell depletion, and GvHD prophylaxis, that modify the T-cell homeostasis, particularly in the early period post-transplant.

- **The early and sustained increase of T_{SCM} CD8 anticipated the development of cGvHD, after *in vivo* T-cell depleted RIC allo-HSCT with ATG and CsA/MMF**. T_{SCM} correspond to a naïve-derived subset, with unique characteristics, that provide a potential reservoir of memory T-cells throughout life[72,73][343]. Previous studies have shown that IL-7 and IL-15 are necessary for the generation of human T_{SCM} from naïve precursors *in vitro* and that T_{SCM} prevail early post transplant, when these homeostatic cytokines are particularly abundant[74]. In this setting, we speculate that the allogeneic stimulation of donor-infused naïve T-cells leads to the initial expansion of T_{SCM} CD8, that are maintained through self-renewal[52]. The use of ATG and CsA as GvHD prophylaxis in this setting, results in a profound $CD4^+$ lymphodepletion, with further increased levels of circulating IL-7 and/or IL-15, which might favour T_{SCM} expansion. The factors beyond the extensive proliferation of T_{SCM} CD8 observed in cGvHD patients remain elusive.

- **Following low dose rIL-2, the emergence of low frequency/new Tregs clones was the only variable that correlated with cGvHD improvement**. This result emphasizes the role of alloantigen recognition by Tregs, during thymopoiesis post allo-HSCT, and the subsequent expansion of specific Treg clones, for an optimal Treg function. The relevance of allo-specific Tregs in maintaining tolerance was highlighted in pre-clinical studies[344], and further confirmed in the context of clinical transplant, where allo-specific Tregs demonstrated superior immunosuppressive capacity[345,346]. Results from this study confirm that reversal of cGvHD is largely dependent on the recognition of Ags by allo-specific Tregs.

An important limitation of our studies is the use of peripheral blood samples to study the the dynamics of circulating T-cells post allo-HSCT. The use of peripheral blood is an easy and noninvasive method; however, the peripheral blood contains only a very small

fraction of the total body T-cells[2]. Besides, the peripheral T-cell dynamics may not reflect the modifications in target tissues[10], and may be affected by several factors unrelated to cGvHD, such as the conditioning regimen, thymic involution, type of graft, stem cell dose, T-cell depletion, HLA mismatching, GvHD prophylaxis and treatment. Despite such limitations, there was some consistency on the data obtained from the different studies, which validates our results, and provides some clues into cGvHD pathogenesis and possible therapeutic targets.

Overall, our findings add significantly to the current knowledge of T-cell homeostasis post allo-HSCT and point to abnormalities in T-cell immunity that may be implicated in the development of cGvHD. Future work is needed before incorporating these findings into the clinical practice. With this in mind, we propose the following future research:

- **To clarify the homeostasis of naïve Tregs in the early period post-transplant**, in different transplant settings. Using the cryopreserved serum of patients included in each studied cohort, we will measure the plasma levels of IL-2 at early timepoints, and correlate with the proliferation and absolute numbers of naïve Tregs. We anticipate significantly lower plasma levels of IL-2, compared to HD, that correlate with decreased Treg expansion, and increased risk for loss of peripheral T-cell tolerance. The prophylactic administration of low-dose IL-2 after allo-HSCT has already been tested in clinical trials[347–349]. However, the studies evaluating the plasma levels of IL-2 post allo-HSCT in humans are scarce[258].IL-2 is mainly produced by activated $CD4^+$ Tcons, which are considerably low in the post-transplant setting due to the conditioning regimen and GvHD prophylaxis. By addressing this question, we are also improving our knowledge on the effect of selected conditioning and GvHD prophylaxis regimens on $CD4^+$ Tcon.

- **To clarify the homeostasis of T_{SCM} CD4 and CD8 and investigate the allo-reactivity of T_{SCM} CD8 in the initiation of cGvHD.** Using cryopreserved serum of patients included in the RIC_Lisbon cohort, we will measure the plasma levels of the main cytokines involved in T_{SCM} homeostasis, namely IL-7 and IL-15, at

different timepoints post-transplant, and correlate to proliferation and absolute numbers of T_{SCM} within $CD4^+$ and $CD8^+$ T-cells. To further investigate the suggested role of T_{SCM} CD8 in cGvHD development, we will collect fresh blood samples from patients before and after RIC allo-HSCT with ATG, and perform *in vitro* assays to test the reactivity of T_{SCM} CD8 towards patient-derived antigens presented by host-APC.

- **To characterize the TCRs from IL-2 expanded Tregs and identify the target antigens**. The relevance of antigen-specificity for Treg function in human disease is poorly understood, largely due to the lack of knowledge regarding the specific antigens recognized by Tregs in the thymus and the way antigen-specific Tregs prevent disease. In mice, Tregs with restricted TCR have shown to promote tolerance and prevent GvHD, while sparing GvL[344]. In humans, allo-specific Tregs have shown an increased potential for a targeted and potent GvHD treatment[346]. However, most antigens involved in cGvHD pathology are unknow, which limits the applicability of this targeted therapy. Recently, the team from João Lacerda Lab has elaborated and tested a protocol to expand human allogeneic specific Treg *in vitro.* Interestingly, they have found that allo-specific Tregs could recognize matched HLA alleles in both APCs from the original donor and APCs from partially matched donors[350]. With this future work we aim to study potential target antigens involved in cGvHD, to further generate antigen-specific Tregs, with specific TCRs or chimeric antigen receptor (CAR), able to induce transplantation tolerance[351].

Our main goals with the future research focus on the identification of both, a biological variable involved in cGvHD initiation, and Treg-specific antigens involved in Treg function. Considering the chronicity of this disease, we anticipate that an early intervention, such as cytokine therapy or selective Treg-based therapy, can ensure a balanced T-cell recovery and promote immune tolerance post allo-HSCT, without compromising the immune competence of the host or the GvL effect.

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LIST OF PUBLICATIONS

Peer reviewed articles

A phase 1 study of donor regulatory T-cell infusion plus low-dose interleukin-2 for steroid-refractory chronic graft-vs-host disease.

Jennifer Whangbo[1], Sarah Nikiforow[2], Haesook T Kim[2], Jonathan Wahl[2], Carol G Reynolds[3], Sharmila Chamling Rai[4], Soomin Kim[5], Andrew Thomas Burden[2], Ana Alho[6], João F Lacerda[7], Edwin P Alyea[8], Corey S Cutler[9], Vincent T Ho[2], Joseph H Antin[2], Robert J Soiffer[2], Jerome Ritz[2], John Koreth[2]

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Maturation and Phenotypic Heterogeneity of Human CD4$^+$ Regulatory T cells from birth to adulthood and after Allogeneic Stem Cell Transplantation

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Functional Analysis of Clinical Response to Low-dose IL-2 in Patients with Refractory Chronic Graft-*versus*-Host Disease

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Naïve and Stem Cell Memory T cell subset recovery reveals opposing reconstitution patterns in CD4$^+$ and CD8$^+$ T cells in Chronic Graft *versus* Host Disease

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Low-dose IL-2 selectively activates subsets of CD4$^+$ Tregs and NK cells

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Circulating T follicular helper cells with increased function during chronic graft-*versus*-host disease

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Unbalanced recovery of regulatory and effector T cells after allogeneic stem cell transplantation contributes to chronic GVHD

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Manuscript in preparation

Early predominance of effector memory CD8$^+$ T-cells over true naïve and central memory Tregs results in chronic GvHD after myeloablative allo-HSCT

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Communications

ORAL PRESENTATION

Reconstituição imune T na Doença de Enxerto Contra Hospedeiro Crónica após alotransplante de dador não relacionado: análise de três grupos clínicos.

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Reunião da Sociedade Portuguesa de Hematologia, Vilamoura, Portugal, 15-17 Novembro, 2018

POSTER PRESENTATIONS

Impact of True Naïve and Stem Cell Memory recovery on chronic graft-*versus*-host disease after allogeneic hematopoietic stem cell transplantation.

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44th Annual Meeting of the European Group for Blood and Marrow Transplantation; Lisboa, Portugal.

Increased Naïve, Stem Cell Memory and Terminally Differentiated CD8$^+$ T cells in Chronic Graft *versus* Host Disease.

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59th ASH Annual Meeting & Exposition, Atlanta, GA, USA. Blood 2017 130:3260; December 10, 2017.

T cell reconstitution following allo-HSCT in three different patient groups: regulatory T cell dynamics is compromised in chronic GvHD patients

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XI CAML PhD meeting, 24-26 Maio 2017, Edifício Egas Moniz, FMUL

Treg and Tcon dynamics after Allo-HSCT: cGvHD is associated to decreased Naïve and Stem Cell Memory Subsets with a concomitant increase in Terminally Differentiated T Cell Subsets

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58th ASH Annual Meeting & Exposition, San Diego, California, USA. Blood 2016 128:2229; December 3, 2016.

Long-Term Homeostatic Effects of Daily Low-Dose IL-2 on $CD4^+Foxp3^+$ Regulatory T Cells in Patients with Active Chronic Graft-*versus*-Host Disease

Jennifer Whangbo[1,2], John Koreth[3], Haesook T. Kim[4], Katharine Dusenbury[1], Ana Cristina Alho[1], Carol G Reynolds[1], Marie J Chammas[1,] Kyle T Jone[5], Sarah Nikiforow[5],

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57th ASH Annual Meeting & Exposition, Orlando, USA. Blood 126 (23), 3133; December 3, 2015.

Maturation and Phenotypic Diversity of Human CD4$^+$ Regulatory T Cells in Umbilical Cord Blood and Peripheral Blood from Healthy Donors

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57th ASH Annual Meeting & Exposition, Orlando, USA. Blood 2015 126:2357; December 3, 2015.

Homeostatic Reconstitution of CD4+Regulatory and Conventional T cell subsets in Adult Patients after allogeneic Hematopoietic stem cell transplantation (HSCT).

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56th ASH Annual Meeting & Exposition, San Francisco, California, USA. Blood 124 (21), 2496; December 6, 2014.

Major conferences attended

44th Annual Meeting of the European Group for Blood and Marrow Transplantation; Lisboa, Portugal; 18-21 March, 2018

42th Annual Meeting of the European Group for Blood and Marrow Transplantation; Valência, Spain; 3–6 April 2016

58th ASH Annual Meeting & Exposition, San Diego, California, USA. December 3-6, 2016

56th ASH Annual Meeting & Exposition, San Francisco, California, USA. December 6-9, 2014

ANNEXES

Publications Included in the Thesis

Regular Article

TRANSPLANTATION

Unbalanced recovery of regulatory and effector T cells after allogeneic stem cell transplantation contributes to chronic GVHD

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Key Points

- Homeostatic recovery after allogeneic HSCT favors the production, expansion, and survival of effector T cells over CD4Tregs.
- Unbalanced reconstitution of regulatory and effector T-cell subsets contributes to the development of chronic graft-versus-host disease.

The development and maintenance of immune tolerance after allogeneic hematopoietic stem cell transplantation (HSCT) requires the balanced reconstitution of donor-derived CD4 regulatory T cells (CD4Tregs) as well as effector CD4 (conventional CD4 T cells [CD4Tcons]) and CD8 T cells. To characterize the complex mechanisms that lead to unbalanced recovery of these distinct T-cell populations, we studied 107 adult patients who received T-replete stem cell grafts after reduced-intensity conditioning. Immune reconstitution of CD4Treg, CD4Tcon, and CD8 T cells was monitored for a 2-year period. CD3 T-cell counts gradually recovered to normal levels during this period but CD8 T cells recovered more rapidly than either CD4Tregs or CD4Tcons. Reconstituting CD4Tregs and CD4Tcons were predominantly central memory (CM) and effector memory (EM) cells and CD8 T cells were predominantly terminal EM cells. Thymic generation of naive CD4Tcon and CD8 T cells was maintained but thymic production of CD4Tregs was markedly decreased with little recovery during the 2-year study. T-cell proliferation was skewed in favor of CM and EM CD4Tcon and CD8 T cells, especially 6 to 12 months after HSCT. Intracellular expression of BCL2 was increased in CD4Tcon and CD8 T cells in the first 3 to 6 months after HSCT. Early recovery of naive and CM fractions within each T-cell population 3 months after transplant was also strongly correlated with the subsequent development of chronic graft-versus-host disease (GVHD). These dynamic imbalances favor the production, expansion, and persistence of effector T cells over CD4Tregs and were associated with the development of chronic GVHD. (*Blood*. 2016;127(5):646-657)

Introduction

Successful allogeneic hematopoietic stem cell transplantation (HSCT) relies on engraftment of donor hematopoietic stem cells and full reconstitution of a donor-derived immune system in the recipient. Importantly, the reconstituting immune system must include critical regulatory elements as well as highly diverse populations of effector cells. This key feature of immune reconstitution is needed to provide a broad array of adaptive immune effector cells capable of recognizing external pathogens and antigens on recipient tumor cells while suppressing responses to antigens expressed on normal recipient cells. Previous studies have demonstrated that phenotypic and functional recovery of donor T cells is often delayed for months to years after allogeneic HSCT.[1-3] Although most studies have focused on reconstitution of effector T cells, several studies have also examined recovery of CD4 regulatory T cells (CD4Tregs).[5-9] These studies suggest that CD4Treg deficiency can enhance alloreactivity and promote graft-versus-host disease (GVHD).[10-14] Conversely, prompt recovery of CD4Tregs can prevent GVHD while also supporting recovery of a broad T-cell repertoire.[12,15] These results suggest that balanced recovery of CD4Tregs, conventional CD4 T cells (CD4Tcons), and CD8 T cells is needed to control alloimmunity and establish immune tolerance. However, the mechanisms that maintain this balance and regulate the recovery of each T-cell population in vivo are not fully understood.[16,17]

In healthy individuals, the T-cell compartment is maintained at a relatively constant number and functional state by homeostatic mechanisms that regulate the generation, expansion, and survival of each T-cell population.[18,19] Following HSCT, the recovery of peripheral T cells is a dynamic process that also relies on homeostatic signals to restore each T-cell population to normal steady-state levels. As donor T cells engraft, antigen-specific responses also contribute to T-cell recovery after transplant. In patients who receive T-replete stem cell grafts with conditioning regimens that do not include antithymocyte globulin, mature donor T cells in the stem cell product contribute to the early phase of T-cell recovery after transplant.[20,21] Subsequently, T cells derived from donor hematopoietic stem cells and lymphoid progenitors also contribute to T-cell reconstitution.[22] When exposed to lymphopenic conditions and antigen stimulation, naive T cells proliferate and acquire phenotypic and functional features of memory

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T cells.[23,24] The homeostatic controls that regulate each T-cell population are distinct, and this may result in an unbalanced recovery of the total T-cell pool.[20,25,26] Finally, prophylactic administration of immune-suppressive agents to prevent GVHD affects the ability of T cells to respond to homeostatic signals as well as specific antigens and also profoundly affects immune reconstitution.

To examine reconstitution of CD4Tregs, CD4Tcons, and CD8 T cells, we prospectively monitored immune recovery in a cohort of 107 adult patients who underwent allogeneic HSCT. Within each major T-cell population, we identified subsets that expressed differentiation markers of naive, central memory (CM), effector memory (EM), and terminal EM (TEMRA) T cells.[27-29] To define homeostatic characteristics of each subset, we characterized cells for expression of functional markers of recent thymic emigration, cell proliferation, and survival. Taken together, our prospective analysis of T-cell reconstitution identified several factors that contribute to delayed recovery of CD4Tregs relative to other T-cell subsets. This imbalance appears to support the development of chronic GVHD after allogeneic HSCT.[16,17]

Methods

Patients and sample collection

This study included 107 adult patients who underwent allogeneic HSCT at the Dana-Farber Cancer Institute and Brigham and Women's Hospital (Boston, MA) between June 2010 and August 2012 (Table 1). All patients received reduced-intensity conditioning with fludarabine (120 mg/m^2) plus IV low-dose busulfan (3.2 mg/kg in 66 patients; 6.4 mg/kg in 41 patients) followed by transplantation of unmodified stem cell grafts. No patients received antithymocyte globulin for GVHD prophylaxis or low-dose interleukin-2 (IL-2) for treatment of chronic GVHD. Fresh blood samples were obtained at 8 different time points (1, 2, 3, 6, 9, 12, 18, 24 months) after transplant. Samples obtained after relapse were not included for analysis. Samples were also obtained from 15 healthy individuals (13 males and 2 females) with a median age of 54 years (range, 20-69 years). Written informed consent was obtained from patients and healthy donors prior to sample collection, in accordance with the Declaration of Helsinki. Protocol approval was obtained from the Human Subjects Protection Committee of the Dana-Farber/Harvard Cancer Center.

Flow cytometry

A panel of directly conjugated monoclonal antibodies (Mabs) (supplemental Table 1, available on the *Blood* Web site) was used to define functionally distinct T-cell subsets and homeostatic characteristics of each subset. Three major T-cell populations, CD4Treg, CD4Tcon, and CD8 T cells, were defined as CD3$^+$CD4$^+$Foxp3$^+$, CD3$^+$CD4$^+$Foxp3$^-$, and CD3$^+$CD4$^-$CD8$^+$, respectively. Within each population, subsets were defined as follows: naive T cells (CD45RA$^+$CD62L$^+$), CM (CD45RA$^-$CD62L$^+$), EM (CD45RA$^-$CD62L$^-$), and CD8 TEMRA T cells (CD8$^+$CD45RA$^+$CD62L$^-$). Within the CD4 T-cell population, coexpression of CD45RA and CD31 was used to define recent thymic emigrants (RTEs).[30,31] Proliferation was measured by expression of Ki67.[32] Susceptibility to apoptosis was assessed by intensity of surface membrane expression of CD95 (Fas) and intracellular expression of BCL2.[9,33] Prior to analysis, peripheral blood mononuclear cells were purified by density gradient centrifugation. peripheral blood mononuclear cells were incubated with fluorochrome-conjugated Mabs directed against cell surface antigens for 20 minutes at room temperature. Thereafter, surface-stained cells were fixed for 30 minutes, permeabilized with FOXP3 Fix/Perm buffer (eBioscience), and stained with Mabs directed against intracellular antigens for 30 minutes at 4°C. Cells were acquired in a FACSCanto II flow cytometer (BD) and analyzed using FlowJo software (Tree Star). An example of the gating/analysis strategy for simultaneous assessment of T-cell populations and homeostatic markers is shown in supplemental Figure 1.

Table 1. Patient characteristics

Patient characteristics	N	%
Total	107	
Age, median (range), y	62 (19, 73)	
Patient-donor sex		
M-M	47	43.9
M-F	18	16.8
F-M	23	21.5
F-F	19	17.8
Diagnosis		
AML	37	34.6
MDS	24	22.4
NHL	12	11.2
MPD	7	6.5
CLL/SLL/PLL	6	5.6
ALL	5	4.7
Mixed MDS/MPD	4	3.7
CML	2	1.9
Hodgkin disease	3	2.8
MM/PCD	4	3.7
Anemia/red cell disorder	2	1.9
Immunodeficiency	1	0.9
Disease risk index		
Low	17	15.9
Intermediate	46	43
High	37	34.6
Donor HLA type		
Matched unrelated	66	61.7
Matched related	30	28
Mismatch unrelated	11	10.3
Stem cell source		
PBSCs	99	92.5
Bone marrow	7	6.5
Bone marrow and PBSCs	1	0.9
Acute GVHD prophylaxis		
Tac-Sir-MTX	74	69.2
Tac-Sir	24	22.4
Tac-MTX	8	7.5
Sir-MMF-MTX	1	0.9
Acute GVHD grade		
0	63	58.9
I	12	11.2
II	16	15
III	15	14
IV	1	0.9
Chronic GVHD		
None	61	57
Extensive	42	39.3
Limited	4	3.7

ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; CMV, cytomegalovirus; F, female; HLA, human leukocyte antigen; M, male; MDS, myelodysplastic syndrome; MM, multiple myeloma; MMF, mycophenolate mofetil; MPD, myeloproliferative disease; MTX, methotrexate; NHL, non-Hodgkin lymphoma; PBSC, peripheral blood stem cell; PCD, plasma cell dyscrasias; PLL, prolymphocytic leukemia; Sir, sirolimus/rapamycin; SLL, small lymphocytic lymphoma; Tac, tacrolimus.

Statistical analysis

Patient baseline characteristics and immunophenotype data were analyzed primarily descriptively. Immunophenotype data were compared using the Wilcoxon rank-sum test for unpaired group comparison and the Wilcoxon signed-rank test for paired comparison. Correlation studies were assessed using the nonparametric Spearman test. All tests are 2-sided at the significance level of 0.05 and multiple comparisons are not considered. All statistical analyses were performed using SAS version 9.2 (SAS Institute) and R version 3.1.3 (the CRAN project). All graphs were made using Prism software (GraphPad). Heatmaps were generated using GENE-E (http://www.broadinstitute.org/ cancer/software/GENE-E).

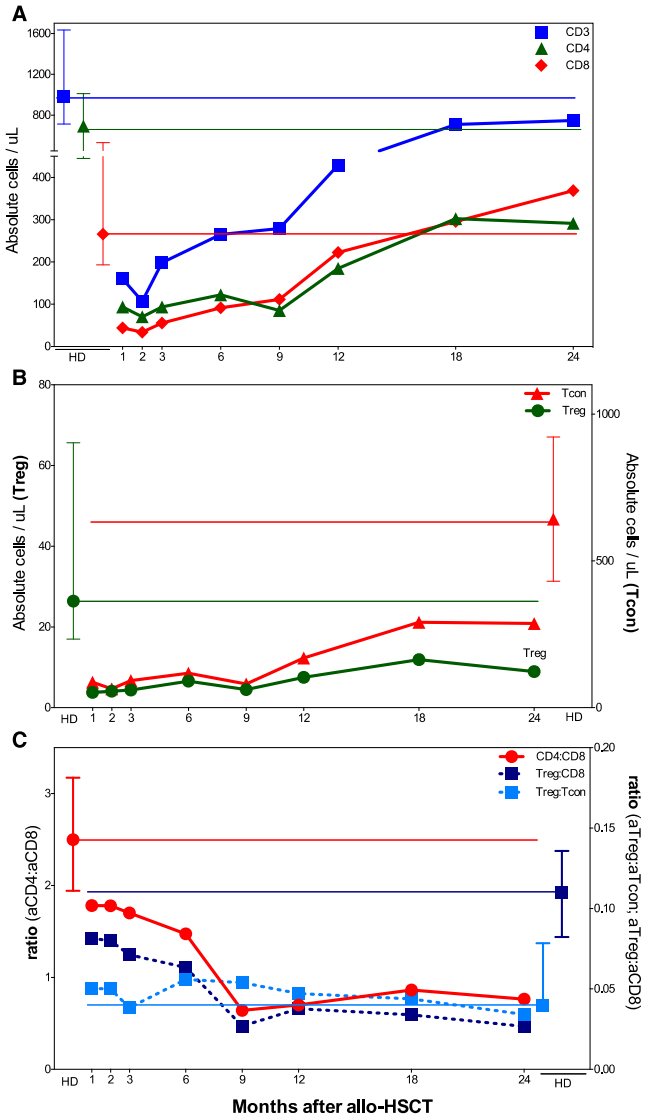

Figure 1. Reconstitution of major T-cell populations after allogeneic HSCT. The recovery of each T-cell population in peripheral blood was assessed prospectively by flow cytometry. The median cell counts per microliter for each population are represented at each time point, as well as the corresponding median value in healthy donors (HD), with the interquartile range (IQ) range (whisker bars). (A) Median $CD3^+$ (blue), $CD4^+$ (green), and $CD8^+$ (red) T-cell counts. (B) Median CD4Treg (green) and CD4Tcon (red) counts. (C) Median CD4:CD8, Treg:CD8, and Treg:Tcon ratios. To compare the patterns of CD4Tregs and CD4Tcons in the same graph, different scales were used in panels B and C. aCD4, absolute CD4; aCD8, absolute CD8; aTcon, absolute Tcon; aTreg, absolute Treg.

Results

Patient characteristics

Clinical characteristics of the patients included in the study are summarized in Table 1. The median age was 62 years (range, 19-73 years), 97% had hematologic malignancies (and most were classified with intermediate [43%] or high [34.6%] disease risk), 89.7% underwent transplantation from HLA-matched donors, and 92.5% received filgrastim-mobilized peripheral blood stem cells. In 92.5% of patients, GVHD prophylaxis included sirolimus combined with a calcineurin inhibitor, with or without methotrexate and mycophenolate mofetil. Following transplant, 80% of patients achieved complete donor chimerism in peripheral blood by day +30, 25 patients (23.3%)

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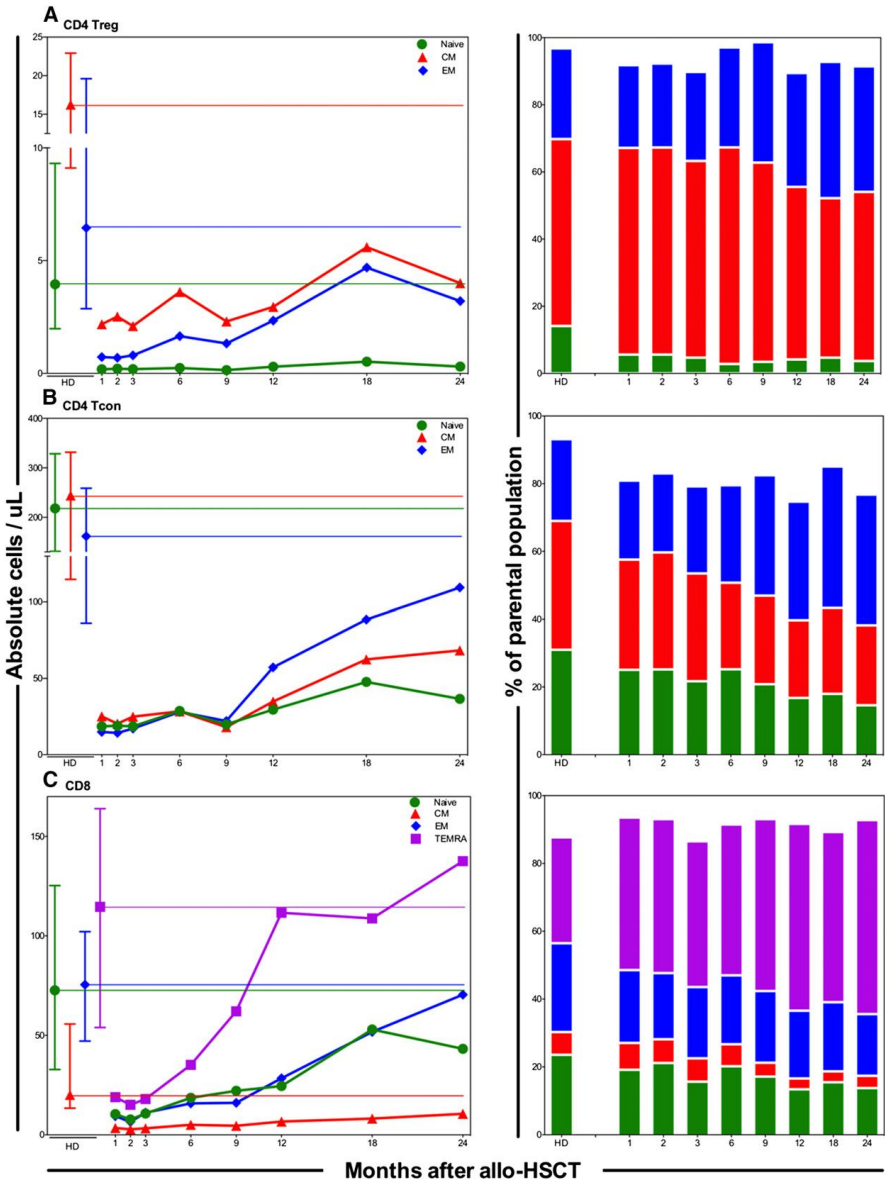

Figure 2. Reconstitution of naive and memory T-cell compartments. Absolute numbers (cells per microliter) of each subset in peripheral blood are shown on the left and relative percentages at each time point are shown on the right. Graphs on the left show median cell counts per microliter for each population and the corresponding median in HDs, with the IQ range (whisker bars). Bar graphs on the right show the median percentage of each population and the corresponding median in HDs. (A) CD4Treg subsets: naive (green), CM (red), EM (blue). (B) CD4Tcon subsets: naive (green), CM (red), EM (blue). (C) CD8 T-cell subsets: naive (green), CM (red), EM (blue), TEMRA (purple).

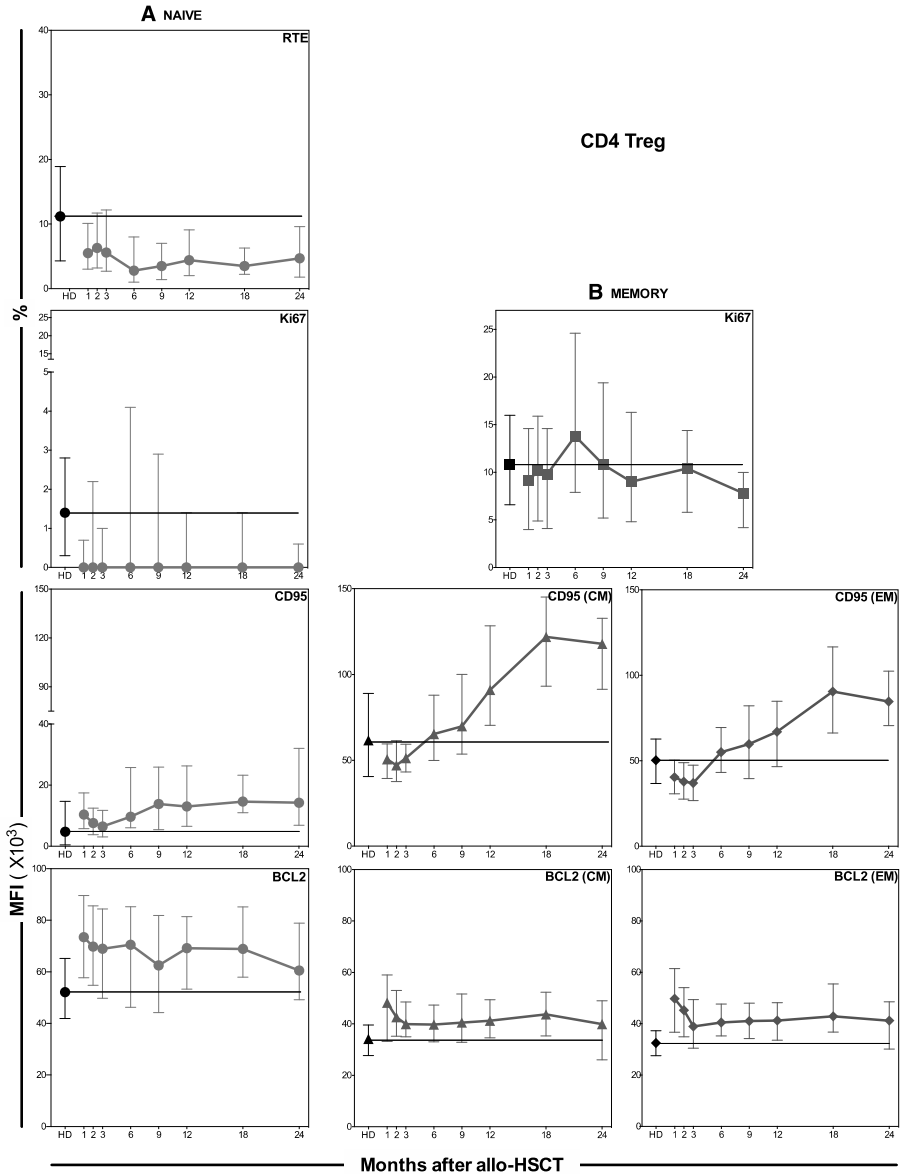

Figure 3. Dynamic profile of CD4Treg subsets. Phenotypic markers of thymic production, proliferation, and susceptibility to apoptosis were measured at different time points over a 2-year period. Results are shown for (A) naive and (B) memory subsets of CD4Tregs. Individual graphs show the percentage of RTEs, the percentage of Ki67 (proliferation), and the MFIs of CD95 and BCL2. Median values and the IQ range (whisker bars) are shown for each parameter. Results in HD are shown in black. MFI, mean fluorescence intensity.

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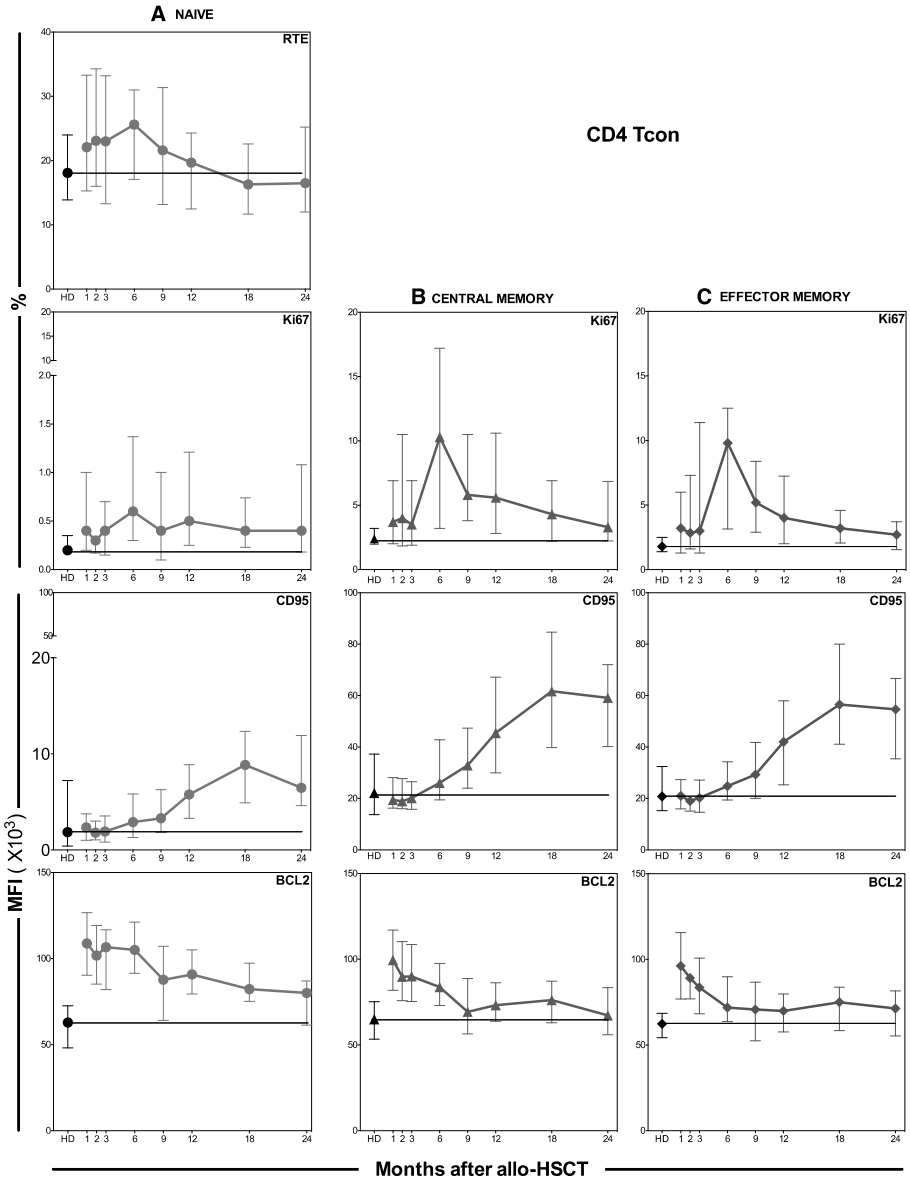

Figure 4. Dynamic profile of CD4Tcon subsets. Phenotypic markers of thymic production, proliferation, and susceptibility to apoptosis were measured at different time points over a 2-year period. Results are shown for (A) naive, (B) memory, and (C) EM subsets of CD4Tcons. Individual graphs show the percentage of RTEs, the percentage of Ki67 (proliferation), and the MFIs of CD95 and BCL2. Median values and the IQ range (whisker bars) are shown for each parameter. Results in HD are shown in black.

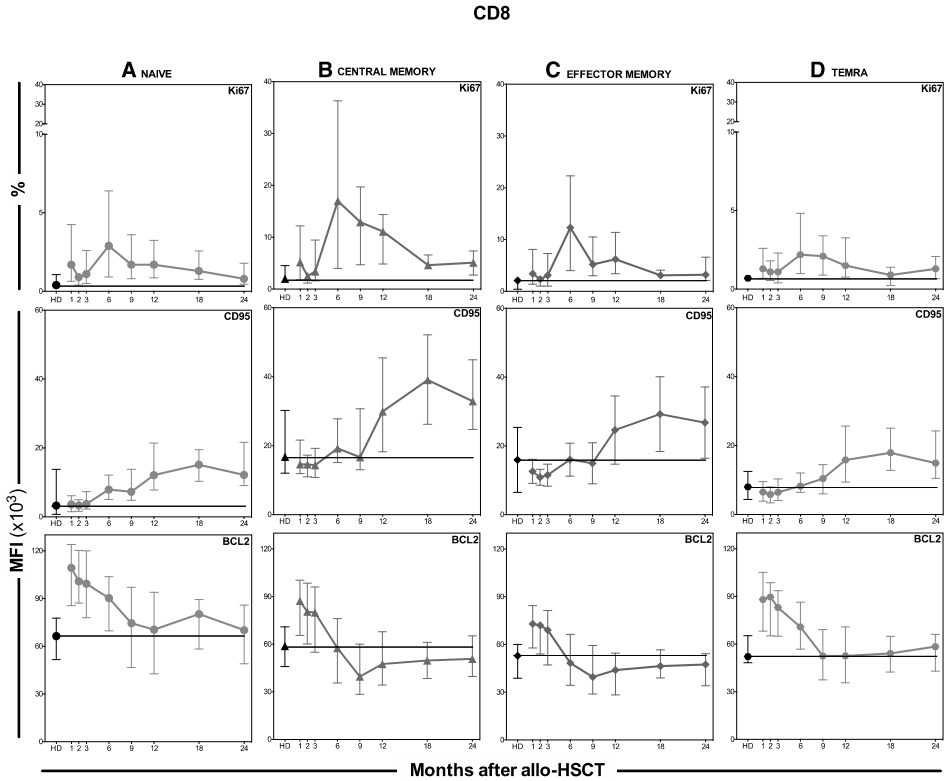

Figure 5. Dynamic profile of CD8 T-cell subsets. Phenotypic markers of proliferation and susceptibility to apoptosis were measured at different time points over a 2-year period. Results are shown for (A) naive, (B) CM, (C) EM, and (D) TEMRA subsets. Individual graphs show the percentage of Ki67 (proliferation) and the MFIs of CD95 and BCL2. Median values and the IQ range (whisker bars) are shown for each parameter. Results in HD are shown in black.

developed grade II-IV acute GVHD, 46 patients (42.9%) developed chronic GVHD, and 10 patients (9.3%) developed both acute and chronic GVHD. Relapse within the 2-year period of follow-up occurred in 46 patients (42.9%).

Kinetics of T-cell reconstitution after HSCT

Recovery of the major T-cell populations is summarized in Figure 1. CD3 T cells gradually increased and approached the normal range 18 months after transplant (Figure 1A). Delayed T-cell recovery was primarily due to slow CD4 T-cell reconstitution. CD8 T cells reached normal levels 12 months after transplant but CD4 T cells remained below normal throughout the 2-year follow-up period ($P < .001$). Within the CD4 T-cell population, both CD4Tregs and CD4Tcons increased gradually, but neither subset reached normal levels ($P < .001$) 2 years after HSCT (Figure 1B). Despite the more rapid recovery of CD8 T cells, CD4 T-cell numbers were higher than CD8 T-cell counts for the first 6 months after HSCT. By 9 months, CD8 T-cell counts were higher than CD4 T-cell counts resulting in an inverse CD4:CD8 ratio (Figure 1C). Due to the similar pace of reconstitution of CD4Tregs and CD4Tcons, the Treg:Tcon ratio remained relatively stable and within normal range throughout the follow-up period. In contrast, CD4:CD8 and Treg:CD8 ratios decreased in the first 9 months and remained below normal ($P < .001$) throughout the follow-up period (Figure 1C).

Within CD4Treg, CD4Tcon, and CD8 T-cell populations, we determined the fraction of cells that had a naive, CM, or EM phenotype (Figure 2). Within CD8 T cells, we also defined a TEMRA subset. Within each T-cell population, memory cells represented the predominant subsets. This was evident at early as well as late time points and the relative distribution at all time points was similar to what was found in healthy adults (Figure 2, right panels). More detailed analysis of CD4Treg reconstitution (Figure 2A) revealed a gradual increase in both CM and EM fractions with very little recovery of naive CD4Tregs during the entire follow-up period. In healthy donors, naive cells represent a relatively small fraction (median: 14.1%) of circulating CD4Tregs. Nevertheless, the naive Treg fraction was significantly decreased throughout the posttransplant period ($P < .001$) compared with healthy donors. In the first 6 months, CM CD4Tregs comprised the major CD4Treg subset. Beginning 9 months after HSCT, there was a gradual increase in the EM CD4Treg subset (Figure 2A, right panel). Analysis of CD4Tcon recovery showed no increase in the numbers of

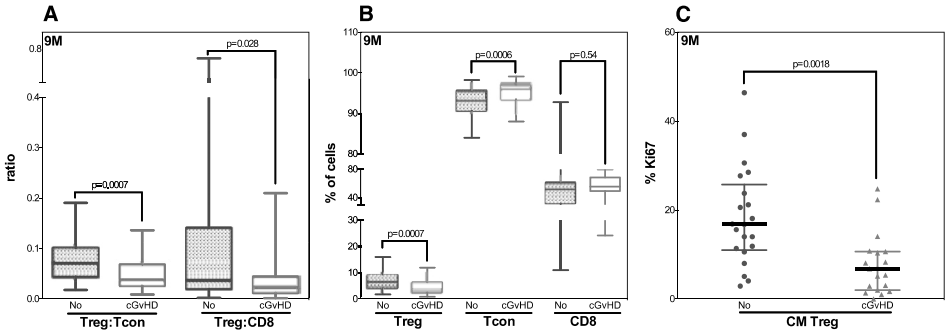

Figure 6. Reconstitution of CD4Tregs, CD4Tcons, and CD8 T cells according to cGVHD status 9 months after transplant. (A) CD4Treg:CD4Tcon and CD4Treg:CD8 ratios. (B) The percentages of CD4Tregs, CD4Tcons, and CD8 T cells. (C) Proliferation (%Ki67) in CM CD4Tregs. Each figure compares patients with (red) and without (blue) cGVHD. Box plots in panels A and B depict the 75th percentile; median and 25th percentile values and whiskers represent maximum and minimum values. In panel C, dots depict the number of patients (n = 20 for each group) and the whiskers correspond to the 75th and 25th percentile. cGVHD, chronic GVHD.

circulating cells in the first 9 months after HSCT (Figure 2B). Beginning 12 months after HSCT, there was a gradual increase in all CD4Tcon subsets but the greatest increase occurred within the EM CD4Tcon subset. Notably, the frequency of naive CD4Tcons was relatively normal early post-HSCT and gradually decreased as the EM CD4Tcon subset increased 9 to 24 months post-HSCT (Figure 2B, right panel). Analysis of CD8 T-cell recovery showed that all subsets began to increase in number 6 months post-HSCT (Figure 2C). Rapid CD8 recovery occurred primarily as a result of increasing numbers of CD8 TEMRA that achieved normal levels 9 months post-HSCT and comprised >50% of all CD8 T cells. Naive and EM CD8 subsets also showed substantial recovery. In contrast to CD4 T cells, the CM subset represented a small fraction of CD8 T cells and this subset showed very little recovery (Figure 2C, right panel).

Thymic-dependent T-cell reconstitution

In CD4 T cells, RTEs can be identified within the naive T-cell subset by coexpression of CD31 and CD45RA (RTE: Figures 3-4).[30,31] In healthy donors, the frequency of RTE is greater within CD4Tcons (median = 18%) than within CD4Tregs (median = 11%; $P = .001$). In the first 3 months after transplant, the percentage of RTE CD4Tregs was within the lower limits of normal but subsequently decreased below normal from 6 to 24 months post-HSCT (Figure 3A, RTE). In contrast, the RTE CD4Tcon percentage was within the high normal range 1 to 6 months post-HSCT and remained at normal levels as CD4Tcon recovery continued (Figure 4A, RTE). Taken together, these results indicate that thymic production of CD4 T cells strongly favors CD4Tcons over CD4Tregs for at least 2 years after transplant.

Reconstitution of CD4Tregs

T-cell recovery relies on proliferation of mature T cells as well as generation of naive cells from lymphoid progenitor cells. Persistence of differentiated cells is also dependent on their susceptibility to apoptosis. In our study, these factors were evaluated by measuring expression of Ki67 (a marker of proliferation), cell surface CD95 (a marker of extrinsic pathway apoptosis), and intracellular BCL2 (a marker of intrinsic pathway apoptosis).[34] Concurrent analysis of each major T-cell population revealed important similarities as well as differences unique to each major T-cell population. Within the CD4Treg population, naive CD4Tregs exhibited very low levels of proliferation with no evidence of recovery over a 2-year period (Figure 3A). CD95 expression in naive CD4Tregs was relatively normal in the first 6 months post-HSCT and then remained stable at levels above normal for the next 18 months. BCL2 expression in naive CD4Tregs was above normal for the entire 2-year period. Although proliferation of naive CD4Tregs was very low, proliferation of memory CD4Tregs was consistently normal both early and late post-HSCT (Figure 3B). This is remarkable because steady-state proliferation of memory CD4Tregs in healthy donors is normally 9- to 10-fold higher than naive Tregs. In the first 2 to 3 months post-HSCT, memory CD4Tregs expressed relatively low levels of CD95 and relatively high levels of BCL2 compared with healthy donors. Subsequently, expression of CD95 gradually increased to very high levels 12 to 24 months post-HSCT. BCL2 expression also remained high 6 to 24 months post-HSCT in memory CD4Tregs. Expression of CD95 and BCL2 was very similar in CM and EM CD4Treg subsets.

Reconstitution of CD4Tcons

Within the CD4Tcon population, assessment of Ki67, CD95, and BCL2 expression revealed several changes during post-HSCT recovery (Figure 4). Proliferation of naive, CM, and EM subsets was consistently at or above normal at all time points (Figure 4, Ki67 panels). Within the CM and EM subsets, proliferation was markedly increased at 6 to 12 months post-HSCT. Expression of CD95 was relatively normal in all CD4Tcon subsets 1 to 9 months post-HSCT but then increased to very high levels 12 to 24 months post-HSCT (Figure 4, CD95 panels). Expression of BCL2 was elevated in all CD4Tcon subsets in the first 6 months post-HSCT (Figure 4, BCL2 panels). BCL2 levels gradually decreased to just above normal levels in CM and EM CD4Tcon subsets but remained elevated in naive CD4Tcons.

Reconstitution of CD8 T cells

Although recovery of CD8 T cells was more rapid than CD4Tcons, patterns of proliferation and expression of CD95 and BCL2 were remarkably similar in these 2 populations as peripheral counts gradually recovered after HSCT (Figure 5). Proliferation of all CD8 subsets remained normal or above normal throughout the 2-year study period (Figure 5, Ki67 panels). High levels of proliferation were observed at 6 to 12 months post-HSCT in CD8 CM and EM subsets. Expression of CD95 was relatively normal in all subsets 1 to 9 months post-HSCT

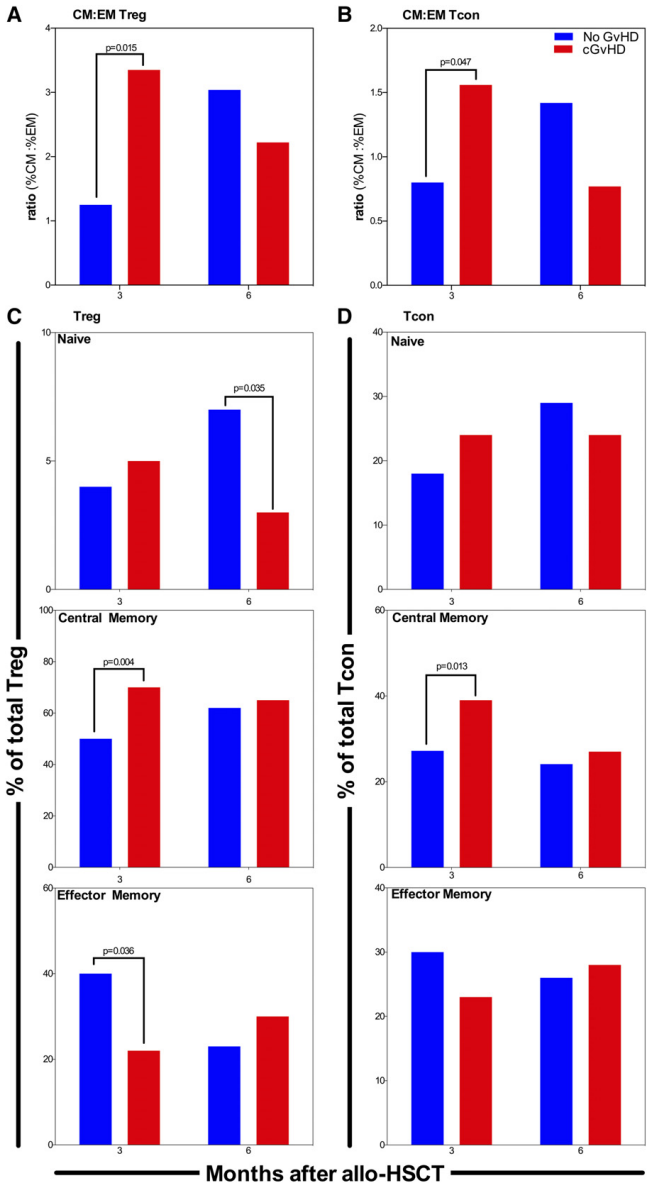

Figure 7. Differentiation characteristics of CD4Tregs, CD4Tcons, and CD8 T cells associated with cGVHD. Percentages of naive, CM, and EM subsets were determined at 3 and 6 months after transplant. Results were compared in patients who developed (red) or did not develop (blue) cGVHD. (A) CM:EM ratio in CD4Tregs. (B) CM:EM ratio in CD4Tcons. (C) CD4Tregs: Median percentage of the naive, CM, and EM. (D) CD4Tcons: Median percentage of the naive, CM, and EM. (E) Frequency profile of the entire T-cell compartment at 3 months after transplant. Data are presented as a heatmap using hierarchical clustering after standardization of each row. Top bar indicates subsequent cGVHD outcome (green, cGVHD; yellow, no cGVHD). Blue, white, and red represent low, intermediate, and high subset frequency.

and subsequently gradually increased in all subsets as CD8 counts recovered (Figure 5, CD95 panels). BCL2 expression was relatively high in all subsets in the first 3 to 6 months post-HSCT and then gradually returned to normal levels 9 to 24 months post-HSCT (Figure 5, BCL2 panels).

Aberrant T-cell dynamics in chronic GVHD

In our cohort, 46 patients (43%) developed chronic GVHD and 61 patients (57%) did not develop chronic GVHD during the 2-year follow-up period. Immune reconstitution of total CD4Treg, CD4Tcon

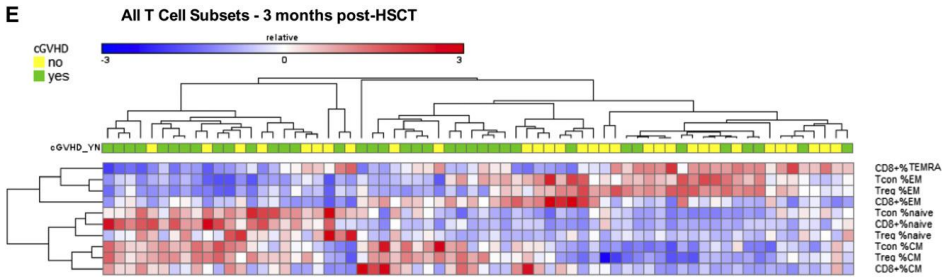

Figure 7. (Continued).

and CD8 T cells was similar between groups during the first 6 months post-HSCT (data not shown). Forty patients were analyzed 9 months post-HSCT. By this time, 20 had developed chronic GVHD and 20 remained free of any clinical manifestations of chronic GVHD. As shown in Figure 6A, the ratio of both Tregs:Tcons and Tregs:CD8 was significantly greater in patients who had not developed chronic GVHD compared with patients who had developed chronic GVHD ($P = .0007$ and $P = .028$, respectively). The significant difference in Tregs:Tcons and Tregs:CD8 primarily reflects a significantly lower percentage of CD4Tregs and a higher percentage of CD4Tcons in patients with chronic GVHD ($P = .0007$ and $P = .0006$, respectively) (Figure 6B). The percentage of CD8 was not different between patients with and without chronic GVHD ($P = .54$). Central memory CD4Tregs represent the predominant Treg subset at this time and proliferation of this CD4Treg subset was significantly lower in patients with chronic GVHD ($P = .0018$) (Figure 6C).

As naive T cells encounter antigen and differentiate into memory T cells, CM cells remain relatively immature whereas EM cells acquire functional characteristics of increased tissue homing and increased cytolytic capacity. Examination of the ratio of CM:EM at 3 and 6 months post-HSCT revealed changes during this period that were significantly different in patients who developed or did not develop chronic GVHD. As shown in Figure 7A-B, the CM:EM ratio was significantly higher 3 months post-HSCT in patients who develop chronic GVHD. This shift was most evident in CD4Tregs but was also present in CD4Tcons. As shown in Figure 7C-D, the switch in predominance between CM and EM subsets is driven primarily by the increase in CM cells in patients who develop chronic GVHD. The fraction of CM cells is significantly increased in both CD4Tregs and CD4Tcons at 3 months post-HSCT in patients who subsequently develop chronic GVHD but there is little change in the CM fraction at 6 months post-HSCT and the percentage of CM is similar in patients with and without chronic GVHD at this time point. Also evident 6 months post-HSCT is the selective increase in naive CD4Tregs in patients who do not develop chronic GVHD.

Differences in CD4Treg and CD4Tcon subsets noted 3 months after HSCT led us to undertake a more detailed analysis of all T-cell subsets at this early time point comparing patients who would subsequently develop chronic GVHD with those that remained free of chronic GVHD. The unsupervised hierarchical clustering of the percentage of naive, CM, and EM T cells is shown in the Figure 7E heatmap. Patient clusters on the right are characterized by increased fractions of more differentiated subsets in all 3 major T-cell populations represented by CD8 TEMRA as well as EM within CD4Treg, CD4Tcon, and CD8 T-cell populations. In contrast, patient clusters on the left are characterized by increased frequency of less differentiated subsets in all 3 major T-cell populations represented by naive and CM subsets within CD4Treg, CD4Tcon, and CD8 T-cell populations. Patients who subsequently develop chronic GVHD predominate within the latter group of patients that have high percentages of naive and CM T cells and low percentages of TEMRA and EM T cells 3 months after HSCT. In contrast, patients who do not develop chronic GVHD are characterized by having high percentages of differentiated T-cell subsets and low percentages of naive and CM subsets 3 months after HSCT. Further analysis of 3-month data excluding patients who developed acute GVHD did not change these findings.

Discussion

Full reconstitution of T-cell immunity after allogeneic HSCT requires the balanced recovery of both effector and regulatory T-cell populations. To coordinate recovery of CD4Tregs, CD4Tcons, and CD8 T cells, we monitored immune reconstitution in serial blood samples from a large cohort of 107 adult patients who received T-replete stem cell products; results were compared with 15 healthy donors. In addition to measuring absolute recovery of each major T-cell population, we evaluated reconstitution of naive and memory subsets within each population. In the first year after transplant, T-cell recovery is primarily driven by homeostatic proliferation in response to persistent lymphopenia. However, antigen-specific responses as well as generation of new T cells from donor hematopoietic precursor cells also play important roles. To evaluate these various factors that influence immune recovery, we concurrently identified RTEs and the fraction of proliferating cells within each subset. Although T cells can persist for prolonged periods, activated proliferating cells also become more susceptible to apoptosis. As surrogate markers for susceptibility to apoptosis, we quantified the intracellular expression of BCL2 and surface expression of CD95 (Fas) within each subset.[9,26,34] These markers do not provide a complete picture of cellular dynamics because they cannot measure critically important transitions from one phenotype to the next as T cells mature. Nevertheless, this comprehensive phenotypic analysis allowed us to simultaneously evaluate the response of both regulatory and effector T cells to both homeostatic and antigen-specific signals in vivo during a 2-year period of immune reconstitution after allogeneic HSCT and identify significant differences that contribute to the subsequent development of chronic GVHD.

Consistent with previous reports, CD3 T cells did not reach normal levels until 1 to 1.5 years after transplant. Within the T-cell compartment, CD8 T cells recovered more rapidly, and neither CD4Tregs nor CD4Tcons achieved normal levels during the 2-year observation period. Within CD4Tregs and CD4Tcons, naive cells

were the slowest to recover. As a result, reconstituting CD4Tregs were predominantly CM cells and CD4Tcons were predominantly EM cells. Within the CD8 T-cell population, recovery was primarily due to expansion of terminal EM cells but naive CD8 T cells also recovered to normal levels 1.5 years after transplant.

To examine production of new T cells from lymphoid progenitor cells, we monitored $CD4^+CD45RA^+CD31^+$ RTEs. As RTEs proliferate, CD31 expression is lost and these cells become indistinguishable from other naive CD4 T cells.[31,35] In healthy adults, limited availability of IL-2 in the thymic microenvironment favors production of CD4Tcons.[36,37] Early after transplant, CD4Tcon RTEs are maintained within the high normal range but very few CD4Treg RTEs are produced for at least 2 years. CD8 RTEs do not express a unique phenotype, but the frequency of naive CD8 T cells is relatively normal after transplant. Thus, thymic production of CD4Tregs is selectively compromised for prolonged periods whereas maturation of new T cells is markedly skewed to maintain production of CD4Tcons and CD8 T cells at relatively normal frequencies.

Following transplant, mature T cells undergo homeostatic proliferation to expand the number of cells available for immune surveillance. In our cohort, proliferation, measured by expression of Ki67, of both naive and memory CD4Tcons and CD8 T cells was maintained at relatively normal levels in the first 3 months after transplant. Subsequently, increased proliferation of memory cells was noted 6 months posttransplant within both CD4Tcons and CD8 T cells. Proliferation of other CD4Tcon and CD8 T-cell subsets was also normal or above normal but the increased proliferative thrust at 6 months was not observed in these subsets. In contrast, proliferation of memory CD4Tregs is only maintained at normal levels and proliferation of newly generated naive CD4Tregs is markedly reduced. In fact, proliferation of naive CD4Tregs is not detectable in the majority of patients.

Within each T-cell population, proliferation of mature T cells is balanced by apoptosis of activated cells. In our cohort, expression of BCL2 was relatively high in the first 3 to 6 months after transplant and then returned to normal levels in all CD4Tcons and CD8 T cells. In contrast, CD95 expression was normal in the first 6 to 9 months and subsequently increased 12 to 24 months after transplant. Within CD4Tregs, BCL2 expression was only increased in the first 2 months after transplant in CM and EM subsets. BCL2 expression is increased for a longer period in naive CD4Tregs but this represents a very small fraction of the CD4Treg population. CD95 expression is relatively normal in CD4Tregs in the first 6 to 9 months posttransplant and subsequently increases to high levels 12 to 24 months after transplant. When compared with other T cells, lower expression of BCL2 early after transplant and higher expression of CD95 at later time points suggests that CD4Tregs are more susceptible to apoptosis and therefore likely have shorter survival in vivo.

Having identified differences in T-cell recovery that favored expansion of CD4Tcons and CD8 T cells over CD4Tregs, we examined whether this dynamic imbalance was associated with the development of chronic GVHD. In the first 6 months post-HSCT, recovery of CD4Tregs, CD4Tcons, and CD8 T cells is similar in patients who subsequently developed or did not develop chronic GVHD. However, at 9 months posttransplant, some patients have already developed chronic GVHD and the ratio of CD4Tregs: CD4Tcons and CD4Tregs:CD8 T cells was significantly lower in patients with chronic GVHD. The lower CD4Treg:CD4Tcon ratio reflects both lower CD4Tregs and greater CD4Tcons, but the lower CD4Treg:CD8 ratio is entirely due to a lower percentage of CD4Tregs. A major driver of this imbalance appears to be the significantly lower proliferation of CM CD4Tregs in patients who develop chronic GVHD. Patients who develop chronic GVHD begin immune-suppressive therapy and some of these changes may therefore reflect effects of this therapy. Nevertheless, a variety of dynamic factors support the generation, expansion, and survival of T effector cells over CD4Tregs and contribute to the inability to establish immune tolerance after allogeneic transplant.

As we monitored T-cell reconstitution, we also observed that altered CD4Treg and CD4Tcon differentiation associated with development of chronic GVHD could be observed as early as 3 months following transplant. At this early time point, the great majority of T cells are CD4Tcons but all T-cell populations, including CD4Tregs, CD4Tcons, and CD8 T cells, were skewed toward predominance of naive and CM subsets in patients who subsequently developed chronic GVHD. This skewing was less evident 6 months after transplant and began to reverse in CD4Treg and CD4Tcon populations as patients begin to develop chronic GVHD (data not shown). Further studies will be needed to fully understand the immunologic causes of these shifts in T-cell differentiation after transplant. Previous studies in murine models have shown that T cells capable of promoting GVHD are primarily contained within the naive T-cell compartment.[38] Moreover, a recent clinical trial demonstrated that depletion of naive T cells from the stem cell product had little effect on the incidence of acute GVHD but appeared to markedly reduce the incidence of chronic GVHD.[39] In part, early recovery of naive T cells in our cohort reflects increased thymic production of CD4Tcons and CD8 T cells. Homeostatic proliferation and increased survival also plays a role in the expansion of naive T cells in the early posttransplant period. In this setting, robust expansion of relatively immature T cells in the early posttransplant period appears to expand the pool of potential alloreactive T cells and increase the risk of subsequent development of chronic GVHD. These naive cells do not immediately respond to alloantigens and do not increase the risk of acute GVHD, perhaps because of concurrent immune-suppressive therapy. Nevertheless, increased early expansion of relatively immature T cells appears to set the stage for subsequent development of chronic GVHD when immune-suppressive medications are reduced and unbalanced recovery of CD4Tregs is not sufficient to promote immune tolerance. Sequential tracking of specific T-cell populations is now possible through T-cell receptor sequencing and further studies using this approach may help elucidate the role of early expansion of naive T-cell populations in the subsequent development of GVHD.[40]

In summary, prospective monitoring of T-cell reconstitution revealed several homeostatic imbalances that appear to contribute to the development of chronic GVHD. In this setting, interventions that selectively promote the generation, expansion, or persistence of CD4Tregs may improve the balanced recovery of effector and regulatory T-cell populations and promote immune tolerance after transplant.[41,42] Various methods to selectively enhance CD4Tregs after transplant, including adoptive CD4Treg infusions and administration of low-dose IL-2, are already being evaluated in clinical trials.[43-47] The clinical effectiveness of these interventions may depend on their long-term ability to alter the homeostatic balance of regulatory and effector T cells after transplant.

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Authorship

Contribution: A.C.A., J.F.L., and J.R. conceived and designed the study; R.J.S., S.N., C.S.C., J.K., V.T.H., P.A., J.H.A., and E.P.A. provided patients; M.J.C., C.G.R., T.R.M., J.W., E.F., and A.C.A. collected and assembled data; A.C.A. and J.R. analyzed and interpreted data; H.T.K. analyzed and interpreted data and performed statistical analysis; and all authors wrote the manuscript.

Conflict-of-interest disclosure: The author declares no competing financial interests.

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Unbalanced recovery of regulatory and effector T cells after allogeneic stem cell transplantation contributes to chronic GVHD

Ana C. Alho, Haesook T. Kim, Marie J. Chammas, Carol G. Reynolds, Tiago R. Matos, Edouard Forcade, Jennifer Whangbo, Sarah Nikiforow, Corey S. Cutler, John Koreth, Vincent T. Ho, Philippe Armand, Joseph H. Antin, Edwin P. Alyea, Joao F. Lacerda, Robert J. Soiffer and Jerome Ritz

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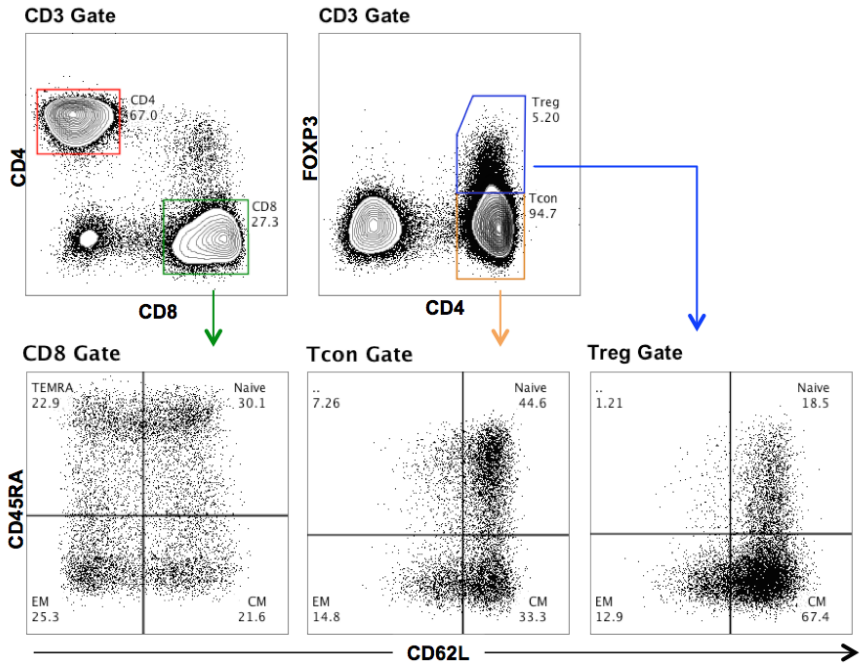
Figure 1. Gating strategy for analysis of T cell subsets



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Naive and Stem Cell Memory T Cell Subset Recovery Reveals Opposing Reconstitution Patterns in CD4 and CD8 T Cells in Chronic Graft vs. Host Disease

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The success of allogeneic hematopoietic stem cell transplantation (allo-HSCT) in the treatment of hematological malignancies remains hampered by life-threatening chronic graft vs. host disease (cGVHD). Although multifactorial in nature, cGVHD has been associated with imbalances between effector and regulatory T cells (Treg). To further elucidate this issue, we performed a prospective analysis of patients undergoing unrelated donor allo-HSCT after a reduced intensity conditioning (RIC) regimen containing anti-thymocyte globulin (ATG) and the same GVHD prophylaxis, at a single institution. We studied T cell subset homeostasis over a 24-month follow-up after HSCT in a comparative analysis of patients with and without cGVHD. We also quantified naive and memory T cell subsets, proliferation and expression of the apoptosis-related proteins Bcl-2 and CD95. Finally, we assessed thymic function by T cell receptor excision circle (TREC) quantification and T cell receptor (TCR) diversity by TCRVβ spectratyping. While the total number of conventional CD4 (Tcon) and CD8 T cells was similar between patient groups, Treg were decreased in cGVHD patients. Interestingly, we also observed divergent patterns of Naive and Stem Cell Memory (SCM) subset recovery in Treg and Tcon compared to CD8. Patients with cGVHD showed impaired recovery of Naive and SCM Tcon and Treg, but significantly increased frequencies and absolute numbers of Naive and SCM were observed in the CD8 pool. Markedly increased EMRA CD8 T cells were also noted in cGVHD. Taken together, these results suggest that Naive, SCM and EMRA CD8 play a role in the emergence of cGHVD. Reduced Naive and recent thymic emigrant Tcon and Treg in cGVHD was likely due to impaired thymic output, as it was accompanied by decreased CD4 TREC and TCR diversity. On the other hand,

CD8 TCR diversity was similar between patient groups. Furthermore, no correlation was observed between CD8 TREC content and Naive CD8 numbers, suggesting limited thymic production of Naive CD8 T cells in patients after transplant, especially in those developing cGVHD. The mechanisms behind the opposing patterns of CD4 and CD8 subset cell recovery in cGVHD remain elusive, but may be linked to thymic damage associated with the conditioning regimen and/or acute GVHD.

Keywords: chronic graft vs. host disease, hematopoietic stem cell transplantation, T lymphocyte, stem cell memory, Naive T cell, immune reconstitution

INTRODUCTION

Despite the recent advances with patient-tailored therapies, allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been increasingly used in the USA and in Europe for the treatment of hematological malignancies (1, 2). However, this technique is not without risks and is frequently accompanied by serious complications such as graft vs. host disease (GVHD) and infections, which are major causes of morbidity and mortality post-transplant (3). GVHD results from the recognition of patient tissues by donor-derived effector cells. Acute GVHD (aGVHD) typically occurs early after transplant, has markedly inflammatory manifestations and is thought to be primarily mediated by mature T lymphocytes infused with the graft (4). On the other hand, chronic GVHD (cGVHD) usually occurs later after transplant and resembles an auto-immune disease (5), affecting specific target organs, primarily the eyes, mouth, gastrointestinal tract, liver, skin, lungs, musculoskeletal, and genitourinary systems (6). Chronic GVHD has a complex pathophysiology. Thymic damage resulting both from the conditioning regimen and acute GVHD likely has an impact on normal T cell development (7, 8), and self-reactive T cells are believed to play a pivotal role in the development of cGVHD. Moreover, there appears to be a deficit in the regulatory T cell pool, contributing to the loss of immunologic tolerance post-transplant. A pivotal role for autoreactive B cells and the production of self-reactive antibodies has also been clearly associated to cGVHD pathogenesis, whereby self-reactive B lymphocytes are activated due to increased levels of BAFF (9, 10). In the end, excessive macrophage activation leads to fibroblast proliferation and collagen deposition, which is a hallmark of cGVHD (11).

The imbalances in regulatory (Treg) and effector T cells (12) appear to be central to cGVHD pathogenesis. This has led to clinical trials investigating the effect of low-dose rhIL-2 in patients with cGVHD in order to induce Treg expansion *in vivo* (13, 14). Also with the aim of increasing the Treg pool, we and others are conducting clinical trials of donor Treg infusion in patients with moderate and severe cGVHD (www.tregeneration.eu).

The involvement of donor T cells in the pathophysiology of GVHD led to the development of *ex vivo* (T cell-depleted grafts) and *in vivo* (anti-thymocyte globulin; ATG) T cell depletion approaches that significantly reduce GVHD incidence (5). ATG also delays immune reconstitution post-transplant through the depletion and/or function modification of T, B and NK cells (15). However, ATG does not completely abrogate the emergence of cGVHD (16–18), which attests to the multifactorial nature of this condition. On the other hand, thymic ablation has been shown to prevent cGVHD (8), suggesting a significant role for *de novo* thymic-derived T cells in this pathology.

In this study, we aimed at further investigating the biology of cGVHD and its effects on T cell homeostasis. Given the role that T cell immunity plays in cGVHD, we prospectively evaluated T cell reconstitution and thymic function in a homogenous patient population undergoing allo-HSCT after a reduced intensity conditioning (RIC) regimen containing ATG. We assessed the kinetics of T cell reconstitution after allo-HSCT and performed a comparative analysis of patients developing cGVHD vs. those who did not.

MATERIALS AND METHODS

Patients and Sample Collection

We prospectively monitored 57 patients undergoing allo-HSCT at Hospital de Santa Maria (Centro Hospitalar Universitário Lisboa Norte) from unrelated donors after a RIC regimen containing fludarabine 30 mg/m^2/day for 5 days (D-8 to D-4), melphalan 70 mg/m^2/day for 2 days (D-3 and D-2), and ATG (thymoglobulin) 4–6 mg/Kg (total dose) divided in 2–3 days, according to HLA compatibility. GVHD prophylaxis consisted of cyclosporine A (CsA) plus mycophenolate mofetil (MMF) in all patients. CsA and MMF were initiated on D-1 with CsA at 3 mg/kg/day intravenously (*iv*) twice daily and MMF at 2 g/day (*iv* or *per os*). CsA blood levels were monitored to target levels of 200 ng/mL. In the absence of GVHD, immune prophylaxis was tapered and discontinued between months 6 and 9 post-transplant.

Our center has acquired a significant experience with the administration of thymoglobulin in unrelated donor allo-HSCT over the last decade and maintained the same protocol in the present patient cohort.

Abbreviations: aGVHD, acute GVHD; ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; cGVHD, chronic GVHD; CMV, cytomegalovirus; F, female; G, grade; GVHD, Graft versus Host Disease; HL, Hodgkin lymphoma; HLA, human leukocyte antigen; M, male; MDS, myelodysplastic syndrome; MF, Myelofibrosis; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; PBSC, peripheral blood stem cell.

Seventeen patients were excluded due to early disease relapse or death from either infection or aGVHD in the first 9 months post-transplant. Chronic GVHD diagnosis and staging were performed according to the 2014 NIH criteria (19). Patient and donor characteristics are shown in **Table 1**. Peripheral blood was collected on months 1, 2, 3, 6, 9, 12, 18, and 24 after HSCT, and from five healthy controls (HC). Up to 2 weeks of variance in sample collection was allowed for patient sample collection.

Written informed consent was obtained before sample collection in accordance with the Declaration of Helsinki. This study obtained approval from the Ethics Committee of Lisbon Academic Medical Center (ref. 459/13).

TABLE 1 | Patient/donor characteristics.

	cGVHD	No cGVHD
Patients	18	22
Gender	6 F; 12 M	12 F; 10 M
Age	51 (30–67)	46 (19–69)
DIAGNOSIS		
AML	7	10
ALL	0	4
CLL	1	0
CML	2	2
HL	1	0
NHL	4	3
MDS	2	0
MF	1	0
MM	0	3
CMV Positive	14	18
DONORS		
Gender	11 F; 7 M	9 F; 13 M
Age	35 (21–49)	32 (20–57)
Female Donor to Male Patient	6	4
CMV Pos	12	15
HLA MATCHING		
10/10	8	9
9/10	9 (3A, 1B, 4C, 1DQB1)	12 (8A, 1B, 2C, 1DQB1)
8/10	1 (DRB1+DQB1)	1 (A+DQB1)
SOURCE OF GRAFT		
BM	5	11
PBSC	13	11
aGVHD	17/18	9/22
GI	5	2
GII	6	4
GIII	6	3
GIV	–	–
cGVHD	18	–
Mild	7	–
Intermediate	4	–
Severe	7	–
Day of cGVHD Onset	230 (149–455)	–
Follow Up (days)	682 (286–1,051)	483 (90–840)

Flow Cytometry

Flow cytometry analysis was performed on fresh whole blood, at pre-determined fixed time-points as indicated above, using the Human Regulatory T Cell Whole Blood Staining Kit (eBioscience), according to the manufacturer's instructions and immediately acquired on a LSR Fortessa (BD Biosciences).

The following monoclonal antibodies were used: Bcl-2 (Bcl-2/100) and CD31 (WM-59) FITC; Ki-67 (20Raj1) and CD95 (DX2) PE; CD3 (OKT3) PerCPCY5.5, CD45RA (HI100) and CD25 (M-A251) PE-CY7; CD4 (RPAT4) APC; CD127 (eBioRDR5) and CD62L (DREG-56) APCeFlour780 and FoxP3 (PCH101) e450 (all from eBioscience, except for CD25 PE-CY7, Becton Dickinson). Daily flow cytometer quality control monitoring was performed using Cytometry Setup and Tracking Beads (Becton Dickinson). Eight peak calibration Rainbow Beads (Becton Dickinson) were used to ensure stable fluorescence measurements throughout the study. Data was analyzed without prior knowledge of cGVHD status, using FlowJo9®. For quantification of Ki-67, Bcl-2, and CD95 within T cell subsets a cutoff of 50 events was used as a valid data point for statistical analysis. Five healthy control samples were stained in the same conditions.

CD4 and CD8 T Cell Receptor (TCR) Repertoire Analysis

TCR Diversity was evaluated with a TCRβgene (TRB) complementarity-determining region 3 (CDR3) spectratyping assay, performed on patient samples from either early (month 3) or late (month 9 or 12) time-points post-HSCT, as previously described (20). Cryopreserved PBMC from a separate set of 8 healthy controls, 10 patients with, and 10 without cGVHD were thawed, stained for CD3, CD4 and CD8, and FACSorted (BD FACSAria III) into CD4 and CD8 T cells. Total RNA isolated from each cell fraction (AllPrep, Qiagen) was used for first-strand cDNA synthesis primed with an equivolume mixture of random hexamers and oligo (dT) (Invitrogen Superscript III). TCRβ transcripts were amplified with TRBV family specific primers and a common TRCB reverse primer (21, 22). A run-off reaction with a second TRCB FAM-labeled primer was used to extend these products. Each fluorescent TRBV-TRBC fragment was separated using capillary electrophoresis-based DNA automated sequencer. Data were analyzed with GeneMapper (Thermo Fischer Scientific) for size, peak count, and fluorescent intensity determination. Profiles of transcript TRB repertoires were classified based on peak count, distribution shape and relative fluorescence intensity (RFI) of each peak (% RFI = 100 × clonal peak area/total peak area) (23).

Signal-Joint TCR Excision Circle (sjTREC) Quantification in CD4 and CD8 T Cells

sjTREC sequences were analyzed with a multiplex qPCR assay (24) in CD4 and CD8 T cells using the same samples purified for the diversity studies. sjTREC sequence copy numbers were extrapolated from standard curves obtained by 10-fold serial dilutions of a triple-insert plasmid containing sjTREC and TRAC fragments in a 1:1:1 ratio (kind gift from L. Imberti, Spedali Civili of Brescia, Italy). Detected sjTREC copies were genome

normalized with the mean quantity of T cell receptor α-chain sequences. Results were expressed as sjTREC copies per 10^6 CD4 or CD8 T lymphocytes.

Statistical Analysis

The patients' clinical data shown in **Table 1** was compared using Fisher's exact test and the Mann–Whitney test when comparing continuous data. Data obtained for patients with and without cGVHD were compared at each time-point using the Mann-Whitney test on GraphPad Prism®. To analyze slopes of reconstitution early (up to 6 months) and late (from 6 months onwards) post-HSCT, we used linear mixed effects (LME) models over those two periods. In this approach, the data of all patients over time is analyzed together, with patient as the random effect, while time and cGVHD (yes/no) are covariates. In each case, we tested also for an interaction term of cGVHD with time (i.e., different slopes of reconstitution). Previous aGVHD, gender, and source of graft were further included as covariates. In all cases, statistical significance was assessed at $\alpha = 0.05$.

RESULTS

Patient Characteristics

All patients underwent allo-HSCT from an unrelated donor and received the same conditioning and GVHD prophylaxis regimens, as described in the methods section. Patient and donor characteristics are summarized in **Table 1**. The most frequent underlying disease within patients included in the analysis was AML (42.5%) and 60% of transplants used PBSC as the source of graft. After a 24-month follow-up post-HSCT, 18 patients developed cGVHD while 22 did not (No cGVHD). Median cGVHD onset day was 230. Patients without cGVHD had a shorter follow-up compared to cGVHD patients. Seventeen out of 18 cGVHD patients had previously developed aGVHD, in contrast to 9 out of 22 in the No cGVHD group. No significant differences were identified when comparing the patient characteristics between groups (**Table 1**), with the exception of the higher incidence of aGVHD in patients with cGVHD ($p = 0.0006$). Five healthy controls (HC), with a median age of 43 (range 36–45), were also studied.

Distinct Treg, Tcon, and CD8 Reconstitution Patterns After HSCT

Treg numbers were low in both patient groups up to month 6 after HSCT (**Figure 1A**). From months 9 to 18, Treg were decreased in cGVHD vs. No cGVHD patients. Analysis of *ex vivo* proliferation using intracellular Ki-67 staining revealed significantly decreased proliferation from months 3 to 18 in patients developing cGVHD as compared to No cGVHD, suggesting that reduced Treg numbers in cGVHD may be partly due to reduced homeostatic proliferation (**Figure 1B**).

Lower Treg numbers in cGVHD were not associated with increased susceptibility to apoptosis as assessed by Bcl-2 and CD95 expression levels, as these proteins were expressed at similar levels in both patient groups (**Figures 1C,D**). These data suggest that apoptosis mediated by these pathways did not play a major role in the low Treg numbers observed in cGVHD.

We further quantified conventional CD4 T (Tcon; Foxp3$^-$ CD4) and CD8 T cell numbers and found no major differences between patients with and without cGVHD (**Figure 1A**). We did observe significant reductions in Tcon and CD8 proliferation, as assessed by Ki-67 staining, in cGVHD compared to No cGVHD (**Figure 1B**). On the other hand, no clear trend for altered Bcl-2 or CD95 expression in cGVHD was observed (**Figures 1C,D**).

Impaired Naive and SCM Treg Reconstitution in cGVHD

Quantification of Naive/memory Treg subsets was performed by flow cytometry as described in the methods using CD45RA, CD62L, and CD95 surface markers. We identified Central Memory (CM) cells as CD45RA$^-$ CD62L$^+$, Effector Memory (EM) as CD45RA$^-$ CD62L$^-$ and CD45RA-expressing Terminal effectors (EMRA) as CD45RA$^+$ CD62L$^-$. We further used CD95 to distinguish Naive, CD45RA$^+$ CD62L$^+$CD95$^-$, from the Stem Cell Memory (SCM) subset, CD45RA$^+$ CD62L$^+$CD95$^+$, as previously described (25) (detailed gating strategy illustrated in **Supplementary Figure 1A**). The percentages of each subset within Treg from patients with and without cGVHD is summarized in **Figure 2A**. A detailed visualization of the overall data showing percentage and absolute counts for each subset in all time-points for both patient groups and healthy controls, as well as the corresponding significances, is displayed in **Supplementary Figure 1B**.

The most prevalent subset within Treg was CM, followed by EM, naïve, and SCM, while EMRA were an extremely rare subset (**Figure 2A**). We observed decreased Naive Treg percentages and absolute counts in cGVHD as compared to No cGVHD throughout the follow-up (**Figures 2A,C**). The only exception was at Month 1 when Naive Treg frequency and absolute counts were increased in cGVHD vs. No cGVHD patients (**Figures 2A,C**). Despite this, Naive Treg remained low in patients developing cGVHD while they increased in the absence of cGVHD. This trend, reflecting an inability of Naive Treg to recover in patients developing cGVHD, was confirmed in a LME analysis of the first 6 months post-HSCT. This analysis showed significantly different slopes estimated for the recovery of Naive percentages, whereby Naive Treg percentages only increased in the absence of cGVHD (**Figure 2B**). From month 9 onwards, Naive Treg percentages and counts were lower in patients developing cGVHD as compared to patients that remained free from cGVHD (**Figures 2A,C**).

Similar to Naive Treg, the recovery of the SCM Treg subset was impaired in patients developing cGVHD. This was the case for percentages and for absolute counts, which were significantly reduced in cGVHD from month 9 to 18 (**Figures 2A,D**). The relative proportion of the remaining memory subsets was largely unaltered by cGVHD (**Figure 2A**). CM and EM Treg absolute counts were either similar or reduced in cGVHD, whereas EMRA Treg were similar or increased, but no sustained statistical significances were noted (refer to **Supplementary Figures 1B,C** for details).

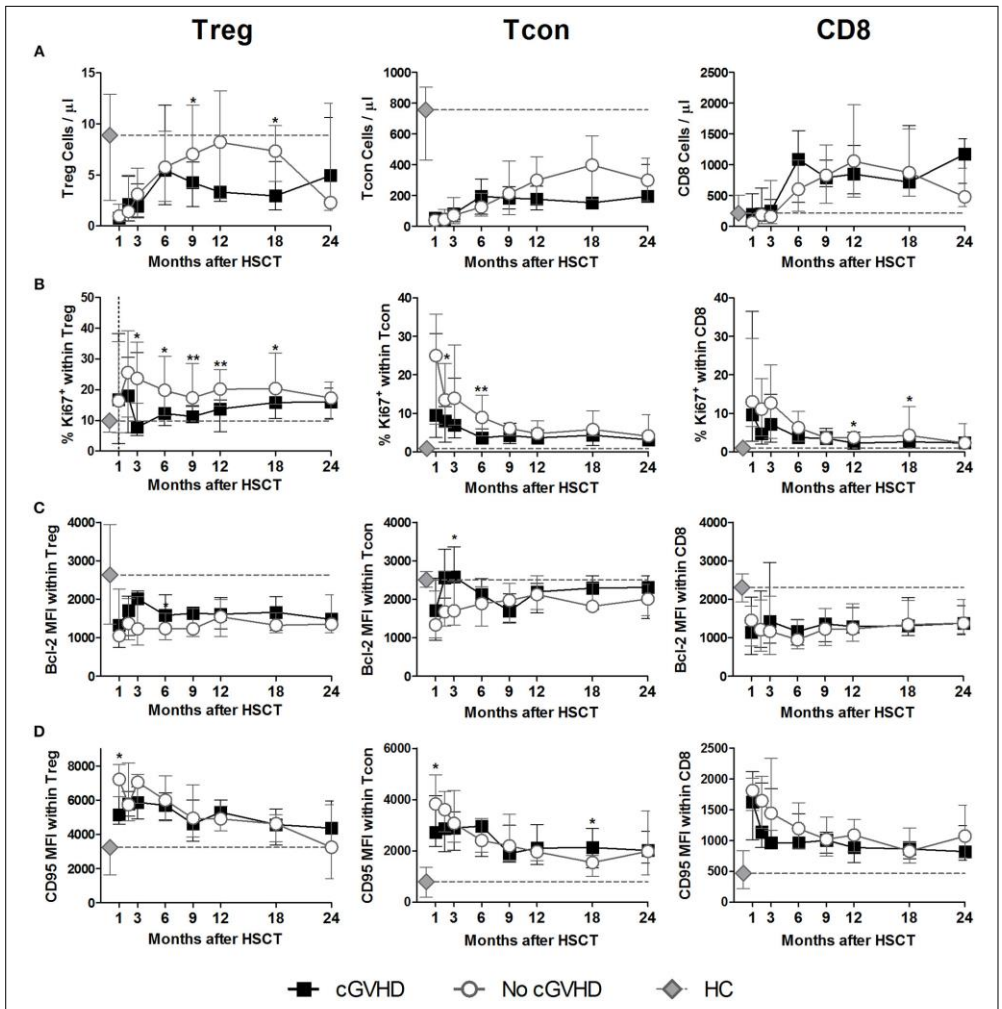

FIGURE 1 | Treg, Tcon and CD8 Homeostasis following HSCT. Patients were divided into No cGVHD (gray lines and open circles) and cGVHD (black lines and squares). Healthy controls (HC) are also shown (gray diamonds and dotted gray lines). **(A)** Absolute counts per microliter for Treg ($CD3^+$ $CD4^+$ $CD25^{bright}$ $Foxp3^+$ $CD127^{low}$), Tcon ($CD3^+$ $CD4^+$ $Foxp3^-$), and CD8 ($CD3^+$ $CD4^-$) T cells. **(B)** Frequency of Ki-67^+ cells within $CD3^+$ $CD4^+$ $Foxp3^+$ Treg, Tcon, and CD8 T cells. Median fluorescence intensity (MFI) for Bcl-2 **(C)** and CD95 **(D)** within $CD3^+CD4^+$ $Foxp3^+$ Treg, Tcon and CD8 T cells. Symbols represent median values and whiskers represent interquartile range (IQR). Asterisks denote statistically significant differences between patient groups (*$p = 0.01$ to 0.05; **$p = 0.001$ to 0.01).

In summary, cGVHD was associated with impaired Treg recovery, particularly of the Naive and SCM Treg subsets, and lower Treg proliferation as compared to the No cGVHD group.

Impaired Naive and SCM Tcon Reconstitution in cGVHD

Chronic GVHD was associated with a marked impairment in Naive Tcon recovery as compared to patients who did not develop

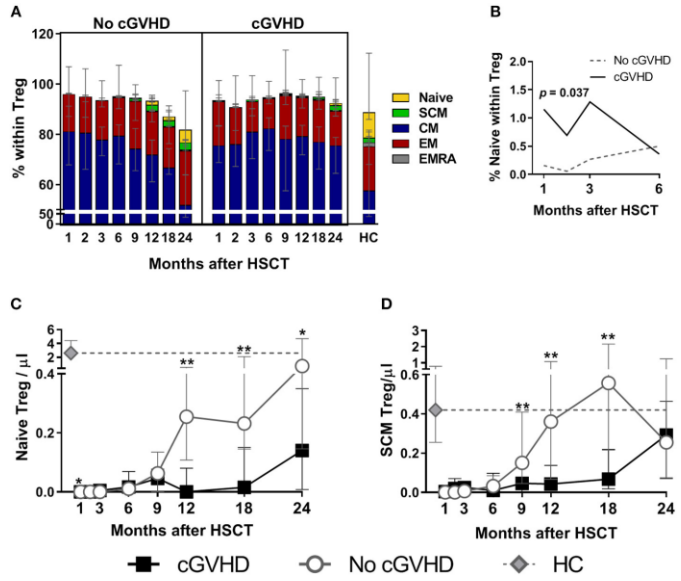

FIGURE 2 | Treg subset reconstitution in cGVHD. Treg were identified as CD4$^+$ Foxp3$^+$ within a CD3$^+$ lymphocyte gate. Naive and memory subsets were identified as shown in **Supplementary Figure 1. (A)** The median percentage for each subset within Treg is shown for patients with and without cGVHD, as well as for HC. **(B)** Naive Treg percentages in the first 6 months after HSCT in patients with cGVHD (black line) and No cGVHD (dotted gray line), illustrating the results of the LME analysis for this subset. Absolute counts of Naive **(C)** and SCM **(D)** Treg in patients without cGVHD (gray lines and open circles), with cGVHD (black lines and squares) and HC (gray diamonds and dotted gray lines). Symbols represent median values and whiskers represent IQR. Asterisks denote statistically significant differences between patient groups (*p = 0.01 to 0.05; **p = 0.001 to 0.01).

cGVHD. This became apparent from month 9 onwards in terms of both frequency and absolute counts (**Figures 3A,C**). Although differences between patient groups only reached significance after month 9, the LME analysis revealed that the percentage of Naive Tcon up to month 6 was better represented by a model with significantly different slopes for cGVHD and No cGVHD (p = 0.008) (**Figure 3B**). Hence, during the first 6 months after HSCT, Naive Tcon percentages increased in patients who did not subsequently develop cGVHD, while they remained stable in patients who develop cGVHD. This translated, at later time-points, into significant differences in Naive Tcon frequencies and numbers between patient groups from months 12 to 24 (**Figures 3A,C**; **Supplementary Figures 1B,C**). Impaired Naive Tcon recovery in cGVHD was mimicked by decreased SCM Tcon levels, reaching significance at month 18 for SCM Tcon counts (**Figures 3A,D**).

CM and EM were the most abundant Tcon subsets post-HSCT and were largely similar between patient groups, while EMRA showed a non-significant tendency to be increased in cGVHD (**Figure 3A**). Percentages and absolute counts for all Tcon subsets are detailed in **Supplementary Figures 1B,C**.

In summary, we did not observe significant disparities in whole Tcon recovery between patients developing cGVHD and those who did not. However, the composition of the Tcon pool after HSCT reveals an impairment in Naive and SCM subsets in patients who develop cGVHD, similar to what was observed in Treg.

Increased CD8 Naive, SCM, and EMRA in cGVHD

The reconstitution of Naive and memory subsets within the CD8 T cell pool differed greatly between patient groups (**Figure 4**) and had a strikingly different pattern to that observed in Tcon and Treg. This was particularly prominent not only for the least abundant Naive and SCM CD8 subsets, but also for the more abundant, terminally differentiated EMRA subset.

Interestingly, we observed significantly increased Naive CD8 percentages and counts in cGVHD vs. No cGVHD during the initial post-transplant period (**Figures 4A,C**). This statistical significance disappeared from month 12 onwards, as Naive CD8 increased in patients who did not develop cGVHD. Such accelerated Naive CD8 T cell recovery in the absence of cGVHD at later time-points was also revealed by the LME analysis of months 9 to 24 post-HSCT. This analysis showed a significant difference in the slopes for Naive CD8 percentages between the

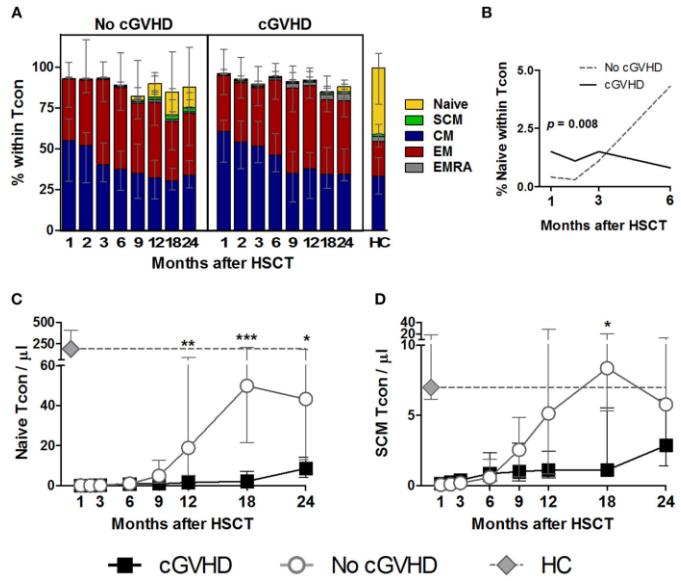

FIGURE 3 | Naive and memory Tcon reconstitution in cGVHD. Tcon were identified as $CD4^+$ $Foxp3^-$ within a $CD3^+$ lymphocyte gate. Naive and memory subsets were identified as shown in **Supplementary Figure 1. (A)** The percentage of each subset within Tcon cells is shown for patients with and without cGVHD, as well as HC. **(B)** Percentage of Naive within Tcon in the first 6 months after HSCT in patients with cGVHD (black line) and No cGVHD (dotted gray line), illustrating the results of the LME analysis for this subset. Naive **(C)** and SCM **(D)** Tcon absolute counts in patients without cGVHD (gray line and circles), patients with cGVHD (black line and squares) and HC (gray diamonds and dotted gray lines). Median values and IQR are shown. Asterisks denote statistically significant differences between patient groups (*$p = 0.01$ to 0.05; **$p = 0.001$ to 0.01; ***$p = 0.0001$ to 0.001).

two patient groups (**Figure 4B**). While in patients with cGVHD the percentage of Naive CD8 stabilized, in the absence of cGVHD the frequency of these cells increased over time.

Interestingly, the SCM CD8 subset was also increased in patients developing cGVHD vs. No cGVHD, starting from the early time points of the follow-up period, both in percentages and absolute counts (**Figures 4A,D**).

Despite such prominent differences in Naive and SCM subsets, these were always the least abundant populations. Within the most frequent populations the EMRA showed the most striking differences between patient groups. Hence, terminally differentiated EMRA CD8 cells were increased in percentage and counts from the very early post-transplant period in patients developing cGVHD (**Figures 4A,E**), while the remaining memory subsets, CM and EM, showed an opposite pattern to that observed in Naive, SCM and EMRA, with significant reductions in percentages being noted in patients developing cGVHD (**Figure 4A**; **Supplementary Figures 1B,C**).

In summary, CD8 T cell reconstitution in cGVHD was associated with persistently increased Naive, SCM and EMRA subsets. This became apparent very early after HSCT, suggesting a possible involvement of these subsets in disease development.

The Effects of Acute GVHD on SCM Tcon and CD8

In our patient cohort, all but one of the patients developing cGVHD had a history of previous aGVHD, while within the group of 22 patients who did not develop cGVHD, 9 had developed acute GVHD (aGVHD). In order to clarify the contribution of aGVHD and the associated therapies to differences in T cell reconstitution between patient groups, we tested the effect of adding aGVHD as a covariate in the LME analysis. This analysis showed that during the first 6 months after HSCT, SCM Tcon and CD8 percentages and counts were significantly affected by aGVHD ($p < 0.01$). No impact of aGVHD was observed from month 6 onwards.

To complement this analysis, we stratified the No cGVHD group into acute only (Ac GVHD, $n = 13$) and no GVHD at all (No GVHD, $n = 9$). The only patient in our cohort who did not have aGVHD prior to cGVHD was excluded from this analysis. Hence, the chronic GVHD group consisted of patients with acute and chronic GVHD (Ac & Ch GVHD, $n = 17$). Month 24 was excluded from this analysis due to insufficient number of data points.

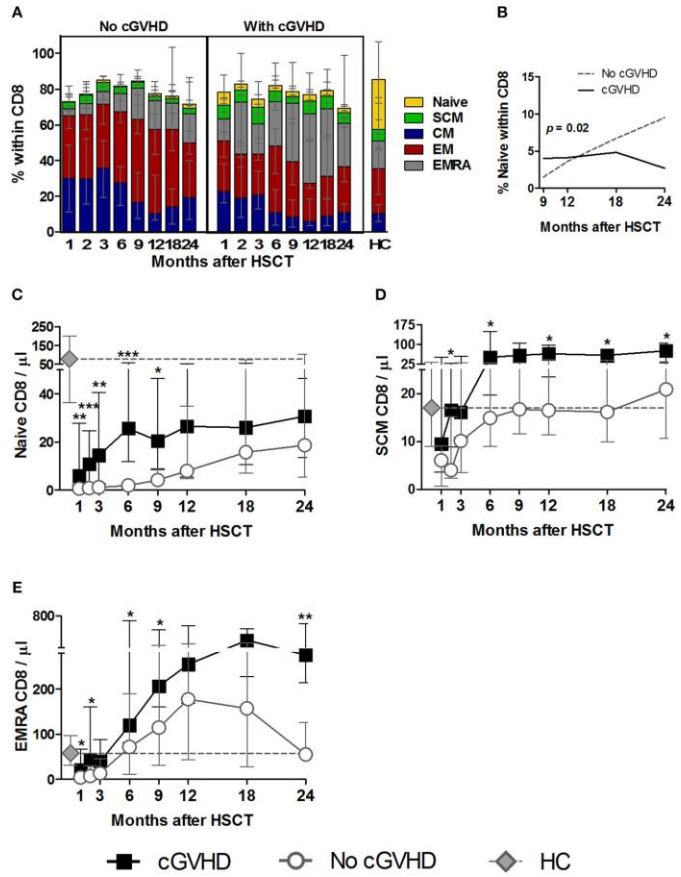

FIGURE 4 | Naive and memory CD8 reconstitution in cGVHD. CD8 T cells were identified as $CD3^+$ $CD4^-$ lymphocytes. Naive and memory subsets were identified as shown in **Supplementary Figure 1. (A)** Percentage of each subset within CD8 cells for patients with and without cGVHD, as well as HC. **(B)** Naive CD8 percentages from month 9 to 24 after HSCT in patients with cGVHD (black line) and no cGVHD (dotted gray line), illustrating the results of the LME analysis for this subset. Absolute counts of Naive **(C)**, SCM **(D),** and EMRA **(E)** CD8 cells in patients without cGVHD (gray circles and dotted lines), patients with cGVHD (black squares and lines) and HC (gray diamonds and dotted gray lines). Symbols represent median values and whiskers represent IQR. Asterisks denote statistically significant differences between patient groups (*$p = 0.01$ to 0.05; **$p = 0.001$ to 0.01; ***$p = 0.0001$ to 0.001).

Stem Cell Memory (SCM) Tcon numbers were significantly increased during the first 3 months in GVHD vs. No GVHD patients (**Figure 5A**). At later time-points, this pattern was reversed and while SCM Tcon reconstituted in the No GVHD and Ac GVHD groups, they became significantly reduced in the Ac & Ch GVHD patient group. A similar pattern was observed when analyzing SCM percentages within Tcon (data not shown). Overall, these data suggest that GVHD development is associated to early increases in SCM Tcon.

For CD8 T cells, initial time-points showed a similar reconstitution pattern to Tcon, whereby patients with GVHD showed significantly increased SCM as compared to No GVHD (**Figure 5B**). Interestingly, from month 6 onwards patients with aGVHD showed similar SCM CD8 counts as patients who did not develop any form of GVHD. On the other hand, patients who subsequently develop cGVHD appear to sustain a significant increase in this population throughout the follow-up period when compared to the other two groups of patients. A similar

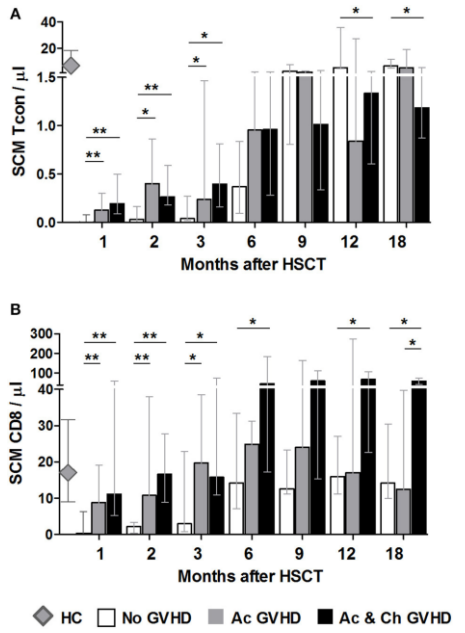

FIGURE 5 | The effect of acute GVHD on SCM Tcon and CD8 reconstitution. SCM absolute counts for Tcon **(A)** and CD8 **(B)** in patients without any form of GVHD (No GVHD, white bars), patients with acute GVHD only (Ac GVHD, gray bars), patients with acute and chronic GVHD (Ac & Ch GVHD, black bars) and healthy controls (HC, gray diamonds). Bar graphs shows median values and IQR. Asterisks denote statistically significant differences between patient groups (*$p = 0.01$ to 0.05; **$p = 0.001$ to 0.01).

pattern was observed when analyzing SCM percentages within CD8 (data not shown).

These data suggest that GVHD development is associated to early increase in the SCM subset after transplant. Furthermore, we observed a sustained increase in this CD8 subset in patients who subsequently develop cGVHD, further pointing to a possible role for these cells in disease development.

Chronic GVHD Is Associated With Reduced CD4 TCR Vβ Diversity

TCR repertoire diversity was analyzed in purified CD4 and CD8 T cells from patients at early (month 3) and late time-points (months 9 or 12) post-HSCT by TCRVB spectratyping (**Figure 6**).

Early post-HSCT, we observed no significant differences in TCR diversity between patient groups in either CD4 or CD8 cells (**Figures 6B,D**). There were few VB families displaying polyclonal distributions, while those with skewed, oligoclonal, and monoclonal distributions prevailed (**Figures 6A,C**). When early and late time-points were compared within each patient group, significant increases in TCR diversity were observed, suggesting *de novo* CD4 and CD8 T cell production during patient follow-up.

However, when patients developing cGVHD were compared to No cGVHD, a significant reduction in CD4 TCR diversity in cGVHD was observed at later time-points (**Figure 6B**). This was reflected in decreased prevalence of polyclonal Gaussian profiles and the appearance of numerous skewed profiles (**Figure 6A**), translating into decreased TCRVB peaks within CD4 T cells in cGVHD (**Figure 6B**).

Similarly to CD4, we observed an increase in TCR diversity from early to late time-points in CD8. However, the median number of CDR3 peak count within the VB families did not show statistically significant differences between patient groups (**Figure 6D**). This was likely the result of the observed high prevalence of skewed distributions in CD8 T cells from both patient groups (**Figure 6C**).

Chronic GVHD Negatively Impacts Thymic Function

We next analyzed sjTREC content as an estimate of thymic activity in purified CD4 and CD8 T cells at early (month 3) and late (months 9 or 12) time-points post-HSCT. Unfortunately, insufficient cell numbers precluded the analysis at month 3. sjTRECs were significantly reduced in CD4 (**Figure 7A**) and CD8 (**Figure 7C**) T cells in cGVHD vs. No cGVHD patients at late time-points.

Importantly, sjTREC content and Naive counts from all patients were positively correlated in CD4 (**Figure 7B**) but not in CD8 T cells (**Figure 7D**). Furthermore, Naive CD4 counts were positively correlated with sjTRECs in No cGVHD, but not in cGVHD (data not shown). In CD8 T cells, no significant correlation was observed between sjTREC content and Naive CD8 counts in either patient group (data not shown).

The presence of a significant correlation between CD4 TREC numbers and Naive CD4 counts at month 9 suggests that the Naive CD4 T cell reconstitution observed in patients who do not develop cGVHD is likely the result of *de novo* thymic production. On the other hand, the increase in Naive CD8 T cells observed in patients with cGVHD may depend more on T cell peripheral expansion.

Recent Thymic Emigrant (RTE) Tcon and Treg in GVHD

The expression of CD31 within Naive CD4 T cells has been reported to contain a population enriched in RTE (26). We therefore quantified this population in our patient cohort as an additional measure of thymic function. We found that patients developing cGVHD had significantly reduced RTE Treg (**Figure 7E**) and Tcon (**Figure 7F**) from month 9 onwards. Furthermore, LME analysis of the first 6 months revealed significantly different slopes for RTE Tcon absolute counts (**Figure 7H**) and percentages ($p = 0.0032$) (data not shown), whereby RTE Tcon increase in the absence of cGVHD with negligible recovery of this population in patients developing cGVHD.

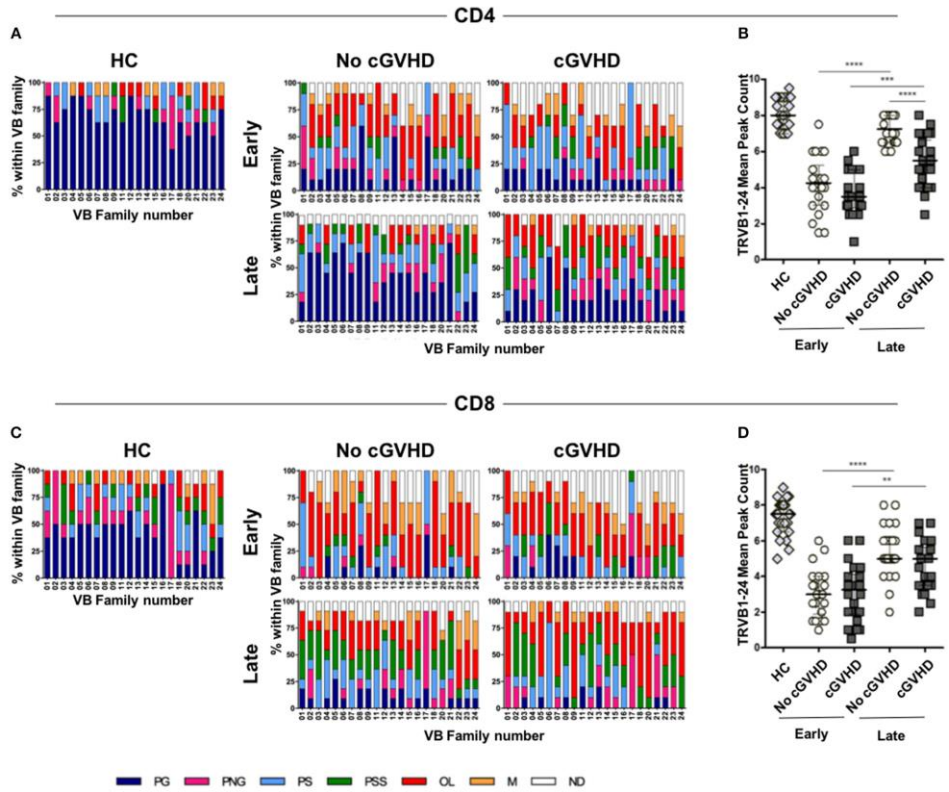

FIGURE 6 | TCR repertoire analysis in HSCT patients and controls. TCRVB diversity analysis was performed on purified CD4 and CD8 T cells from healthy controls (HC) and patients with (cGVHD) and without cGVHD (No cGVHD). Data shown represent values obtained from patient samples early (month 3) and late (months 9 or 12) after HSCT. Clonality analysis of TRB locus transcripts is displayed as profile frequencies of CDR3 spectratypes for TRVB1 to TRVB24 gene families. Profiles were classified on the basis of peak number, distribution shape and relative fluorescence intensity (RFI) as: polyclonal Gaussian (PG, ≥8 peaks with a Gaussian distribution), polyclonal non-Gaussian (PNG, ≥8 peaks with an uneven distribution), polyclonal skewed (PS, 6 peaks or 1 peak with RFI>40%), polyclonal severely skewed (PSS, 1 or 2 peaks with RFI>70%), oligoclonal (O, 2 to 3 peaks), monoclonal (M, 1 peak or a peak with RFI>90%), and not detected (ND). Color stacked columns represent the mean frequency for each of the clonality profiles in CD4 **(A)** and CD8 **(C)** in HC, as well as in patients with and without cGVHD early and late after HSCT. TRVB1-24 CDR3 median peak count in CD4 **(B)** and CD8 **(D)** T cells for HC and patient groups. Asterisks denote statistically significant differences between patient groups (**$p = 0.001$ to 0.01; ***$p = 0.0001$ to 0.001;****$p < 0.0001$).

The addition of aGVHD as a variable to the LME analysis of RTE Treg and Tcon showed that aGVHD was associated with increased RTE Tcon counts and percentages during the first 6 months post-HSCT ($p < 0.01$). No effect was observed at later time-points. Accordingly, when the No cGVHD patient group was split into No GVHD and aGVHD only as described earlier, RTE Tcon were significantly decreased in No GVHD vs. both GVHD patient groups at months 1, 2, and 3 (**Figure 7G**). After month 6, RTE Tcon counts increased in No GVHD and Ac GVHD, while Ac & Ch GVHD patients showed significantly reduced RTE Tcon numbers, suggesting that cGVHD may impact on RTE Tcon reconstitution.

DISCUSSION

Chronic GVHD remains a major hurdle in allo-HSCT. The significant role played by T lymphocytes in cGVHD pathophysiology has been highlighted in studies that identify graft T cells as major mediators in GVHD development, with Naive T cells playing a central role (27). This has led to

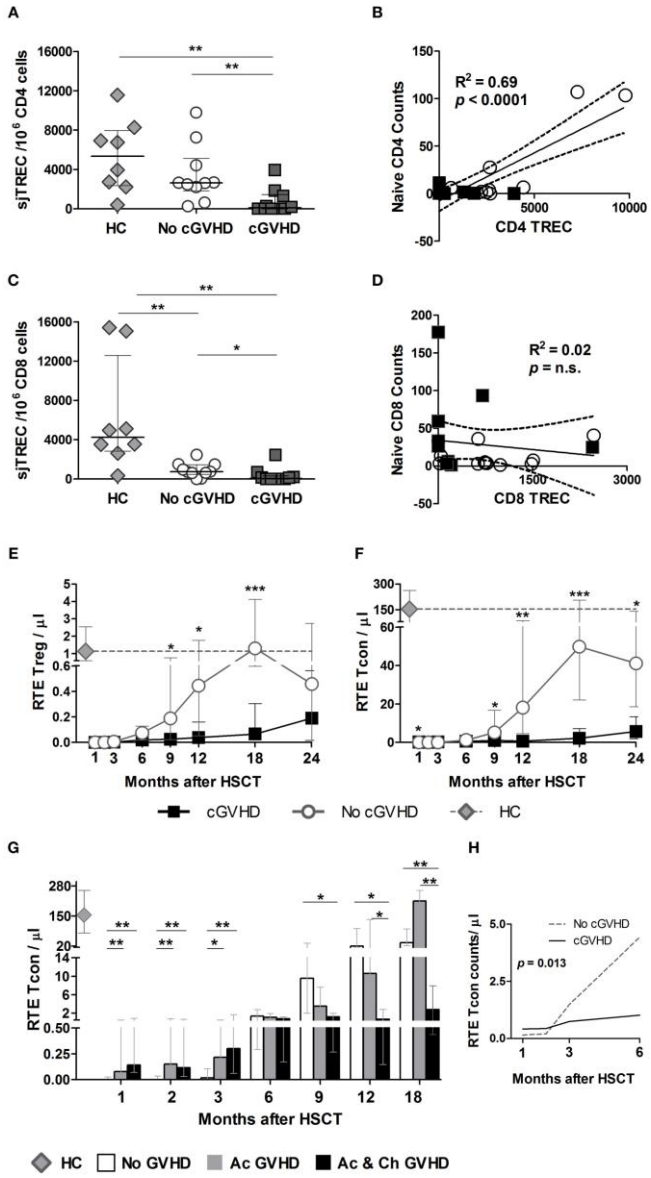

FIGURE 7 | sjTREC content and RTE analysis post-HSCT. sjTRECs were quantified in purified CD4 **(A)** and CD8 **(C)** T cells at months 9 and 12 post-HSCT. Median and IQR values for sjTREC content are shown per 10^6 CD8 or CD4 T cells in healthy controls (HC, gray diamonds), patients without cGVHD (No cGVHD, white

(Continued)

FIGURE 7 | circles), and patients with (cGVHD, dark gray squares). Linear regression analysis between Naive CD4 **(B)** or CD8 **(D)** absolute counts and sjTREC content where black squares represent cGVHD and while circles No cGVHD patients. Absolute counts of RTE Treg **(E)** and RTE Tcon **(F)** in patients with (black lines and squares) and without (gray lines and circles) cGVHD, as well as in HC (gray diamonds and gray dotted lines). **(G)** RTE Tcon counts in patients without GVHD (No GVHD, white bars), with acute GVHD only (Ac GVHD, gray bars), with acute and chronic GVHD (Ac & Ch GVHD, black bars), and in HC (gray diamond). **(H)** RTE Tcon counts on the first 6 months after HSCT in patients with (cGVHD, black line) and without (No cGVHD, dotted gray line) cGVHD, illustrating the results of the LME for these subsets. Median and IQR values are shown. Asterisks denote statistically significant differences between patient groups (*$p = 0.01$ to 0.05; **$p = 0.001$ to 0.01; ***$p = 0.0001$ to 0.001).

the development of T cell depletion strategies that, despite reducing GVHD incidence, are associated with delayed immune reconstitution and increased relapse (28). In order to further study the role played by naive and memory T cell subsets in cGVHD in a setting of partial *in vivo* T cell depletion, we studied a homogenous cohort of patients undergoing unrelated donor allo-HSCT up to 24 months after transplant. This patient cohort was transplanted at a single institution, received the same ATG-containing RIC and GVHD prophylaxis regimens. Hence, despite the negative effects of some immunosuppressive drugs such as CsA, which negatively impacts on Treg function (29), all patients were under the same regimen during the initial post-transplantation period. After 6–9 Months post-HSCT, CsA was discontinued in patients who did not present cGVHD and therefore findings at these later time points may relate to both cGVHD development and/or the immunosuppressive regimen. In this setting, we observed 45% cGVHD incidence (27.5% moderate and severe), likely associated to the fact that 13 out of the 18 patients received PBSC as the graft source and that the majority of these patients had some degree of HLA mismatch with their donors.

While most reports focus on the effects of ATG on cGVHD incidence and immune reconstitution, we investigated the association between naive and memory T cell homeostasis and cGVHD after an ATG-containing conditioning regimen, which is understudied. All our patients received thymoglobulin in the conditioning regimen, which is one of the available rabbit ATGs. Since all of these lymphocyte depleting products are different, it is possible that immune reconstitution in patients receiving another ATG could be somewhat different.

We performed a phenotypic study of T cell reconstitution evaluating Naive, CM, EM, and EMRA reconstitution at fixed time-points after HSCT. We further extend previous studies looking at T cell subsets in cGVHD by investigating the reconstitution of a recently described T cell subset with unique characteristics. SCM have been described as a subset of memory T cells with a Naive-like phenotype, that includes CD45RA, CD62L, and CCR7, that can be distinguished from Naive T cells by the expression of CD95 (30). SCM T cells originate from *in vivo* priming of Naive T cells and possess stem-cell-like properties, being able to generate other memory subsets (25, 30, 31), and are therefore thought to play a role in the maintenance of long term memory. In addition, increased SCM have been associated to autoimmune conditions (32, 33). In order to study the mechanisms involved in T cell reconstitution, we further evaluated proliferation through Ki-67 expression, susceptibility to apoptosis through Bcl-2 and CD95 levels, and evaluated thymic production of CD4 T cells through the identification of CD31-expressing RTE. At selected time-points, we performed TCR diversity and TREC content analysis. Despite the inherent variability of studies performed in human subjects with a distinct genetic makeup, we report interesting observations that shed some light into the biology of cGVHD in humans.

We observed altered Treg homeostasis in patients developing cGVHD. This is consistent with previous reports from our group and others, in the setting of multiple conditioning and GVHD prophylaxis regimens, showing impaired Treg reconstitution in cGVHD (34–37). Reduced Treg in cGVHD was not associated with altered expression of the apoptosis-related proteins Bcl-2 and CD95, but correlated with decreased proliferation, possibly associated with immunosuppressive therapies. Importantly, we demonstrate a clear association between cGVHD development and severely impaired Naive and SCM Treg reconstitution in this setting. Decreased Naive Treg may result from reduced thymic output following GVHD-induced thymic damage. Indeed, we found significantly decreased levels of the RTE-enriched $CD45RA^{+}CD62L^{+}CD31^{+}$ subset, a population that has been used as an indicator of thymic production, within Treg in cGVHD (26, 38). Decreased SCM Treg may result from decreased Naive Treg and/or differentiation into memory phenotypes.

Overall, our data on Treg subset reconstitution support the hypothesis that imbalances in T cell tolerance play an important role in the biology of cGVHD and reinforces the potential benefits of Treg-restoring therapies, particularly of Naive Treg as this subset is particularly depleted in cGVHD.

In line with the previously reported negative impact of ATG on CD4 T cell reconstitution (39–41), Tcon recovery was impaired in both patient groups. However, we did not find significant differences in total Tcon numbers when comparing patients with and without cGVHD. Nevertheless, we report significantly distinct Tcon subset composition when comparing both patient groups. Notably, Naive and SCM Tcon recovery were impaired in patients developing cGVHD. This was accompanied by a tendency for increased levels of the more differentiated subsets EM and EMRA in patients with cGVHD

T cell reconstitution is thought to occur through peripheral expansion and *de novo* thymic production (42, 43). In order to investigate the pathways leading to impaired Naive Tcon reconstitution, we measured *ex vivo* proliferation throughout the follow-up, using Ki-67 as a proliferation marker. We observed a tendency for reduced Ki-67 expression within total Tcon, as well as in Tcon subsets (data not shown), in patients developing cGVHD. Susceptibility to apoptosis, as assessed by the quantification of Bcl-2 and CD95 expression levels, was similar between patient groups, pointing to decreased

homeostatic proliferation as a contributing factor to impaired Naive Tcon reconstitution.

In order to have a measure for *de novo* thymic production, we quantified sjTREC in total CD4 T cells. TRECs are episomal DNA sequences formed during thymic T-cell development by TCR V-J gene rearrangements that correlate with thymopoiesis, particularly in the absence of extensive proliferation (44). sjTRECs were significantly reduced in CD4 T cells from cGVHD *versus* No cGVHD patients. Interestingly, CD4 sjTREC correlated with Naive CD4 numbers, suggesting that, in the absence of cGVHD, the Naive Treg and Tcon recovery is likely to result from *de novo* thymic production occurring after HSCT (45). In order to further detail the contribution of thymic output to CD4 T cell recovery post-transplant, we quantified RTE Tcon. We show that from month 9 onwards, RTE Tcon increased in the absence of cGVHD while patients who develop cGVHD show a severely impaired RTE reconstitution. It is therefore likely that a combination of decreased proliferation and impaired thymic output leads to impaired Naive Tcon and Treg reconstitution in cGVHD. In addition, CD4 TCRVB diversity was significantly reduced in patients, more so in cGVHD, further pointing to impaired thymic production in patients developing cGVHD.

When accessing the role played by acute GVHD in RTE Tcon recovery after HSCT, we found increased RTE Tcon at months 1–3, both in Acute GVHD and in Acute and Chronic GVHD patients, as compared to No GVHD. This suggests a potential deleterious role of RTE Tcon early after HSCT. It is unclear if these cells are of thymic origin or result from cytokine-driven proliferation of infused RTE (46). We speculate that RTE Tcon detected early after HSCT may contain self-reactive clones, while RTE Tcon present at later time-points in No cGVHD result from adequate thymic selection processes.

Overall, we observed impaired Naive and SCM Tcon reconstitution in cGVHD, associated with reduced peripheral expansion. In addition, decreased TREC content and RTE Tcon recovery further suggest that cGVHD results from alterations in *de novo* thymic production. Intriguingly, we found that cGVHD development was associated to increased RTE Tcon early after HSCT through yet undescribed mechanisms.

It is noteworthy that we report a distinct picture emerging for CD8 T cells. EM, EMRA and CM were the most abundant CD8 subsets in the post-transplantation period. Interestingly, cGVHD associated with increased Naive, SCM and EMRA as compared to patients who did not develop cGVHD.

Stem Cell Memory (SCM) have been reported to differentiate from Naive, to be increased in autoimmune disease (32, 33) and to induce lethal GVHD in mouse studies (47). Our findings of increased Naive and SCM CD8 in cGVHD vs. No cGVHD raise the possibility that Naive CD8 T cells may differentiate into SCM and be involved in GVHD development by yet undescribed mechanisms. Interestingly, when the effects of previous aGVHD were taken into consideration in our statistical analysis, we observed that during the first 6 months after transplant SCM Tcon and CD8 were significantly affected by aGVHD. The stratification of patient groups into No GVHD, Acute GVHD only and Acute & Chronic GVHD confirmed that during these initial stages after HCST patients developing GVHD show increased numbers of these cells. A sustained increase in this population in patients developing cGVHD was observed for CD8 but not Tcon, further suggesting a potential role for CD8 SCM is cGVHD development.

Other studies have shown that the thymus is a direct target of GVHD (7) resulting on the emergence of self-reactive clones and the autoimmunity associated to cGVHD (48, 49). We observed a high prevalence of skewed and oligoclonal TCR repertoires in CD8 from both patient groups. Interestingly, the significantly reduced CD8 sjTREC content in cGVHD vs. No cGVHD patients suggests reduced thymic output and/or a high level of homeostatic proliferation observed in these patients.

Our data raises the possibility that increased Naive and SCM CD8 present from early time-points after transplant in cGVHD patients, may originate from defective negative selection mechanisms resulting from thymic tissue damage, leading to the output of self-reactive CD8 clones that may further differentiate and mediate disease. Given the increased levels of homeostatic T cell proliferation in the post-transplantation period, we cannot exclude the possible contribution of cell proliferation to the observed increase in Naive and SCM CD8.

We therefore extend on the observations by Alho et al. (34) that reported increased Naive T cell subsets in patients developing cGVHD, by comparing patients with and without cGVHD at month 3 after HSCT (34). We now show that this occurs not only within Naive CD8 but also in the SCM CD8 T cell subset, by detailing increases in these cell populations in patients who develop cGVHD in a cohort of patients undergoing unrelated HSCT after an ATG-containing conditioning regimen. We further show that this occurs in CD8 T cells in a sustained manner from month 1 to 9 in both percentages and absolute counts. In addition, by stratifying our patient cohort with regards to previous history of acute GVHD, we observed that increased SCM in Tcon and CD8 occurs early after HSCT in patients developing aGVHD. Of note, we did not observe any statistically significant impact of adding aGVHD as a co-variate within the Treg subsets.

In summary, we show that after an ATG-containing RIC regimen, cGVHD development after allo-HSCT is associated with reduced Treg recovery, particularly of Naive and SCM subsets. We speculate that this is likely due to reduced thymic production within the CD4 T cell compartment, where significantly decreased TCRVB diversity is observed in patients developing cGVHD. We also show that cGVHD development is associated to increases in Naive, SCM and EMRA CD8 T cells. This becomes apparent early after HSCT and persists throughout our 24-month follow-up, suggesting a potential involvement of these cells in the development of cGVHD.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics Committee of Lisbon Academic Medical Center with written informed consent from all subjects. All subject gave written informed consent in

accordance with the Declaration of Helsinki. The protocol was approved by Ethics Committee of Lisbon Academic Medical Center (ref. 459/13).

AUTHOR CONTRIBUTIONS

MS analyzed data and wrote the paper. RA designed experiments, performed experiments, analyzed data, and wrote paper. IF, SB, and AR performed experiments. AV performed cell sorting and flow cytometry support. PP and NC collected clinical data. DL performed the spectratyping and TREC analysis. RR performed statistical data analysis. AA and AS analyzed data. CM, FL, and RM recruited patients. JR and JL conceived and designed the study. JL recruited patients, designed experiments, and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00334/full#supplementary-material

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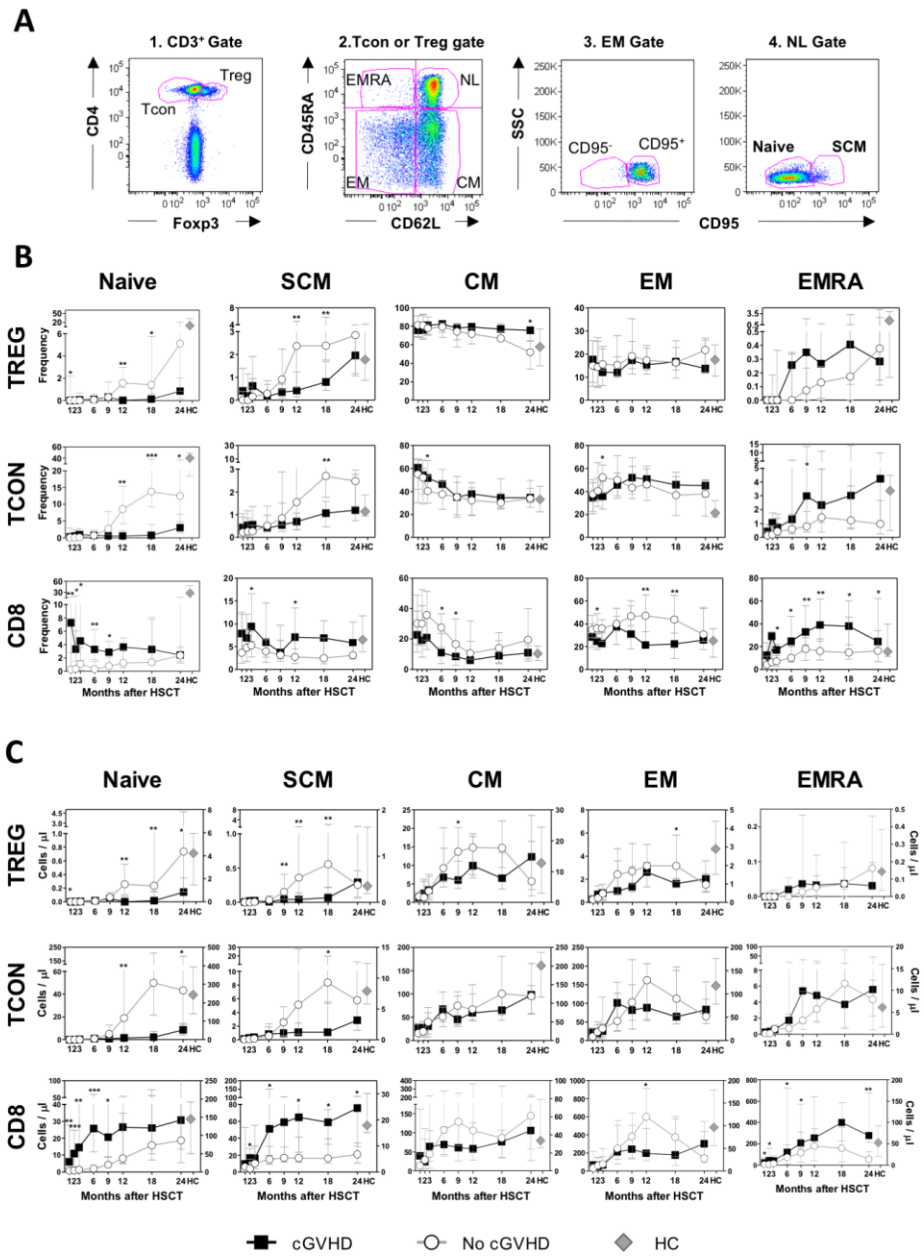

Supplemental Figure 1. Treg, Tcon and CD8 subset reconstitution in cGVHD and No cGVHD patients **(A)** Flow cytometry plots illustrating the gating strategy used to identify Naive and Memory subsets. Treg were identified as $CD4^+$ $Foxp3^+$, Tcon as $CD4^+$ $Foxp3^-$ and CD8 as $CD4^-$, all within $CD3^+$ lymphocytes. T cell populations were further divided into subsets according to CD45RA and CD62L expression: Central memory (CM, $CD45RA^-$ $CD62L^+$), Effector Memory (EM, $CD45RA^-$ $CD62L^-$), $CD45RA^+$ Effector Memory (EMRA, $CD45RA^+$ $CD62L^-$), Naive–like (NL, $CD45RA^+CD62L^+$) cells. Stem Cell Memory (SCM) as $CD45RA^+$ $CD62L^+$CD95+ and Naive as $CD45RA^+$ $CD62L^+CD95^-$ **(B)** Median percentages for each subset within Treg, Tcon and CD8 cells. **(C)** Median absolute cell number per ∝l for Treg, Tcon and CD8 subsets. No cGVHD: grey lines and circles; with cGVHD: black lines and squares; HC: grey diamonds and dotted lines. Median counts from patients are shown on the left and HC on the right y axis. Symbols represent median values ± IQR. (*p=0.01 to 0.05; **p=0.001 to 0.01).

REGULAR ARTICLE

blood advances

Functional analysis of clinical response to low-dose IL-2 in patients with refractory chronic graft-versus-host disease

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Key Points

- Low-dose IL-2 selectively improves diversity of the CD4Treg TCR repertoire in patients with cGVHD.
- Clinical response to IL-2 is associated with rapid turnover of the TCR repertoire and emergence of CD4Tregs with new TCR clonotypes.

Patients with chronic graft-versus-host disease (cGVHD) have a paucity of regulatory CD4 T cells (CD4Tregs) that mediate peripheral tolerance. In clinical trials, daily low-dose interleukin-2 (IL-2) has been administered safely for prolonged periods in patients with steroid-refractory cGVHD. Peripheral CD4Tregs expand dramatically in all patients during IL-2 therapy but clinical improvement was observed in ~50% of patients. Here, we examined the impact of low-dose IL-2 therapy on functional T-cell markers and the T-cell repertoire within CD4Tregs, conventional CD4 T cells (CD4Tcons), and $CD8^+$ T cells. IL-2 had profound effects on CD4Tregs homeostasis in both response groups including selective expansion of the naive subset, improved thymic output, and increased expression of Ki67, FOXP3, and B-cell lymphoma 2 within CD4Tregs. Similar changes were not seen in CD4Tcons or CD8 T cells. Functionally, low-dose IL-2 enhanced, in vitro, CD4Treg-suppressive activity in both response groups, and all patient CD4Tcons were similarly suppressed by healthy donor CD4Tregs. High-throughput sequencing of the T-cell receptor β (TCRβ) locus demonstrated that low-dose IL-2 therapy increased TCR repertoire diversity and decreased evenness within CD4Tregs without affecting CD4Tcons or CD8 T cells. Using clone-tracking analysis, we observed rapid turnover of highly prevalent clones in CD4Tregs as well as the conversion of CD4Tcons to CD4Tregs. After 12 weeks of daily IL-2, clinical responders had a greater influx of novel clones within the CD4Treg compartment compared with nonresponders. Further studies to define the function and specificity of these novel CD4Treg clones may help establish the mechanisms whereby low-dose IL-2 therapy promotes immune tolerance.

Introduction

Chronic graft-versus-host disease (cGVHD) is the leading cause of nonrelapse morbidity and mortality following conventional allogeneic hematopoietic stem cell transplantation, occurring in 60% to 70% of adults and 20% to 50% of children surviving >100 days posttransplant.[1-3] Previous studies have shown that patients with active cGVHD have poor reconstitution of regulatory CD4 T cells (CD4Tregs), which function to suppress autoreactive and alloreactive immune responses.[4-8] Interleukin-2 (IL-2) is the primary homeostatic regulator of CD4Tregs in vivo and normally functions to support differentiation of CD4Tregs in the thymus as well as CD4Treg expansion in the periphery. IL-2 also promotes FOXP3 expression in CD4Tregs and enhances the regulatory functions of these cells.[9,10] Importantly, CD4Tregs do not secrete IL-2 but constitutively express a high-affinity IL-2 receptor. This allows

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CD4Tregs to respond to low levels of IL-2 secreted by activated effector T cells. Previous clinical trials at our center demonstrated that daily administration of low-dose IL-2 for prolonged periods is safe, well tolerated, and leads to preferential in vivo expansion of CD4Tregs with a clinical response rate of 50% to 60% in patients with steroid-refractory cGVHD.[11,12] Interestingly, all patients exhibited similar quantitative increases in circulating CD4Tregs even though only one-half of the patients had clinical improvement. We previously identified some predictors of clinical response, which included earlier IL-2 initiation (after transplantation and after cGVHD onset) and a higher ratio of CD4Tregs to conventional CD4 T cells (Treg:Tcon) at baseline and after week 1 of IL-2 therapy.[11]

In the present studies, we examined homeostatic and functional effects of IL-2 therapy to identify intrinsic T-cell factors that were associated with clinical improvement of cGVHD. Daily low-dose IL-2 led to preferential expansion of naive CD4Tregs and enhanced CD4Treg-suppressive function, but this did not distinguish response groups. We also performed T-cell receptor β (TCRβ) sequencing of CD4Tregs, CD4Tcons, and CD8 T cells and found that low-dose IL-2 increased diversity within CD4Tregs without affecting CD4Tcons or CD8 T cells. Notably, greater turnover of novel CD4Treg clones during IL-2 therapy was associated with a better clinical response.

Methods

Patients and sample collection

Samples used in this study were obtained from adult patients with steroid-refractory cGVHD enrolled in a phase 2 study of daily IL-2.[11] Samples were also obtained from 24 healthy individuals. Patients and healthy donors (HDs) were enrolled in clinical research protocols approved by the Human Subjects Protection Committee of the Dana-Farber/Harvard Cancer Center. Written informed consent was obtained before sample collection, in accordance with the Declaration of Helsinki.

Flow cytometry analysis

Blood samples were obtained at: baseline; 1, 2, 4, 6, 8, and 12 weeks during IL-2; and every 8 weeks while receiving extended-duration IL-2. Using cell surface markers, CD4Tregs were defined as $CD3^+CD4^+CD25^{med\text{-}high}CD127^{low}$, CD4Tcons as $CD3^+CD4^+CD25^{neg\text{-}low}CD127^{med\text{-}high}$, and $CD8^+$ T cells as $CD3^+CD4^-CD8^+$. Fifty microliters of whole blood (15% EDTA) was incubated with fluorophore-conjugated monoclonal antibodies as previously described.[11] For intracellular marker analysis, CD4Tregs, CD4Tcons, and CD8 T cells were defined as $CD3^+CD4^+Foxp3^+$, $CD3^+CD4^+Foxp3^-$, and $CD3^+CD4^-CD8^+$, respectively.[7] Subsets within each population were defined as: naive T cells ($CD45RA^+CD62L^+$), central memory (CM; $CD45RA^-CD62L^+$), effector memory (EM; $CD45RA^-CD62L^-$), and CD8 effector memory RA/TEMRA T cells ($CD8^+CD45RA^+CD62L^-$). Within CD4 T cells, coexpression of CD45RA and CD31 was used to define recent thymic emigrants (RTEs).[13,14] Proliferation was measured by expression of Ki67.[15] Susceptibility to apoptosis was assessed by intensity of intracellular expression of B-cell lymphoma 2 (BCL2).[16] Cells were acquired in a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar). Gating and analysis strategies have been described previously.[7]

In vitro suppression assays

$CD4^+$ T cells were isolated by negative selection with magnetic beads (Miltenyi Biotec). CD4Tregs and CD4Tcons were isolated by sorting for $CD4^+CD25^+CD127^-$ and $CD4^+CD25^-$ cells, respectively. CD4Tcons were labeled with CellTrace Violet (Invitrogen) and stimulated with anti-CD3/CD28/CD2–coated beads (Treg Suppression Inspector, human; Miltenyi Biotec), and autologous or allogeneic CD4Tregs were added at a ratio of 1:1. After 4 days, cells were harvested and cell divisions of CD4Tcons were evaluated using flow cytometry. For allogeneic assays, all patient CD4Tregs were tested against the same HD CD4Tcons and all patient CD4Tcons were tested against the same HD CD4Tregs.

TCRβ repertoire analysis

Frozen peripheral blood mononuclear cells from 12 patients (6 clinical responders, 6 nonresponders) and 8 HDs were sorted into CD4Tregs, CD4Tcons, and CD8 T cells. Clinical characteristics of these patients are summarized in supplemental Table 2. Genomic DNA was extracted (Qiagen) from the purified populations; DNA amounts varied with cell type and time point (supplemental Table 1). Survey-level TCRβ sequencing was performed using the ImmunoSEQ platform with subsequent analysis using algorithms developed by Adaptive Biotechnologies.[17] The Hill-based diversity profile and evenness indices were used for measures of TCRβ repertoire diversity.[18] Diversity and evenness were first calculated with α ranging 1 through 10, followed by principal component analysis (PCA) on the profile. PCA uses an orthogonal transformation to convert a set of correlated diversity levels across all α levels into a set of linearly uncorrelated variables, called principal components (PC1, PC2, …PC10). This transformation is defined such that the first principal component (PC1) has the largest possible variance that accounts for most of the variability in the data. In this analysis, PC1 explained 95.5% variability in diversity and 88% in evenness. We therefore used PC1 for the analysis of diversity and evenness. All diversity levels were log2 transformed prior to PCA and a small weight was multiplied to diversity values in CD4Tregs only as the CD4Treg diversity was correlated with the DNA amount. The weight was $(400\ ng/\text{the DNA amount in each sample})^{0.15}$, where 400 ng is the maximum amount of DNA. TCR overlap between samples was examined using the Jaccard index, defined as the size of the intersection divided by the size of the union of the sample sets.[19]

Statistical analysis

Data analysis was performed primarily descriptively. Phenotypic and functional data were compared using the exact Wilcoxon-rank-sum test for unpaired group comparison and the Wilcoxon-signed-rank test for paired comparison. Correlation analysis was performed using Pearson correlation coefficients. For missing samples at baseline, 1 or 12 weeks after IL-2 treatment in FOXP3 and BCL-2 expression levels, multiple imputation technique[20] was used to impute the missing data under the assumption of missing at random. All tests were 2-sided at the significance level of 0.05 and multiple comparisons were not considered. All statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC) and R version 3.1.3 (the CRAN project).

Results

Changes in naive and memory T-cell subsets in response to low-dose IL-2

All evaluable patients in the previously reported phase 2 study, regardless of clinical response, exhibited expansion of peripheral blood CD4Tregs which peaked at 4 weeks. Increased Treg numbers

were sustained above baseline throughout the initial 12 weeks of therapy and during extended-duration IL-2 therapy.[11] Here, we examined changes in naive and memory subsets within the CD4Tregs, CD4Tcons, and CD8 T-cell populations during IL-2 therapy (Figure 1A-C). Prior to starting IL-2, patients with cGVHD had lower absolute numbers of circulating CD4Tregs but the proportion of naive CD4Tregs was similar compared with normal HDs (median, 9% vs 12.1%; $P = .34$) (Figure 1A). The proportion of EM CD4Tregs did not differ with HDs, but patients with cGVHD had a higher proportion of CM CD4Tregs compared with HDs (median, 61% vs 41.7%; $P = .0005$). Low-dose IL-2 initially led to expansion of the CM CD4Tregs at week 1. Naive CD4Treg expansion was evident by week 4, leading to a significant increase above baseline after 12 weeks of IL-2 therapy (median, 9% to 16.5%; $P = .02$), and achieving levels above HDs by 6 months of extended therapy (median, 22.2% vs 12.1%; $P = .007$). The absolute EM and CM CD4Treg cell counts peaked at week 2 and week 4, respectively, then decreased over time, but remained above baseline levels. In contrast, the absolute naive CD4Treg cell count continued to increase steadily (Figure 1A). Within the CD4Tcons and CD8 T cells, the fraction of naive cells in patients was similar to HDs at baseline and did not change significantly during IL-2 therapy (Figure 1B-C). Patients had a higher proportion of CM CD4Tcons than HDs (median, 29% vs 8%; $P < .0001$), and a lower proportion of EM CD8 T cells than HDs at baseline (median, 20% vs 32%; $P = .006$). These differences persisted throughout IL-2 therapy.

To determine whether expansion of CD4Tregs was simply due to increased proliferation of naive CD4Tregs, we examined Ki67 expression in the CD4Treg subsets. Low-dose IL-2 induced rapid proliferation of both memory and naive CD4Treg subsets with Ki67 expression peaking after 1 week (22% and 25% increase in Ki67$^+$ cells from week 0 to week 1, respectively) (Figure 1D). The expansion of naive CD4Tregs observed after 4 weeks of IL-2 therapy was not associated with an increase in proliferation of this subset.

Naive CD4Tregs can be derived from thymic generation of new cells as well as through proliferation of circulating cells. To examine thymic production of naive CD4Tregs, we used coexpression of CD45RA and CD31 to identify RTEs.[13,14] Low-dose IL-2 preferentially increased thymic output of CD4Tregs, but not of CD4Tcons (Figure 2). Although output of RTE CD4Tregs did not approach the HD range at any time during IL-2 therapy, increased numbers of circulating RTE CD4Tregs were detected after 2 weeks and remained elevated during extended therapy (Figure 2A). At 6 months, the percentage of RTE CD4Tregs increased to a median of 11.4% ($P = .007$ compared with patients at baseline and $P = .001$ compared with HDs). The percentage of RTE CD4Tcons remained stable throughout IL-2 therapy (Figure 2B). During the initial 12-week therapy period, clinical responders and nonresponders had similar increases in absolute CD4Treg, naive CD4Treg, and RTE CD4Treg counts (supplemental Figure 1).

Cellular markers of CD4Treg function

We next examined expression of functional markers in CD4Tregs. Stable, high-level expression of FOXP3 is required for suppressive function whereas low expression levels are associated with decreased suppressive activity.[21,22] At baseline, FOXP3 expression in CD4Treg subsets was similar in memory and naive CD4Tregs (Figure 3A). IL-2 therapy led to a rapid increase in FOXP3 expression, particularly in CM and EM CD4Tregs. At week 1, the median FOXP3 median fluorescence intensity (MFI) in CM CD4Tregs and EM CD4Tregs was higher than the median FOXP3 MFI in naive Tregs ($P = .004$ and $P = .01$, respectively). FOXP3 levels in CD4Treg subsets peaked between weeks 1 and 2 but remained above baseline levels during the entire 12-week treatment period (Figure 3A). Clinical responders and nonresponders had similar changes in FOXP3 expression from baseline to the peak at week 1 (Figure 3B).

A previous study showed that IL-2 therapy led to increased levels of the antiapoptotic BCL-2 protein in CD4Tregs and enhanced resistance of these cells to apoptosis.[23] Here, we found that BCL-2 expression levels were highest in naive CD4Tregs and lower in memory CD4Tregs in HDs and patients (Figure 3C). BCL-2 expression in all CD4Treg subsets increased 1 week after starting IL-2, peaked at week 12, and remained elevated during extended therapy. Clinical nonresponders had a higher increase in BCL-2 expression from week 1 to week 12 within the CM CD4Treg subset but no difference between response groups was seen within the naive or EM CD4Tregs (Figure 3D). There were no significant changes in BCL-2 expression over time in the CD4Tcon or CD8 T-cell subsets (data not shown).

Impact of low-dose IL-2 on in vitro CD4Treg function

To assess CD4Treg function more directly, we tested the ability of CD4Tregs to suppress the proliferation of stimulated CD4Tcons at study baseline and at week 6. Patient CD4Tregs were tested against both autologous CD4Tcons and HD CD4Tcons from the same HD control subject for all in vitro assays. At baseline, patient CD4Tregs had a decreased ability to suppress HD CD4Tcons compared with CD4Tregs from HD control subjects (Figure 4). CD4Tregs from patients suppressed HD CD4Tcon proliferation to a median of 58% compared with a median of 71% by HD CD4Tregs ($P = .009$). By week 6 of IL-2 therapy, patient CD4Treg-suppressive function improved and was indistinguishable from the HD CD4Tregs ($P = .71$). Significant improvement in Treg function was also observed in the autologous suppression assays using patient CD4Tregs and patient CD4Tcons, with suppression to a median of 44% at baseline and 74% at week 6 ($P = .02$). CD4Tcons from patients and HDs were similarly suppressed by HD CD4Tregs at baseline ($P = .26$) and at week 6 ($P = .09$), indicating that CD4Tcons from patients with cGVHD are not more resistant to suppression in this assay.

Patient CD4Treg suppression of HD CD4Tcons did not differ between response groups at baseline ($P = .55$), and both groups showed improved function at week 6. During extended therapy, CD4Treg-suppressive function remained stable without significant change between week 6 and 6 months of IL-2 therapy (supplemental Figure 2). Similarly, low-dose IL-2 did not alter the ability of patient CD4Tcons to be suppressed by HD CD4Tregs at week 6 or after 6 months.

Impact of low-dose IL-2 therapy on TCR repertoire diversity

Quantitative studies of the TCR repertoire have led to important insights into immune reconstitution and development of GVHD after hematopoietic stem cell transplantation.[24-29] We examined the effect of low-dose IL-2 therapy on the TCRβ repertoire of purified

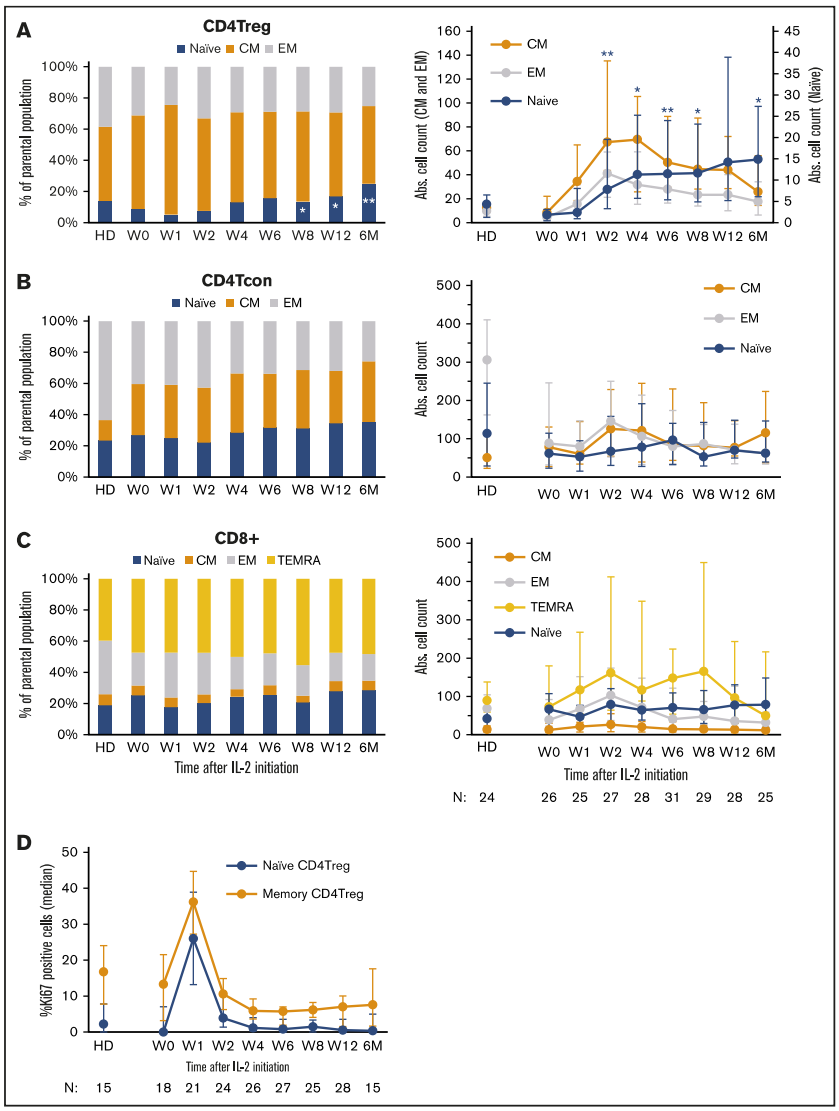

Figure 1. Impact of low-dose IL-2 on naive and memory T-cell compartments. Relative percentages of each subset are shown on the left and absolute (Abs.) cell numbers (cells per microliter) of each subset in the peripheral blood at each time point are shown on the right. Bar graphs on the left show the median percentage of each population and the corresponding median in HDs. Graphs on the right show median cell counts per microliter for each population and the corresponding median in HDs, with the interquartile range (whisker bars). Time points represent weeks (W) and months (M) after IL-2 initiation. Number of patients evaluated at each time point is indicated at the bottom of the image. (A) CD4Treg subsets. $^{*}P < .05$, $^{**}P < .01$, each time point was compared with baseline (W0), 2-sided Wilcoxon signed-rank test. (B) CD4Tcon subsets. (C) $CD8^{+}$ T-cell subsets. (D) Proliferation marker (Ki67) expression in CD4Tregs. The percentage of $Ki67^{+}$ cells within naive (orange) and memory (blue) CD4Tregs is shown for HDs and for patients at multiple time points after IL-2 initiation. Median values and the interquartile range (whisker bars) are shown at each time point.

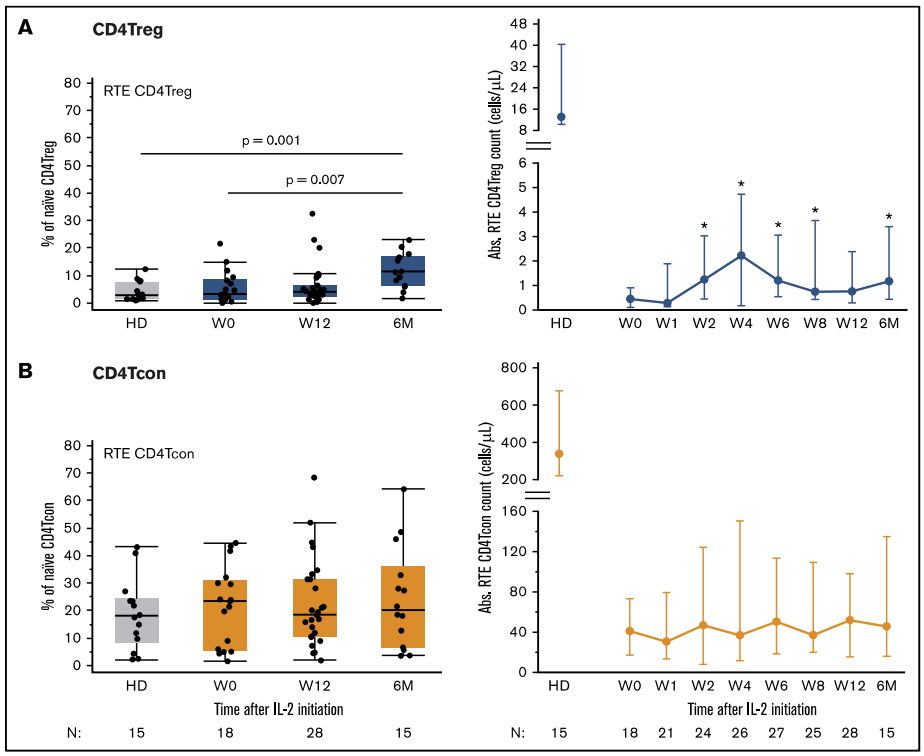

Figure 2. Impact of low-dose IL-2 on thymic production of CD4Tregs and CD4Tcons. Percentage of RTEs is shown on the left and absolute cell numbers (cells per microliter) in the peripheral blood at each time point are shown on the right. Box plots on the left depict the 75th percentile, median, and 25th percentile values; whiskers represent maximum and minimum values. Dots depict individual HDs or patients. Graphs on the right show median cell counts per microliter for each population and the corresponding median in HDs, with the interquartile range (whisker bars). Time points represent weeks (W) and months (M) after IL-2 initiation. Number of patients evaluated at each time point is indicated at the bottom of the image. (A) RTE CD4Tregs in patients (blue) and HDs (gray). *$P < .05$, absolute cell counts compared with baseline (baseline), 2-sided Wilcoxon signed-rank test. (B) RTE CD4Tcons in patients (orange) and HD (gray).

CD4Tregs, CD4Tcons, and CD8 T cells in serial blood samples from 6 clinical responders and 6 nonresponders. We first compared TCR diversity between samples using the Hill-based diversity profile,[18] in which higher values indicate a more diverse distribution of TCRβ sequences. Consistent with previous reports,[25,28,30] we found that CD8 T cells had lower diversity compared with CD4Tregs and CD4Tcons both in HDs ($P = .008$) and in patients ($P \leq .006$ for baseline and week 12; $P = .03$ at 1 year) (Figure 5A). In baseline patient samples, diversity was lower in CD4Tregs compared with HDs ($P = .025$) but not statistically different in CD4Tcons ($P = .18$) and CD8 T cells ($P = .12$). After 12 weeks of IL-2 therapy, CD4Treg diversity increased and was indistinguishable from HDs ($P = .31$) whereas diversity did not change within CD4Tcons and CD8 T cells. CD4Treg diversity increased to similar extents in both response groups, although this comparison was limited by lower amounts of DNA input for CD4Treg samples in clinical responders (supplemental Table 1). We also examined evenness, which represents the extent of clonal expansion of the TCR repertoire by scaling the diversity index to the number of unique TCR clones.[18] High values of evenness indicate that all clones within the population are present at equal frequencies whereas lower values indicate an unequal distribution of clonal frequencies. IL-2 therapy led to significantly decreased evenness in CD4Tregs ($P = .04$ from baseline to week 12) but did not affect CD4Tcons or CD8 T cells (Figure 5B). Together, these observations suggest that low-dose IL-2 therapy both diversifies the clonal composition of CD4Tregs and leads to selective expansion of individual clones.

TCRβ clone tracking during IL-2 therapy

To measure turnover of individual T-cell clones, we focused on the 1000 most abundant productive TCRβ clones present within each cell type after 12 weeks of IL-2 therapy and asked whether these dominant clones were also detected at baseline. We also examined

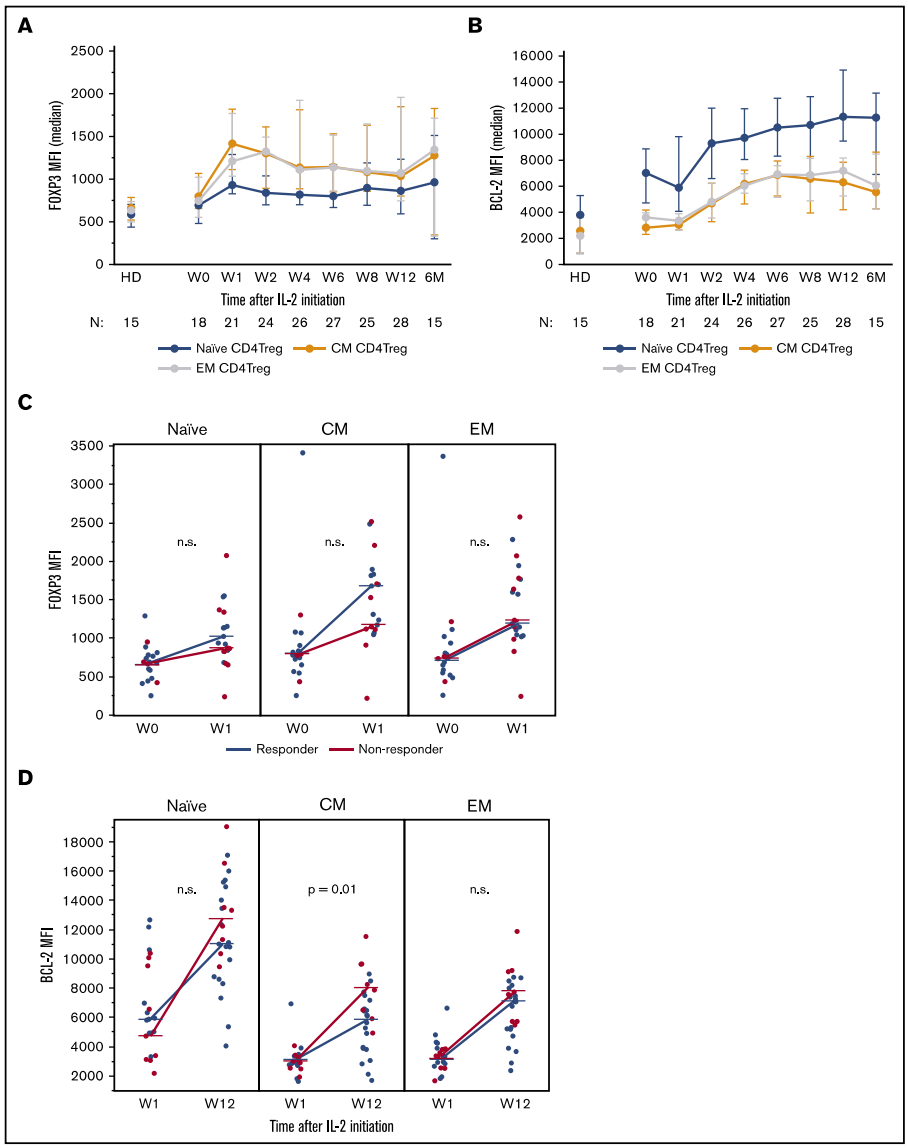

Figure 3. Expression of CD4Treg functional markers during IL-2 therapy. (A) FOXP3 expression in naive and memory CD4Treg subsets. (B) Increase in BCL-2 expression from week 1 (W1) to week 12 (W12) in clinical responders and nonresponders for naive and memory CD4Treg subsets. (C) Increase in FOXP3 expression from baseline (W0) to week 1 (W1) in clinical responders and nonresponders for naive and memory CD4Treg subsets. (D) BCL-2 expression in naive and memory CD4Treg subsets. (A-B) Median values and the interquartile range (whisker bars) are shown for each parameter. Time points represent weeks (W) and months (M) after IL-2 initiation. Number of patients evaluated at each time point is indicated at the bottom of the image. (C-D) Dots depict individual patients. Lines connect the median values at each time point. The Wilcoxon rank-sum test was used to compare responders vs nonresponders. n.s., not significant.

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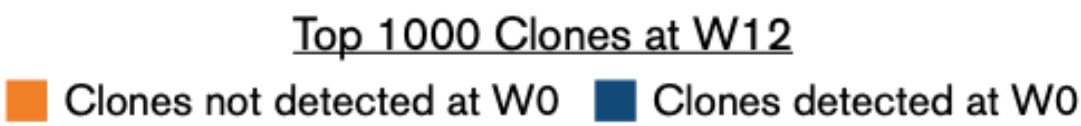

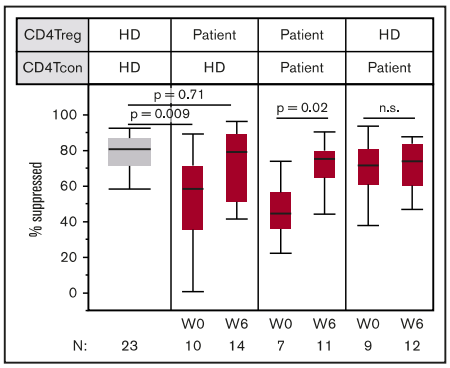

Figure 4. Low-dose IL-2 improves in vitro CD4Treg-suppressive activity. Suppressive activity of patient CD4Tregs was tested against HD CD4Tcons in vitro. Patient CD4Tregs were assessed at baseline (W0) and at week 6 (W6). In addition, the ability of HD CD4Tregs to suppress the proliferation of patient CD4Tcons was assessed at both time points. Box plots depict the 75th percentile, median, and 25th percentile values; whiskers represent maximum and minimum values, except for outliers. The box for HD is shaded in gray. *P* values comparing HD CD4Tregs to patient CD4Tregs are shown at week 0 and week 6. Patient CD4Treg suppression of patient CD4Tcons between baseline and week 6 and suppression of patient CD4Tcons by HD CD4Tregs at baseline and week 6 were also compared; 2-sided Wilcoxon signed-rank test. Number of patients evaluated at each time point is indicated at the bottom of the image. For the HD control column, N represents the number of assays using cells from the same HD.

samples taken 12 weeks apart from an HD (Figure 6A). In the HD, the correlation coefficients between the productive frequencies of the top 1000 clones at the 2 time points were high within all 3 cell types, indicating a low level of clonal turnover during this 12-week interval. In patients receiving IL-2, the correlation coefficients between the productive frequencies of the 1000 most abundant week 12 clones and productive frequencies at baseline (week 0) were significantly lower within CD4Tregs compared with CD8 T cells and CD4Tcons (Figure 6A). This suggests that IL-2 therapy preferentially leads to higher clonal turnover in CD4Tregs. Using clone tracking, a median of 14% (range, 3% to 51%) of the most prevalent CD4Treg clones at week 12 were detected at baseline compared with 38.5% (range, 7% to 75%) of the top 1000 CD4Tcon clones and 36.5% (range, 12% to 90%) of CD8 clones. When examined by response group, clinical responders at week 12 had a significantly higher proportion of clones derived from clones that were not detected at baseline compared with nonresponders in CD4Tregs ($P = .04$) (Figure 6B). There were no differences between response groups for CD4Tcons and CD8 T cells. These results suggest that greater expansion of novel or low frequency clones within the CD4Treg fraction during IL-2 therapy is associated with a better clinical response.

The greatest influx of new clones within CD4Tregs occurred in the first 12 weeks of therapy as TCR overlap between the week 12 and 1-year time points was higher than the overlap between baseline and week 12 (supplemental Figure 3A). Among patients who received extended-duration therapy, a median of 9.5% (range, 3% to 20%) of the dominant CD4Treg clones at week 12 was shared with the baseline population, whereas a median of 34% (range, 12% to 57%) of the dominant CD4Treg clones after 1 year of IL-2 therapy was shared with the week 12 population ($P = .03$) (supplemental Figure 3B).

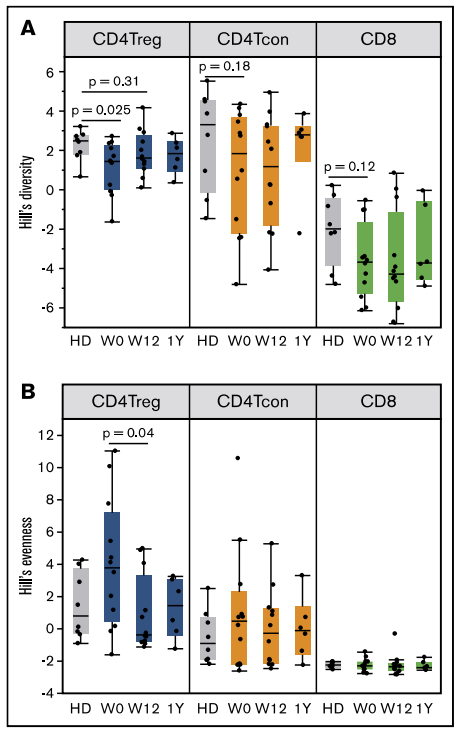

Figure 5. TCR repertoire diversity and evenness in patients receiving low-dose IL-2 and HDs. For patients, diversity measurements were assessed at baseline (W0), week 12 (W12), and 1 year (1Y). (A) Hill-based diversity values for CD4Tregs, CD4Tcons, and CD8 T cells in patients with cGVHD (n = 12) and HDs (n = 8). In HDs, CD8 T cells have lower diversity than CD4Tregs ($P = .008$) and CD4Tcons ($P = .008$). In patients, CD8 T cells have lower diversity than CD4Tregs and CD4Tcons at baseline ($P \leq .001$), week 12 ($P \leq .001$), and 1 year ($P = .03$). Diversity in CD4Tcons and CD8 T cells is similar in patients compared with HDs and does not change with IL-2 therapy. Diversity in CD4Tregs is lower in patients than in HD at baseline ($P = .019$) but is similar to HDs at week 12 ($P = .2$). (B) Hill evenness values for CD4Tregs, CD4Tcons, and CD8 T cells in patients with cGVHD and HDs. Evenness is similar between HDs and patients at baseline in CD4Tcons and CD8 T cells and does not change with IL-2 therapy. In CD4Tregs, evenness is also similar between HDs and patients at baseline. However, within patients, evenness in CD4Tregs decreases significantly from baseline to week 12 ($P = .04$). (A-B) Each dot represents an individual patient. Box plots depict the 75th percentile, median, and 25th percentile values; whiskers represent maximum and minimum values, except for outliers. The boxes for HD are shaded in gray. Two-sided Wilcoxon signed-rank test was used to assess significance.

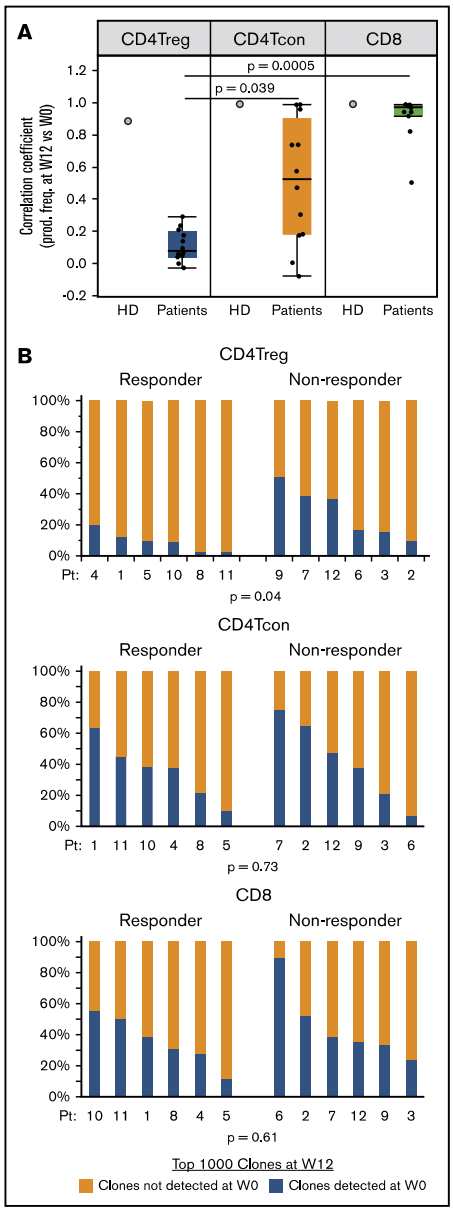

Figure 6. Origin of dominant clones within each T-cell subset at week 12 of IL-2 therapy. (A) Correlation coefficients between the productive frequencies of the top 1000 clones at week 12 and the productive frequencies of the same clones at baseline (week 0) for each cell type are shown for an HD (gray filled circles) and patients (black dots). Each dot represents the correlation coefficient for an individual patient. Box plots depict the 75th percentile, median, and 25th percentile values; whiskers represent maximum and minimum values, except for outliers. The correlation coefficients for CD4Tregs were significantly lower than for CD4Tcons ($P = .039$) and CD8 T cells ($P = .0005$). (B) Origin of the dominant 1000 clones at week 12 (W12) in clinical responders (n = 6; patients 1, 4, 5, 8, 10, 11) vs nonresponders (n = 6, patients 2, 3, 6, 7, 9, 11). For each cell type, bar graphs show the percentage of dominant week 12 clones that were detected (blue) at baseline (W0) and the percentage of dominant week 12 clones that were not detected (orange) at baseline. Each column represents an individual patient. Two-sided Wilcoxon signed-rank test was used to assess significance.

Peripheral conversion of CD4Tcons to CD4Tregs during IL-2 therapy

The frequent detection of new CD4Treg clones after 12 weeks of IL-2 therapy is consistent with the observation that daily low-dose IL-2 increased CD4Treg diversity as well as the number of naive and RTE CD4Tregs. Another potential source of new CD4Treg clonotypes is through peripheral conversion of CD4Tcons into CD4Tregs.[31] To address this possibility, we compared the TCR overlap between productive CD4Treg and CD4Tcon sequences. At baseline, the TCR overlap (Jaccard index) between CD4Tregs and CD4Tcons was low in both patients and HDs (Figure 7A). After 12 weeks of IL-2 therapy, the CD4Treg-CD4Tcon overlap index increased above the baseline level ($P = .03$) whereas there was no change in the CD4Treg-CD8 T-cell overlap. We performed a similar analysis using samples taken 12 weeks apart from an HD and found that CD4Treg-CD4Tcon TCR overlap did not increase (supplemental Figure 4). In all patients, we identified individual CD4Treg clones at week 12 that were detected exclusively in the baseline (week 0) CD4Tcon population (Figure 7B), further supporting the conversion of CD4Tcons into CD4Tregs during IL-2 therapy. However, the calculated TCR overlap index between week 0 CD4Tcon and week 12 CD4Treg samples increased in only some patients (supplemental Figure 5A). Thus, although IL-2 treatment enhances peripheral conversion of CD4Tcons into CD4Tregs, it likely remains a low frequency event. Conversion of CD4Tregs to CD4Tcons occurs to a lesser extent as we could also detect clones within the week 12 CD4Tcon population that were shared exclusively with baseline CD4Tregs (Figure 7B). As expected, there was almost no clone sharing between the CD4Treg and CD8 T-cell populations. There were no differences in CD4Treg-CD4Tcon clone sharing or TCR overlap between response groups (supplemental Figure 5B-C).

Discussion

Previous studies have demonstrated that administration of low-dose IL-2 leads to the rapid increase in peripheral CD4Tregs with little, if any, effect on CD4Tcons or CD8 T cells. We have observed this in patients with steroid-refractory cGVHD and others have seen similar effects in patients with various autoimmune diseases as well as in healthy volunteers.[11,12,32-37] In our studies, clinical responses were documented in ~50% of patients with steroid-refractory cGVHD even though all patients experienced similar increases in absolute numbers of circulating CD4Tregs. To examine this discrepancy between immunologic and clinical responses, we performed a detailed phenotypic and functional characterization of

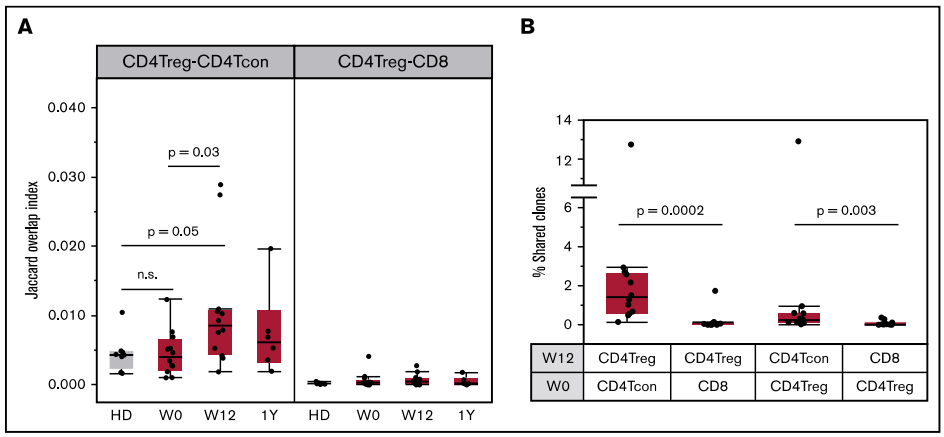

Figure 7. Clone sharing between different T-cell populations. (A) TCR overlap (Jaccard overlap index) between CD4Treg and CD4Tcon repertoires at baseline (W0), week 12 (W12), and 1 year (1Y). Overlap in HDs (gray) is shown for comparison. (B) Percentage of the total unique clones at week 12 (W12) that were detected within different baseline (W0) populations. Each column shows the percentage of week 12 clones within the various cell types that are exclusively shared with a particular baseline population. For example, the first column on the left shows the percentage of week 12 CD4Tregs clones shared exclusively with the baseline CD4Tcon population. There is significantly higher clone sharing between CD4Tregs and CD4Tcons than between CD4Tregs and CD8 T cells. In both panels, each dot represents a patient (n = 12) or an HD (n = 8). Box plots depict the 75th percentile, median, and 25th percentile values; whiskers represent maximum and minimum values, except for outliers. Two-sided Wilcoxon signed-rank test was used to assess significance between the different combinations.

CD4Tregs in serial samples obtained from patients with cGVHD receiving low-dose IL-2 enrolled on a phase 2 clinical trial.[11]

We found that low-dose IL-2 selectively induced several dramatic changes in CD4Tregs. These changes reflect the critical role of IL-2 in CD4Treg homeostasis that has previously been demonstrated in murine models.[38-40] The earliest effect we observed was the rapid proliferation of CM and EM CD4Tregs. This was followed by a more gradual increase in naive CD4Tregs that resulted in part from increased thymic output. IL-2 also induced significant functional effects in CD4Tregs including increased expression of BCL-2 and FOXP3 and enhanced in vitro suppressive activity. Importantly, these changes occurred in clinical nonresponders as well as responders, suggesting that clinical resistance was not because of CD4Treg resistance to the functional and homeostatic effects of IL-2.

Our studies are limited by the number of assays available to measure different CD4Treg functions in vitro. For example, the standard assay used to examine CD4Treg function only measures the ability of CD4Tregs to inhibit proliferation of stimulated CD4Tcons. This likely reflects the ability of CD4Tregs to sequester IL-2 secreted by activated CD4Tcons but does not examine other suppressive mechanisms potentially used by CD4Tregs in vivo. Furthermore, we could only perform our analyses on circulating T-cell populations and did not have access to the target tissues affected by cGVHD. We could not examine the functional activity of tissue-resident CD4Treg or CD4Treg homing to sites of cGVHD inflammation and injury. Although the effects of low-dose IL-2 have been characterized most extensively within CD4Tregs, IL-2 also leads to quantitative and qualitative changes in other cell types including natural killer cells and T follicular helper cells.[41] Further studies are needed to determine whether differences in natural killer and T follicular helper cells in patients receiving IL-2 therapy impact clinical response.

Quantitative assessment of the TCR repertoire provides another tool for investigating T-cell function and the impact of low-dose IL-2 therapy.[42] Using high-throughput TCRβ sequencing, we examined the TCR repertoire of highly purified CD4Tregs, CD4Tcons, and CD8 T cells. At baseline, patients with cGVHD had significantly lower TCR diversity within CD4Tregs compared with HDs. Low-dose IL-2 increased diversity within CD4Tregs to HD levels after 12 weeks of therapy. TCR diversity within CD4Tcons and CD8 T cells was similar between patients and HDs at baseline and did not change with IL-2. Our analysis is limited by the semiquantitative nature of the ImmunoSEQ platform[17,43] and by the low DNA input from CD4Treg samples.

IL-2 therapy increased TCR diversity while decreasing TCR evenness within CD4Tregs during the same period, suggesting expansion of specific CD4Treg clones. We further examined the expansion and contraction of clonotypes during IL-2 therapy by tracking the most abundant TCRβ sequences in serial samples. In contrast to CD4Tcons and CD8 T cells where there was very little change in clonotypes over time, low-dose IL-2 induced rapid and extensive turnover of individual CD4Treg clones. Notably, expansion of CD4Treg clones that were not detectable at baseline occurred more frequently in clinical responders compared with clinical nonresponders after 12 weeks of IL-2 therapy. By tracking individual T-cell clones from baseline to week 12, we also demonstrated that at least some CD4Treg clones that expanded during IL-2 therapy were recruited from the baseline CD4Tcon population. Thus, the increase in CD4Treg diversity during IL-2 therapy appears to be derived not

only from new clones emerging from the thymus and expansion of low-frequency clones, but also from peripheral conversion of CD4Tcons to CD4Tregs. These findings imply that expansion of multiple antigen-specific CD4Treg clones plays an important role in the reversal of cGVHD symptoms and suggests a mechanistic basis for clinical improvement in these patients.

In summary, low-dose IL-2 therapy had profound effects on CD4Treg homeostasis and function in all patients regardless of clinical response. Clinical improvement was associated with the rapid turnover of the TCR repertoire and emergence of CD4Tregs with new TCR clonotypes. These findings are consistent with the observation that CD4Tregs with a monoclonal TCR can suppress diverse effector T-cell populations and can prevent lethal autoimmunity in murine models.[44] Further studies to identify the specific antigens recognized by expanded CD4Tregs may help explain the mechanisms whereby low-dose IL-2 therapy mediates clinical reversal of cGVHD. Characterization of these CD4Treg TCRs and their target antigens may also lead to the development of CD4Treg-based cellular therapies that can induce tolerance and suppress activated immune effector cells. Such therapies will be relevant for the application of low-dose IL-2 in autoimmune diseases as well as in cGVHD.

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Authorship

Contribution: J.S.W., S.N., J.K., and J.R. conceived and designed the study; J.K., E.P.A., P.A., C.S.C., V.T.H., J.H.A., and R.J.S. provided patients; J.S.W., S.N., A.C.A., B.F., S.K., K.D., M.J.F., and C.G.R. collected and assembled data; H.T.K. analyzed and interpreted data and performed statistical analysis; and all authors wrote the manuscript.

Conflict-of-interest disclosure: J.K. reports research funding from Prometheus Laboratories Inc, Millennium Pharmaceuticals Inc, and Miltenyi Biotec GmbH; consulting fees from Amgen, Equillium, and Fortress Biotech; and advisory board fees from Takeda Pharmaceuticals Inc and Kadmon Corp. P.A. reports research funding from Bristol-Myers Squibb, Merck, Affimed, Adaptive, Roche, Tensha, Otsuka, and Sigma Tau; and consulting fees from Bristol-Myers Squibb, Merck, Infinity, Pfizer, and Affimed. J.R. reports research funding from Prometheus Laboratories, Neovii Biotech, Nektar, Merck, and Kite Pharma; and consulting income from Celgene, Avrobio, LifeVault Bio, Draper Laboratory, and TScan Therapeutics. The remaining authors declare no competing financial interests.

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Supplemental Table 1. DNA input amounts for TCR sequencing

Patient	Response group	TP	Cell type	DNA (ng)
1	R	W0	Treg	67
1	R	W0	Tcon	213
1	R	W0	CD8	69
1	R	W12	Treg	52
1	R	W12	Tcon	83
1	R	W12	CD8	45
1	R	1Y	Treg	216
1	R	1Y	Tcon	400
1	R	1Y	CD8	400
2	NR	W0	Treg	21
2	NR	W0	Tcon	280
2	NR	W0	CD8	400
2	NR	W12	Treg	36
2	NR	W12	Tcon	170
2	NR	W12	CD8	279
3	NR	W0	Treg	19
3	NR	W0	Tcon	92
3	NR	W0	CD8	103
3	NR	W12	Treg	53
3	NR	W12	Tcon	100
3	NR	W12	CD8	84
4	R	W0	Treg	15
4	R	W0	Tcon	43
4	R	W0	CD8	45
4	R	W12	Treg	76
4	R	W12	Tcon	119
4	R	W12	CD8	93
4	R	1Y	Treg	36
4	R	1Y	Tcon	155
4	R	1Y	CD8	229
5	R	W0	Treg	11
5	R	W0	Tcon	22
5	R	W0	CD8	17
5	R	W12	Treg	57
5	R	W12	Tcon	45
5	R	W12	CD8	19
5	R	1Y	Treg	35
5	R	1Y	Tcon	389
5	R	1Y	CD8	159
6	NR	W0	Treg	400
6	NR	W0	Tcon	59
6	NR	W0	CD8	400
6	NR	W12	Treg	26
6	NR	W12	Tcon	265
6	NR	W12	CD8	400
7	NR	W0	Treg	101
7	NR	W0	Tcon	400
7	NR	W0	CD8	400
7	NR	W12	Treg	90
7	NR	W12	Tcon	216
7	NR	W12	CD8	400
8	R	W0	Treg	24
8	R	W0	Tcon	48
8	R	W0	CD8	51
8	R	W12	Treg	29
8	R	W12	Tcon	18
8	R	W12	CD8	77
8	R	1Y	Treg	41
8	R	1Y	Tcon	400
8	R	1Y	CD8	169
9	NR	W0	Treg	400
9	NR	W0	Tcon	400
9	NR	W0	CD8	400
9	NR	W12	Treg	180
9	NR	W12	Tcon	400
9	NR	W12	CD8	122
10	R	W0	Treg	51
10	R	W0	Tcon	400
10	R	W0	CD8	350
10	R	W12	Treg	91
10	R	W12	Tcon	341
10	R	W12	CD8	355
10	R	1Y	Treg	27
10	R	1Y	Tcon	400
10	R	1Y	CD8	140
11	R	W0	Treg	14
11	R	W0	Tcon	400
11	R	W0	CD8	400
11	R	W12	Treg	233
11	R	W12	Tcon	400
11	R	W12	CD8	400
11	R	1Y	Treg	400
11	R	1Y	Tcon	400
11	R	1Y	CD8	400
12	NR	W0	Treg	39
12	NR	W0	Tcon	344
12	NR	W0	CD8	128
12	NR	W12	Treg	105
12	NR	W12	Tcon	144
12	NR	W12	CD8	21

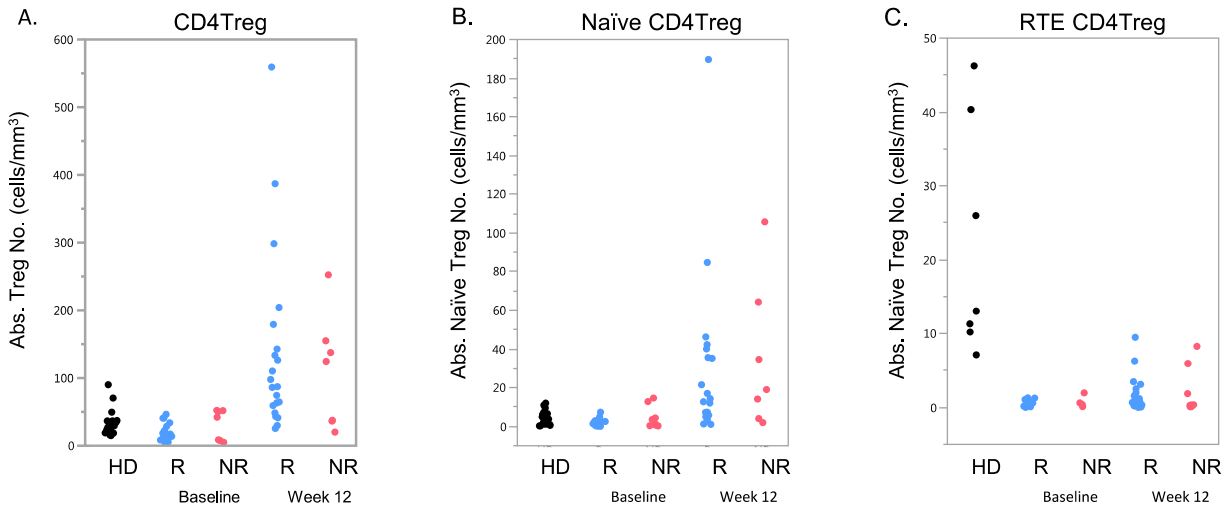
HD	Cell type	DNA (ng)
HD3	Treg	214
HD3	Tcon	400
HD3	CD8	400
HD4	Treg	132
HD4	Tcon	400
HD4	CD8	400
HD7	Treg	400
HD7	Tcon	400
HD7	CD8	400
HD8	Treg	416
HD8	Tcon	400
HD8	CD8	400
HD9	Treg	337
HD9	Tcon	400
HD9	CD8	400
HD10	Treg	222
HD10	Tcon	400
HD10	CD8	400
HD11	Treg	190
HD11	Tcon	400
HD11	CD8	400
HD12	Treg	183
HD12	Tcon	400
HD12	CD8	400

Supplemental Table 2. Patient characteristics for TCRβ sequencing samples

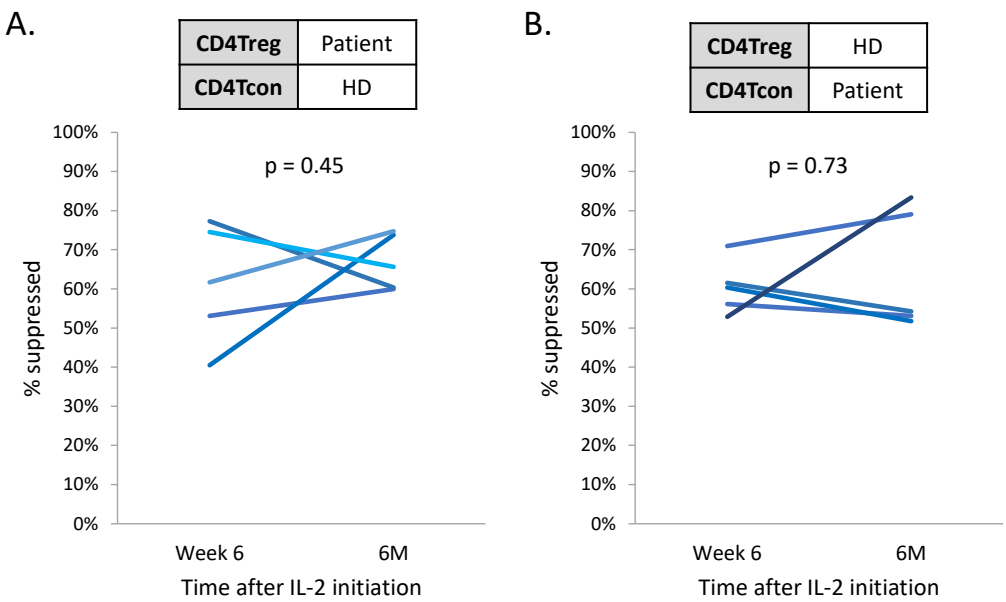
Patient	Age	Response	Patient sex	Donor sex	HLA typing*	Primary Disease	DRI	Conditioning Intensity	Days from HSCT to study	Days from cGVHD to study	Prednisone Dose**	No. of conc. cGVHD tx	No. cGVHD sites
1	44	NR	M	M	8/8 MUD	Mixed MDS / MPD	High	MAC	348	138	40	3	3
2	61	NR	M	F	8/8 MUD	CLL / SLL / PLL	Low	RIC	614	119	30	2	3
3	51	NR	F	F	8/8 MRD	MDS	Very high	MAC	657	399	2.5	2	1
4	68	NR	M	F	8/8 MUD	MDS	High	RIC	1191	1001	40	1	5
5	70	NR	F	F	8/8 MRD	AML	Intermediate	RIC	1163	226	30	2	6
6	34	NR	F	F	8/8 MUD	ALL	Intermediate	MAC	565	235	25	2	5
7	29	R	F	F	8/8 MUD	MDS	Intermediate	MAC	2145	1880	10	2	6
8	44	R	F	M	8/8 MUD	NHL	Low	RIC	389	181	30	3	4
9	53	R	F	M	8/8 MUD	AML	High	RIC	1065	707	17.5	2	3
10	51	R	F	F	7/8MUD	CLL / SLL / PLL	Intermediate	RIC	902	686	20	2	5
11	22	R	F	M	8/8 MUD	AML	Intermediate	MAC	735	348	20	2	6
12	49	R	M	M	8/8 MRD	Other Acute Leukemia	Intermediate	MAC	452	231	15	2	2

*At A, B, C, DRB1. Stem cell source was peripheral blood stem cells for all patients. R: Responder, NR: Non-responder; DRI: disease risk index (Armand et al, Blood 2014)
**At Baseline.

Supplemental Figure 1. Expansion of naïve CD4Treg and RTE CD4Treg is similar between clinical response groups. (A) CD4Treg. (B) Naïve CD4Treg. (C) RTE CD4Treg. Absolute cell counts are shown for each cell type. Each dot represents an individual patient or healthy donor. Black, healthy donor (HD); blue, clinical responders (R); red, clinical non-responders (NR). Two-sided Wilcoxon signed-rank test was used to assess significance between R and NR. There were no significant differences for any of the comparisons.

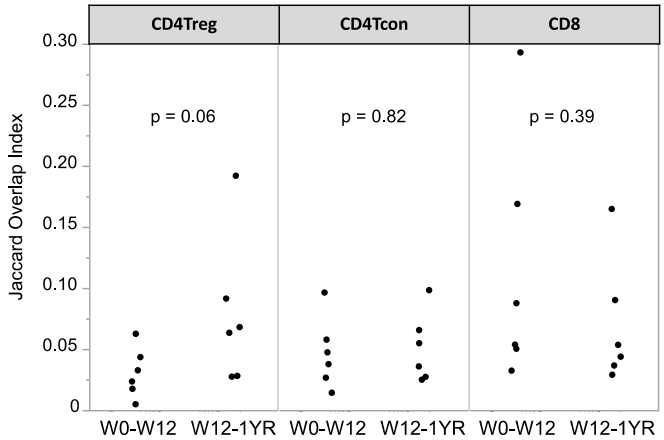


Supplemental Figure 2. *In vitro* CD4Treg suppressive activity remains stable during extended IL-2 therapy. (A) Patient CD4Treg versus HD CD4Tcon. No significant change in patient CD4Treg function from Week 6 to 6 months (6M) in Responders. (B) HD CD4Treg versus patient CD4Tcon. No significant change in patient CD4Tcon suppressibility from Week 6 to 6 months in Responders. HD, healthy donor. Each line represents an individual patient, N = 5.



Supplemental Figure 3. Turnover of CD4Treg clones during extended duration IL-2 therapy. (A) TCR overlap (Jaccard overlap index) between samples at week 12 versus extended duration (W12-1YR) compared with TCR overlap between baseline and week 12 (W0-W12) samples for CD4Treg, CD4Tcon and CD8 T-cells in clinical responders (n = 6). Each dot represents an individual patient. Though not statistically significant, there is a trend toward higher overlap during the extended duration period (W12-1YR) compared with the overlap during the initial 12 weeks of therapy (W0-W12) for CD4Treg.

A.



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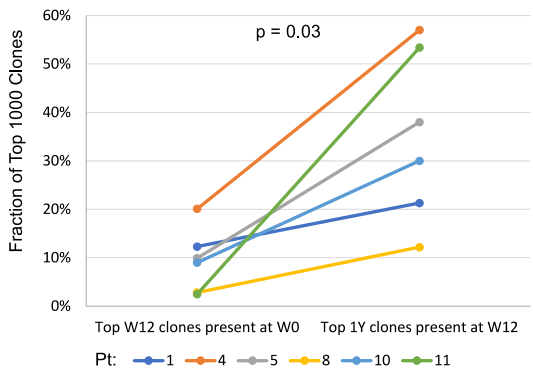
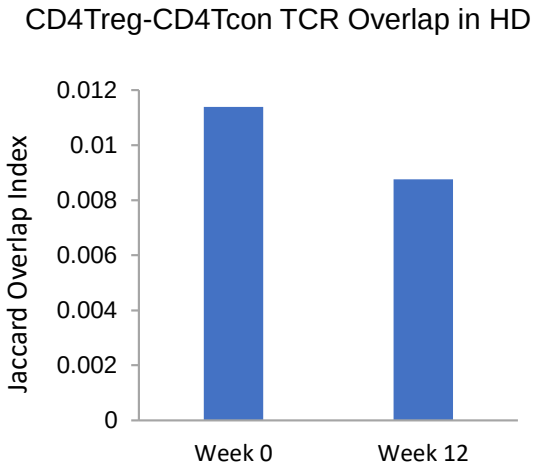
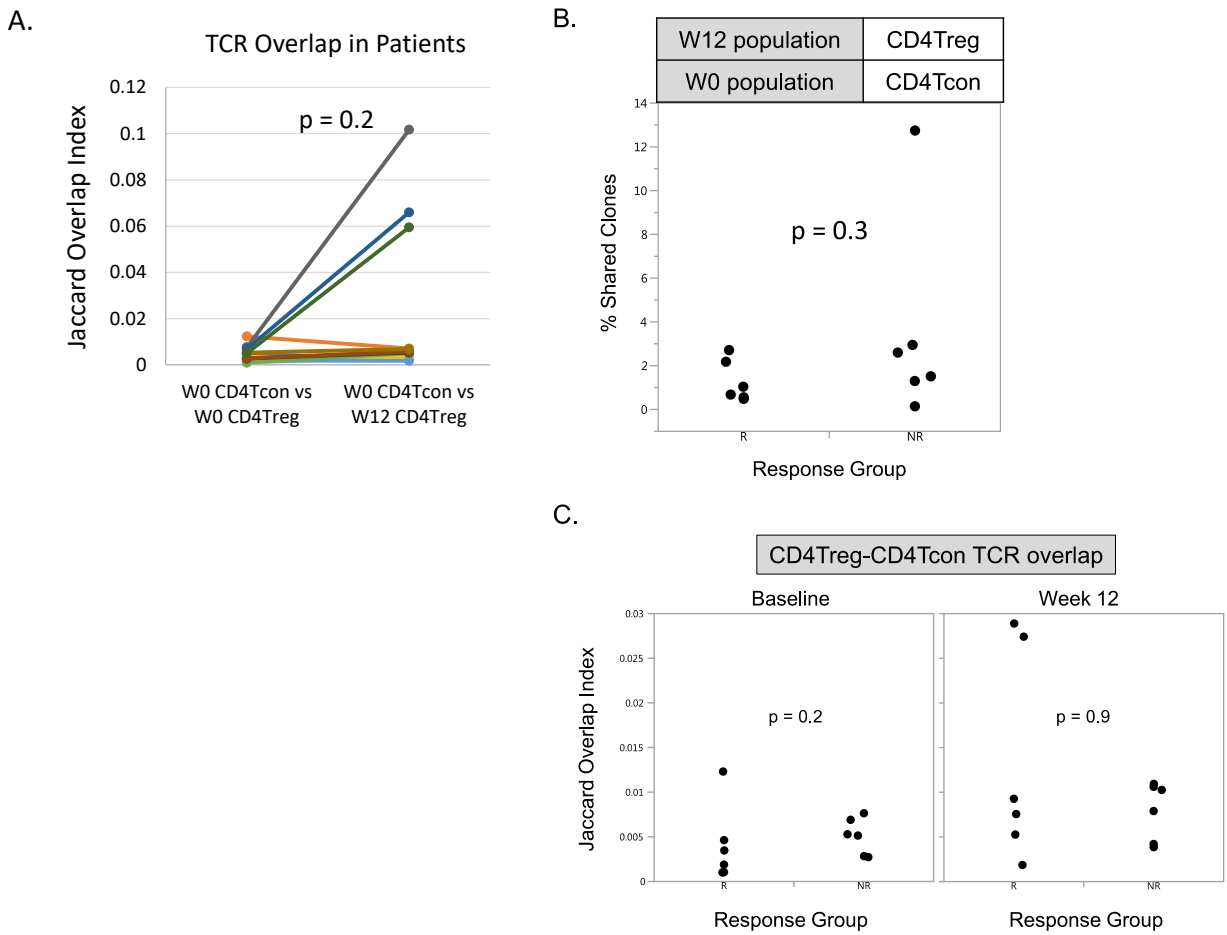


Supplementary Figure 4. TCR overlap in healthy donor control. TCR overlap between CD4Treg and CD4Tcon in a healthy donor (HD) sampled at 2 different time points (week 0 and week 12). TCR overlap (Jaccard overlap index) between the CD4Treg and CD4Tcon samples did not increase during the 12-week interval.



Supplementary Figure 5. Clone sharing and TCR overlap between CD4Treg and CD4Tcon. (A) TCR overlap between baseline (W0) CD4Tcon and W0 and week 12 (W12) CD4Treg in patients. For each patient, the TCR overlap (Jaccard overlap index) is shown between W0 CD4Tcon and W0 CD4Treg compared with the overlap between W0 CD4Tcon and W12 CD4Treg. There is increased TCR overlap of W0 CD4Tcon with W12 CD4Treg in some, but not all patients (p = 0.2). Each line represents a patient (n = 12). (B) Percent of W12 CD4Treg clones shared exclusively with the W0 CD4Tcon population in clinical responders (n = 6) vs non-responders (n = 6). (C) TCR overlap (Jaccard overlap index) between CD4Treg and CD4Tcon repertoires at baseline and W12 in clinical responders vs non-responders. Each dot represents a patient. Two-sided Wilcoxon signed-rank test was used to assess significance between the different combinations.