Universidade de Lisboa
Faculdade de Medicina

Translational research of regulatory T-cells within clinical studies

Tiago dos Reis Matos

Tese orientada pelo Prof. Doutor Luís Ricardo Simões da Silva Graça,
co-orientada pelo Prof. Doutor Jerome Ritz

Tese especialmente elaborada para obtenção do grau de
Doutor em Ciências Biomédicas com classificação de
Domínios Científicos e Tecnológicos de Ciências da Saúde

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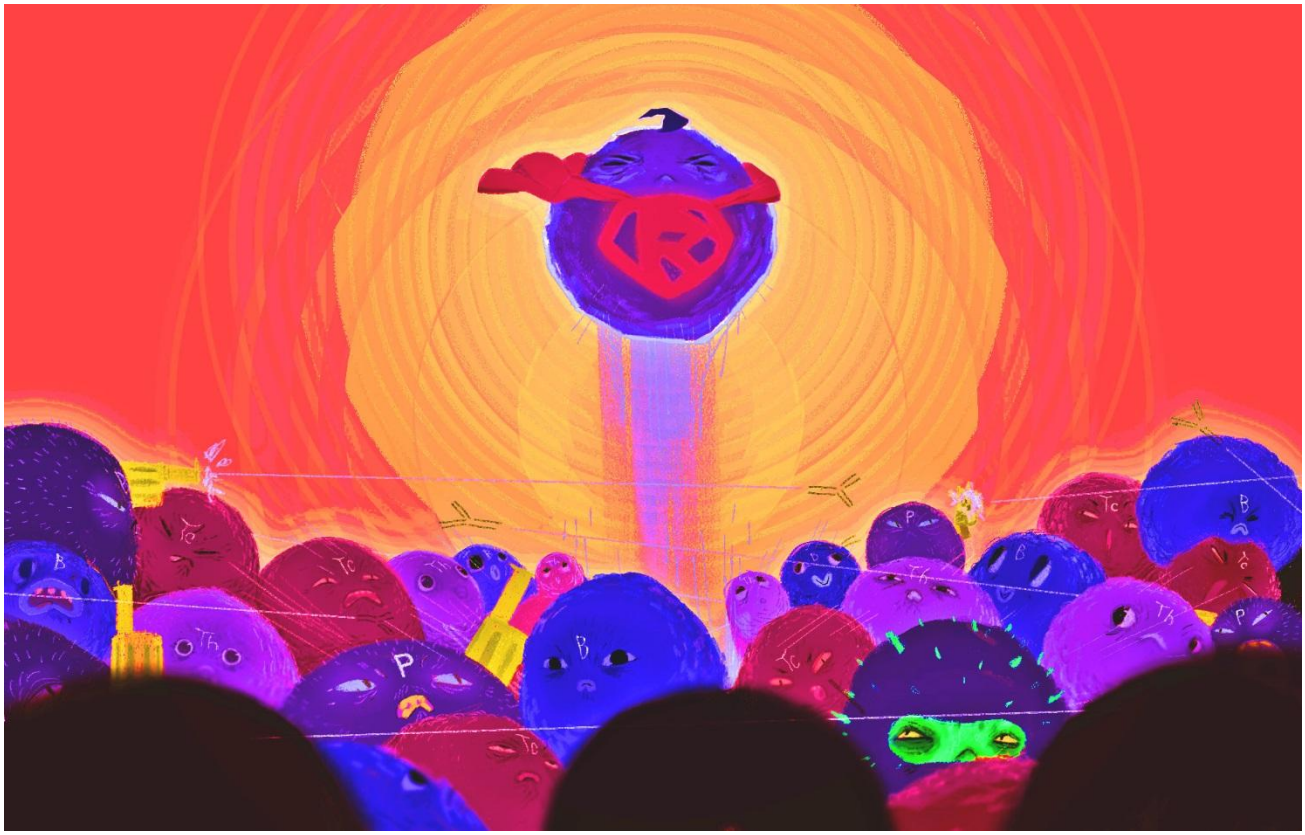
Pelo apoio e compreensão incondicionais, este trabalho é dedicado especialmente aos meus pais e irmã, e a todos os que tornaram este projeto real



What makes Superman a hero is not that he has power, but that he has the wisdom and the maturity to use the power wisely.

Christopher D'Olier Reeve

PREFACE

This thesis focuses primarily on Regulatory T cells (Tregs). We studied in detail these pluripotent and extremely complex cells in various conditions; we describe and characterize them, analyze their development, heterogeneity and functions. Foremost, I aim to convey the reader on why I see these cells as silent heroes within our healthy immune system (as represented in the previous page).

Treg cells are crucial in maintaining peripheral tolerance and immune homeostasis. A reduced number of Tregs or a decline in their functionality, can lead to various pathologies and diseases.

I always had a genuine interest in science. As soon as I started at the University of Medicine (Lisbon), I worked at different research labs to experience and learn about the various fields of biology and medical sciences. In the summer of 2013, I applied for a summer internship at Harvard Medical School. As part of the application, I came across the work of Prof. Jerome Ritz. I was immediately captivated by his research and findings on Tregs. His work was so interesting that I ferociously read his vast bibliography. It was incredible to me, how Tregs, being such a small population, had such a profound effect on the entire immune system and in our health. From genetic diseases, tolerance post-organ or stem cells transplants, autoimmune or inflammatory diseases and even cancer! These cells not only acted on the blood, but their dysfunction also affected multiple solid organs such as the skin, gastrointestinal tract, lungs and liver.

The following years spent at the laboratory of Prof. Jerome Ritz (Boston, USA), were some of the most profoundly enriching years of my career and life. In 2014 we started a collaboration with Prof. Luis Graca from the Institute of Molecular Medicine (Lisboa, Portugal). Prof. Graca had been my Immunology teacher during medical school. Since his course, I was impressed by his character and novel work on Tregs. This collaboration led to my PhD and current thesis.

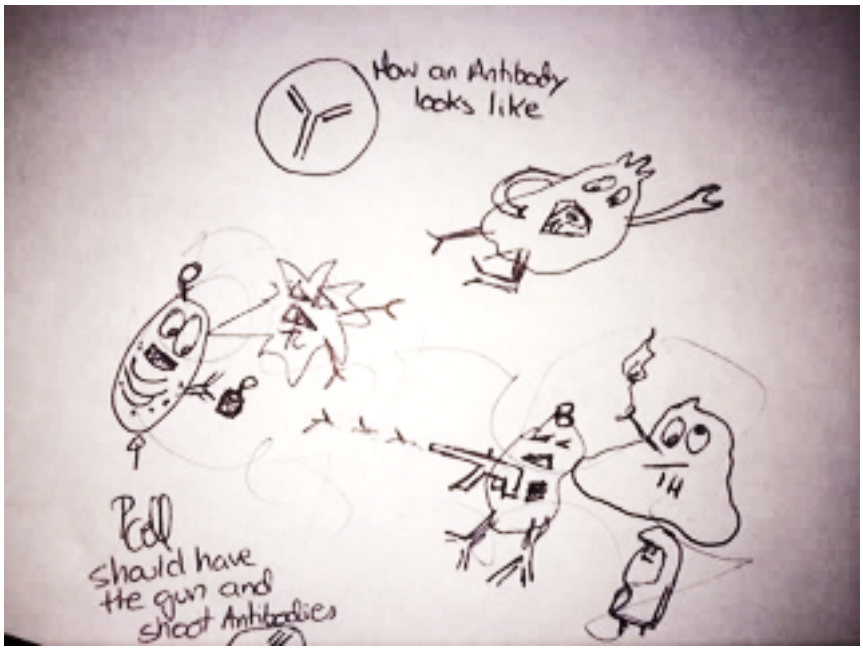
The work and knowledge acquired during my PhD, culminating in this thesis, was only possible thanks to the continued guidance and support of these two incredible mentors; Prof Jerome Ritz and Prof. Luis Graca. I am forever grateful to them!

The figure on the previous page is the results of a collaboration with Kamil Helbin, a young concept Artist and Animator. I met Kamil during the Roche Continents Program, Austria. Roche Continents Program invites the top-talented European students to experience a unique journey exploring sources of inspiration at the intersection of science and art, as well as the creative processes that drive innovation.
Motivated by this incredible meeting, I asked Kamil if it was possible to generate an illustration about the cells that I study. I explained their relevance and function. As we were having coffee, I drew my vision on a napkin (see image below). I was hoping to obtain a visual representation of my idea of Tregs; as heroes of the immune system. An image that I could use during my presentations, to intrigue the audience and leave a lasting memory.

Description of the image from previous page:
On the bottom there are various cells: in blue the "B" cells, in red the cytotoxic T (Tc) cells, in purple the T helper (Th) cells and in violet the plasma (P) cells that are shooting antibodies. Among the many cells, there is one cell in particular that is disguised, trying to remain unnoticed. It has the same round shape and similar color as other cells, but it is in fact an evil cancerous cell. It is camouflaged under a hoodie, just like a criminal! Looking carefully at the eyes, it's possible to see that it is in fact another type of cell; a bright green cell with spikes sticking out of its hoodie. From all the chaos stands out a single cell, a regulatory (R) T cell that will try to keep order and peace among the numerous immune cells. Hereby, we only have one Treg and multiple other cells, because indeed Tregs are a very limited small fraction of all immune cells. Nevertheless, even few Tregs are capable of maintaining tolerance and immune homeostasis.

To me this image is a beautiful example of Scientific Art. Displaying a successful collaboration between two distinct fields, such as Science and Art. An image that I share proudly with my non-immunologist friends and family. It helps me explain what I study and work on, and why I dedicated so many years, weekends and evenings to these fascinating Regulatory T cells.

Furthermore, it emphasizes the importance of collaborations within translational research. As previously mentioned, the most crucial collaboration within this thesis was between the laboratories of Prof Jerome Ritz (Boston, USA) and of Prof. Luis Graca (Lisbon, Portugal). The work in chapter 2 was only possible with the help of specialized bioinformatics from Harvard Medical School (Boston, USA). In chapter 3, we were assisted by skilled statisticians from the Department of Biostatistics and Computational Biology (Boston, USA). For chapter 5, we collaborated with not only with the pharmaceutical industry (Xenicos, The Netherlands), but also with Radboud University (Nijmegen, The Netherlands), University Hospital of Muenster (Muenster, Germany), Utrecht University, (Utrecht, The Netherlands) and The Icahn School of Medicine at Mount Sinai Hospital (New York, NY). In chapter 6, we collaborated with the laboratory of Prof. Jennifer R. Brown, a CLL specialist. Lastly, we are grateful to all the clinicians that cooperated with us, who examined the patients and collected the samples.



Above, the Figure that I drew to show Kamil my concept of regulatory T cells; as heroes of the immune system.

GENERAL INDEX

ABBREVIATIONS

CD	Cluster of Differentiation
BSA	Bovine Serum Albumin
GVHD	Graft-Versus-Host Disease
aGVHD	Acute Graft-Versus-Host Disease
cGVHD	Chronic Graft-Versus-Host Disease
SR-aGVHD	Steroid-Refractory Acute Graft-Versus-Host Disease
GVT	Graft-Versus-Tumor
HLA	Human Leukocyte Antigen
HSCT	Haematopoietic Stem Cell Transplantation
alloHSCT	Allogeneic Haematopoietic Stem Cell Transplantation
IL	Interleukin
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
RTE	Recent Thymic Emigrant
TCR	T Cell Receptor
TREC	T cell Receptor Excision Circle
Treg(s)	Regulatory T cell(s)
Tcon	Conventional T cells
CTLs	Cytolytic T cells
TGF-ß	Transforming growth factor beta
EM	Effector Memory
CM	Central Memory
TEMRA	Terminally Differentiated Effector T Cells
IPEX syndrome	Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked Syndrome
CCR	C-C Chemokine receptor
CLA	Cutaneous Lymphocyte-associated Antigen
CyTOF	Mass Cytometry by Time of Flight
ACCENSE	Automatic Classification of Cellular Expression by Nonlinear Stochastic Embedding
viSNE	Visualization Tool Based on t-Distributed Stochastic Neighbor

	Embedding algorithm
t-SNE	t-Distributed Stochastic Neighbor Embedding
FACS	Fluorescence-activated Cell Sorting
NK	Natural Killer Cells
FoxP3	Forkhead box P3
CB	Cord Blood

ACKNOWLEDGEMENTS

This thesis was only possible thanks to everyone who in one way or another supported, influenced, guided, and challenged me. I have therefore many people to thank, many more than those here mentioned. I am forever grateful to you all!

Dear **Prof. dr. Jerome Ritz,** dear **Jerry**, there will never be enough words to express how thankful I am for all that you have done for me. When I read your work for the first time, in 2013, I became immediately your biggest fan. I was applying for a summer internship of 8 weeks at Harvard Medical School, and your lab was listed as a potential hosting lab. I remember reading your articles with so much enthusiasm and excitement. Your work is truly revolutionary, and I knew then, that I wanted nothing more than to work in your lab, under your guidance! I am so extremely lucky for having the chance to work with you.

Thank you for dedicating your precious time to help me grow. You taught me how to write papers, make presentations, analyze, and present data. You guided me in achieving successful collaborations. You provided me with opportunities to become known among research peers. And when I considered moving to the Netherlands, you gave me strength and support, allowing me to take on a new adventure.

Most of all, thank you for believing in me and giving me a voice. You completely changed my future and I will always be your biggest and most loyal fan. I admire so much the genius that you are, the brilliancy and speed of your thoughts and creativity of ideas. Your determination and hard work are truly inspirational. Working with you was what made me the happiest during my time in Boston and it is what I will miss the most. I hope to make you proud as your mentee and I am forever grateful for being part of your legacy!!
THANK YOU SO SO VERY MUCH!

Dear **Prof. dr. Luis Graca,** dear **Luis**, you have been present throughout my entire academic career, from the early beginning of medical school as the immunology teacher, to now as my Ph.D. mentor. I am so grateful to have your guidance and support during this tumultuous and long journey. From the start, I was captivated by your passion for immunology. As a medical student, studying immunology, I didn't understand why we had to memorize codes with capital letters and numbers (as CD4 and CD8) that corresponded to cells that already had a name (T helper and Cytotoxic T cells). It was simply confusing and made no sense to me! The complexity of immunology, the vast number of still unresolved questions together with your passion and motivation kept me curious and made me ultimately appreciate immunology!

When I was in my 3rd year, I applied for a GAPIC internship within your laboratory, hoping to unveil immunological questions. However, another student got the position for that year. Nevertheless, when I came back to you, years later with the proposal of a Ph.D. you were very welcoming and supportive! Since then, you have supported me unconditionally. You gave me the freedom, guidance, and tools to conduct my own projects and collaborations. You had the patience to help me through my despair when projects failed. You continue to promote my growth as a scientist.

My admiration for you keeps on growing. I'm inspired by your multifaceted success as an Immunologist, the leader of an academic-medical department, and how you are an active member of so many international associations and thereby are shaping the future of Immunology! Thank you for always being there for me, for your support, and for pushing me to go the extra mile! THANK YOU VERY MUCH!

Meus queridos pais, **Maria José** e **Armelim,** muitíssimo obrigado pelo contínuo apoio. Eu sei que este meu percurso de estudos e carreira que escolhi, não tem sido nada fácil para vocês; vivendo em países distantes e em constante mudança... não segui uma carreira médica tradicional, aventurando-me frequentemente em novos projectos, arriscando em novas ideias. Obrigado por me apoiarem nesta que tem sido uma viagem turbulenta. Obrigado pela vossa paciência e apoio mesmo quando não percebem ou quando têm as vossas dúvidas. Obrigado por me incentivarem a estudar quando não queria, e fazer-me ver que o sucesso só é possível com muito trabalho e perseverança. O meu êxito só é possível graças aos melhores pais do mundo! Muitíssimo Obrigado! Amo-vos muito muito!!! Sou feliz graças a vocês

Meu Amorzão, **Martolas**, tu és uma das minhas maiores alegrias. Muito obrigado por toda a tua paciência, ajuda e apoio incondicional. Sei que posso contar sempre contigo e adoro cada momento que estamos juntos. Amo-te tanto! Obrigado pela pessoa que és e o que significas para mim. Tu és parte de mim. Amo-te muito muito muito!

.

RESUMO

As células T reguladoras (Tregs) são essenciais para manter um sistema imunológico saudável. Estas células compõem 5-10% das células CD4 no sangue periférico de indivíduos saudáveis e desempenham um papel crítico na proteção do indivíduo contra danos imunopatológicos após desafios inflamatórios ou imunológicos. Tregs são compostas por sub-populações heterogêneas que, juntas podem suprimir uma ampla gama de células efetoras por meio de vários mecanismos. Os seres humanos apresentam desde o nascimento uma ampla heterogeneidade fenotípica de Tregs, que aumenta durante a infância.

A capacidade das Treg de suprimir as células efetoras permite ao organismo tolerar transplantes e, quando esse equilíbrio cuidadoso é perturbado, pode levar à doença do enxerto contra o hospedeiro (DECH). A heterogeneidade das Tregs também aumenta rapidamente após o transplante de células-tronco hematopoiéticas (TCTH). No entanto, um equilíbrio preciso e coordenado entre várias células do sistema imunológico é necessário para prevenir a rejeição do transplante. Pacientes com DECH, não têm apenas menos células Treg, mas também menos subpopulações de Tregs e maior ativação de células efetoras.

Dois novos tratamentos contra a DECH são a administração de interleucina 2 (IL-2) em dose-baixa ou através de anticorpos conjugados a uma imunotoxina (CD3 / CD7-ricina A). A terapia com dose-baixa de IL-2 induz seletivamente a expansão das Tregs, melhorando as manifestações clínicas da DECH crônica. Esta também promove a homeostase de Treg sem ativar células efetoras. A terapia anti-CD3 / CD7-ricina A, que reduz principalmente as células T e natural killer (NK), tem uma taxa de resposta geral de 60%, com 50% dos doentes atingindo uma resposta completa. A taxa de sobrevivência global em 6 meses é de 60%. Após a eliminação de células efetoras, o sistema imunológico recupera com um repertório de células T diverso, permitindo a tolerância do transplante.
Os efeitos imunológicos selectivos e profundos de ambas as terapias na promoção da tolerância imunológica, podem ser usados numa ampla variedade de situações clínicas. Além disso, a metodologia de pesquisa translacional desenvolvida pode ser reaplicada em estudos clínicos futuros.

No capítulo 1, encontra-se a introdução geral. Começamos pela definição das células Tregs e explicação de como essas células são essenciais para manter a tolerância e preservação da homeostase do sistema imunológico. Em seguida, analisamos a relação entre as células Treg e o TCTH. Tregs têm a capacidade de mediar a tolerância ao transplante e, quando esse equilíbrio cuidadoso é perturbado, pode desencadear DECH. O desequilíbrio entre as células T efetoras e reguladoras que ocorre durante o DECH é explorado em detalhe. Além disso, destacamos a relevância da terapia de IL-2 em dose-baixa para pacientes com DECH e como a terapia recupera positivamente a população de Tregs. Por fim, descrevemos resumidamente os principais objetivos desta tese. No capítulo elaboramos sobre os objetivos e conteúdo da tese.

No capítulo 3, investigamos a diversidade do compartimento das células Treg em recém-nascidos, adultos saudáveis, durante o período de 2 anos após TCTH e em pacientes que sofrem de DECH crónica. Mostramos que os adultos têm quantidades significativamente superiores de Tregs no sangue do que aquelas presentes no sangue do cordão umbilical (CU), mas também têm subpopulações funcionais e de memória adicionais de Tregs que não estão presentes em CU. A quantidade de subpopulações de Tregs funcionais e de memória expande-se do nascimento até à idade adulta. Quanto aos pacientes após TCTH, o repertório subpopulações Treg de memória já se encontra presente após o transplante, enquanto a diversidade de células Treg ainda se expande dentro do período de 2 anos pós-transplante. Os pacientes com DECH demonstram significativamente menos subpopulações distintas de Tregs funcionais, propondo uma correlação entre a falta de heterogeneidade de Tregs com a DECH crónica.

No capítulo 4, demonstramos que a terapia com IL-2 em dose-baixa induz seletivamente a expansão de CD4 Tregs e melhora as manifestações clínicas da DECH crónica. Usando citometria de massa, demonstramos no capítulo 4 que baixas concentrações de IL-2 induzem seletivamente a fosforilação de STAT5 em células Helios CD4Tregs e células $CD56^{bright}$ $CD16^{-}$ NK in vitro e in vivo. Os efeitos da terapia de IL-2 em dose-baixa em células T CD4 convencionais e células T CD8 foram limitados ao aumento da expressão de PD-1 em células T efetoras de memória. Os efeitos seletivos da terapia IL-2 em dose-baixa nas células Helios CD4 Tregs e células NK $CD56^{bright}$ induzem a expressão constitutivamente de receptores de IL-2 de alta afinidade.

Reconhecendo que precisamos urgentemente de terapias mais eficazes para o tratamento de pacientes com doença aguda do enxerto contra hospedeiro, que não reage a esteróides (RE-aDECH) descrevemos**,** no capítulo 5, um ensaio clínico de fase I / II que examina a segurança e eficácia de uma nova terapia biológica para RE-aDECH. Uma combinação de anticorpos anti-CD3 e anti-CD7 separadamente conjugados com uma imunotoxina, a ricina A recombinante (CD3 / CD7-IT). Esta induz a depleção in vivo de células T e células NK, e suprime a ativação do receptor celular em células T. No dia 28 após o início da terapia com CD3 / CD7-IT, a taxa geral de resposta foi de 60%, com 50% atingindo uma resposta completa. A taxa de sobrevida global em 6 meses foi de 60%. O tratamento, que foi administrado durante 1 semana, causou depleção profunda, mas transitória de células T e células NK, seguido por rápida recuperação do sistema imunológico com um repertório diverso de células T e preservação de células T específicas capazes de identificar o vírus Epstein-Barr e citomegalovírus. O tratamento com CD3 / CD7-IT foi seguro e bem tolerado pelos doentes, com uma prevalência relativamente baixa de eventos adversos, os quais foram controláveis e reversíveis, como por exemplo hipoalbuminemia, microangiopática e trombocitopenia.

Por último, estávamos interessados em testar o mesmo painel de citometria de massa usado no capítulo 3, para estudar as células Treg em outras circunstâncias de distúrbio imunológico, não relacionadas ao TCTH. No capítulo 6, avaliamos o idelalisib como terapia de primeira linha para o tratamento da recidiva leucemia linfocítica crónica (LLC). Após um período de acompanhamento de 14,7 meses (em média), a hepatotoxicidade foi considerada um evento adverso frequente e frequentemente grave. Várias linhas de evidência sugerem que essa hepatotoxicidade foi imuno-mediada. Um infiltrado linfocítico foi visto em amostras de biópsia hepática retiradas de 2 indivíduos com transaminase, e os níveis das citocinas pró-inflamatórias CCL-3 e CCL-4 foram maiores em indivíduos com hepatotoxicidade. Uma diminuição nas células T reguladoras do sangue periférico foi observada em pacientes que sofreram de hepatotoxicidade durante a terapia, o que é consistente com um mecanismo imunomediado.

No capítulo 7 concluímos esta tese resumindo e discutindo os resultados.

SUMMARY

Regulatory T cells (Tregs) are essential elements of a healthy immune system. They comprise 5-10% of the peripheral blood CD4 T cell compartment in healthy individuals and play a critical role in protecting their host against immunopathological damage following inflammatory or immunological challenges. Tregs are composed of heterogeneous subsets that together can suppress a wide range of effector cell types through several mechanisms. Humans have since birth Treg maturation and phenotypic heterogeneity, which increases during our childhood.

The Treg capacity to suppress effector cells allows transplantation tolerance, and when this careful balance is disturbed, it can lead to graft-versus-host disease (GVHD). Treg heterogeneity also rapidly increases after hematopoietic stem cell transplantation (HSCT). However, a precise and coordinated balance between several immune cells is required to prevent transplant rejection. Patients with GVHD not only have fewer Treg cells but also fewer Treg subpopulations and higher activation of effector cells.

Two newer possible ways to treat GVHD, are with low-dose interleukin 2 (IL-2) or with antibodies conjugated to an immunotoxin (CD3/CD7-ricin A). Low-dose IL-2 therapy selectively induces the expansion of Tregs, improving the clinical manifestations of chronic GVHD. It promotes Treg homeostasis without activating effector cells.

The anti-CD3/CD7-ricin A therapy, which mostly depletes T and natural killer cells, has an overall response rate of 60%, with 50% achieving a complete response. The 6-month overall survival rate is 60%. After the profound depletion caused by the treatment, the immune system recovers with a diverse T cell repertoire leading to the transplant tolerance.

The profound but highly selective immunologic effects of both therapies in promoting immune tolerance may be used in a wide variety of clinical settings. Furthermore, the translational research methodology developed can be reapplied in future clinical studies, unveiling the defects that contribute to other immune dysfunctions.

Chapter 1 contains a general introduction. We start by defining Treg cells and explaining how these cells are essential for maintaining tolerance and preserving the immune system's homeostasis. Then, we analyzed the relationship between Treg cells and HSCT. Tregs have the ability to mediate transplant tolerance and, when this careful balance is disturbed, it can trigger GVHD. The imbalance between effector and regulatory T cells that occurs during GVHD is explored in detail. In addition, we highlight the relevance of low-dose IL-2 therapy for patients with GVHD and how the therapy positively recovers the Treg population. Finally, we briefly describe the main objectives of this thesis. In chapter 2 we specify the aims and outline of this thesis.

In chapter 3, we investigated the diversity of the Treg cell compartment in newborns, healthy adults, during the 2-year period after HSCT and in patients suffering from chronic GVHD. We have shown that adults have significantly higher amounts of Tregs in their blood than those present in umbilical cord blood (CB), but they also have additional functional and memory subpopulations of Tregs that are not present in CB. The number of subpopulations of functional and memory Tregs expands from birth to adulthood. As for patients after HSCT, the memory Treg subpopulation repertoire is already present after transplantation, while the diversity of Treg cells still expands within the 2-year post-transplant. GVHD patients present significantly fewer distinct subpopulations of functional Tregs, proposing a correlation between the lack of heterogeneity of Tregs and chronic GVHD.

In chapter 4, we demonstrated that low-dose IL-2 therapy selectively induces the expansion of CD4 Tregs and improves the clinical manifestations of chronic GVHD. Using mass cytometry, we demonstrated in chapter 4 that low concentrations of IL-2 selectively induce STAT5 phosphorylation in Helios CD4 Tregs cells and $CD56^{bright}$ $CD16^{-}$ NK cells in vitro and in vivo. The effects of low-dose IL-2 therapy on conventional CD4 T cells and CD8 T cells were limited to increased expression of PD-1 in memory effector T cells. The selective effects of low-dose IL-2 therapy on Helios CD4 Tregs cells and NK $CD56^{bright}$ cells induce the constitutive expression of high-affinity IL-2 receptors.

Recognizing that we urgently need more effective therapies for the treatment of patients with steroid-refractory acute graft-versus-host-disease (SR-aGVHD), we describe, in chapter 5, a phase I / II clinical trial that examines the safety and effectiveness of a new

biological therapy for (SR-aGVHD). A combination of anti-CD3 and anti-CD7 antibodies separately conjugated to recombinant ricin A (CD3/CD7–1T). This induces in vivo depletion of T cells and NK cells, and suppresses the activation of the cellular receptor in T cells. On the 28th day after the initiation of therapy with CD3/CD7-IT, the overall response rate was 60%, with 50% reaching a complete response. The overall 6-month survival rate was 60%. The treatment, which was administered for 1 week, caused profound but transient depletion of T cells and NK cells, followed by rapid recovery of the immune system with a diverse repertoire of T cells and preservation of specific T cells capable of identifying Epstein-Barr virus and cytomegalovirus. Treatment with CD3/CD7-IT was safe and well tolerated by patients, with a relatively low prevalence of adverse events, such as hypoalbuminemia, microangiopathy and thrombocytopenia, which were controllable and reversible.

Finally, we were interested in testing the same mass cytometry panel used in chapter 3, to study Treg cells in other circumstances of immune disorder, unrelated to HSCT. In chapter 6, we evaluated idelalisib as a first-line therapy for the treatment of chronic lymphocytic leukemia (CLL) relapse. After a follow-up period of 14.7 months (on average), hepatotoxicity was considered a frequent and often serious adverse event. Several lines of evidence suggest that this hepatotoxicity was immune-mediated. A lymphocytic infiltrate was seen in liver biopsy samples taken from 2 individuals with transaminitis, and the levels of pro-inflammatory cytokines CCL-3 and CCL-4 were higher in individuals with hepatotoxicity. A decrease in peripheral blood regulatory T cells was observed in patients who suffered from hepatotoxicity during therapy, which is consistent with an immune-mediated mechanism.

In chapter 7 we conclude this thesis by summarizing and discussing the results.

KEYWORDS

Immunology, T cells, Regulatory T cells, Translational research, Clinical studies

PALAVRAS-CHAVE

Imunologia, células T, células T reguladoras, investigação translational, estudos clínicos

CHAPTER 1 - INTRODUCTION

1.1 Immune regulation and Autoimmunity

A crucial evolutionary requisite during the evolution of the mammalian immune system was to be able to protect us from internal malignancies and foreign pathogens, while avoiding deleterious immune reactions against the self.[1] Hence, the immune tolerance had to become a safety system capable of remaining immune unresponsiveness to autoantigens and self-tissues.[2,3] The defect of self-tolerance can cause the immune system to react against own healthy cells prompting autoimmune reactions. This seems to happen mostly due to genetic predisposition, sometimes it occurs for example as response to environmental factors, including exposure to pathogens exhibiting molecular mimicry.[4] The immune tolerance is categorized into central tolerance and peripheral tolerance.[5] Central tolerance refers to the depletion of T and B cell clones with high self-affinity in the primary lymphoid organs: the bone marrow and thymus. As the name suggests, peripheral tolerance occurs in peripheral tissues and in secondary lymphoid organs, such as lymph nodes, spleen and mucosal associated lymphoid tissues. It inactivates autoantigen recognizing B or T cells that have escaped the selection process of central tolerance, by inducing anergy, apoptosis or conversion into immunosuppressive regulatory cells. One of the most studied suppressor immune cells, the regulatory T cells, can very efficiently control autoreactive B and T cells.

1.2 Regulatory T-cells: key players in tolerance and immune homeostasis

Regulatory T cells (Tregs) are the silent heroes within our healthy immune system. Treg cells are as pluripotent as they are complex. Tregs are able to suppress a range of effector cell types through several mechanisms, both direct and indirect, ensuring peripheral tolerance and immune homeostasis.[15,16] Tregs were for the first defined as $CD4^+$ $CD25^+$ T cells that were essential on its own to prevent autoimmune disease in mice after transferring T cell suspensions in thymectomized nude mice.[17] The identification of the interleukin-2 receptor α-chain (CD25) as a highly specific cell markers of Treg cells in mice, promoted the investigation of these same cells in humans. Human Tregs similarly presented in vitro suppressive properties towards effector T cells.[18-24]

1.3 Origin, maturation and definition of regulatory T cells

Regulatory T cells accumulate to 5-10% of the peripheral blood $CD4^+$ T cell compartment in healthy individuals.[15] They originate in the thymus and they are defined and identified by expression of the biomarkers CD3, CD4, high levels of surface CD25, low levels of surface CD127 (α-chain of the interleukin-7 receptor) and intracellular Forkhead box P3 (FoxP3) (table 1). FoxP3 is a forkhead/winged helix transcription factor and responsible for the development and regulation of the immunosuppressive properties of Tregs.[7] Even the induction of FoxP3 expression in $CD4^+$ $CD25^-$ T cells is sufficient to provide suppressive functions to these cells.[25-27]

These cells can be further divided into three main maturation categories: naïve, central memory (CM) and effector memory (EM).[16] The expression of different levels of biomarkers allows us to distinguish stages of Tregs. Naïve Tregs express high levels of CD45RA and low levels of CD45R0 and FoxP3, whereas memory Tregs oppositely are $FoxP3^{hi}CD45RA^-CD45R0^+$.[18,19] In healthy individuals approximately 30% of the regulatory T cell compartment is of naïve nature.[20] As a person ages, the relative amount of naïve Tregs decreases while the proportion of memory Tregs increases. CD45RA naïve Tregs co-express low FoxP3 and low CD25, which relates to their low suppressive capacity until they encounter co-stimulatory antigen and become activated. Activation induces differentiation into effector highly immune suppressive Tregs with high FoxP3 expression levels.

Cutting-edge mass cytometry has recently offered a new insight into the heterogeneity of adult regulatory T cells in peripheral blood, distinguishing more than 22 functionally different subpopulations of Treg cells.[24]

Widely considered the Treg key cytokine, Interleukin-2 (IL-2), plays a critical role in the generation, development, proliferation, expansion, functional activity, and survival of Treg cells.[21,22] This is proven by the rise of systemic autoimmunity in mice deficient in IL-2.[23] Moreover, the number of Tregs is dramatically reduced in these deficient mice, with a decrease of around 50% in the thymus and an even higher 90% in the peripheral lymphoid tissues.

Since Tregs structurally express high levels of CD25 (the IL-2 receptor α-chain and component of the IL-2 receptor), unlike effector T cells, they create a high-affinity receptor for IL-2. However, Treg cells do not produce IL-2, making these cells inherently dependent on exogenous sources for Interleukin-2. This is counteracted by the Treg cell expression of a high-affinity receptor, ensuring these cells respond to low concentrations of IL-2. Hence, CD25 and IL-2 are essential for expansion and maintenance of Tregs.

Marker	Description
Transcription factor	
FOXP3 (forkhead box P3)	Master regulator of Treg development and function
Activation/expansion	
CD25	IL-2 receptor α-chain • Component of the high-affinity IL-2 receptor • Constitutively expressed in murine and human Tregs
OX40 (CD134)	Member of the tumor necrosis factor (TNF) receptor superfamily • Co-stimulatory molecule
GITR	Glucocorticoid-induced TNF receptor • Co-stimulatory molecule
TNFRSF25	TNF receptor superfamily member 25, also known as death receptor 3 (DR3) • Co-stimulatory molecule
CD127	IL-7 receptor α-chain • Human Tregs consistently express lower levels of CD127 than other CD4 + cells • $CD127^{lo/-}$ expression serves as a marker to distinguish Tregs from other CD4 + T cells
Homing	
CD62L	L-selectin
CCR4	Chemokine receptor
CCR6	Chemokine receptor
CCR9	Chemokine receptor
Suppressive and effector function	
CTLA4 (cytotoxic T-lymphocyte-associated protein 4)	Member of the immunoglobulin superfamily • Inhibits T cell activation and proliferation via binding to CD80/CD86
Granzyme A and B	Serine proteases that induce apoptosis by cleaving critical substrates in the target cell
IL-10	Anti-inflammatory cytokine
CD39	Ecto-nucleoside triphosphate diphosphohydrolase • Hydrolyzes adenosine triphosphate and adenosine diphosphate into adenosine monophosphate (AMP)
CD73	Ecto-5′-nucleotidase • Catalyzes the dephosphorylation of extracellular AMP into adenosine • Release of adenosine enhances Treg immunosuppressive functions

Table 1. Key Treg Markers[28]

1.4 Suppression properties of regulatory T cells

Regulatory T cells can suppress effector T cells via four different modes of action[29,30]: 1) the production of inhibitory cytokines such as IL-10 and TGF-ß, 2) cytolysis, by secreting granzyme B, 3) metabolic disruption by depleting IL-2 from effector T cells, and 4) modulation of dendritic cells, which in turn also inhibit effector T cells. However, the type of regulatory mechanism used by Treg cells depends on the specific disease, local inflammatory status and their anatomical localization.

The expression of FoxP3 seems to influence in a dose dependent way the suppression function of Tregs, as noticed in mice studies, where by diminishing the expression of FoxP3 expression mice started developing fatal autoimmune diseases.[31] The intricate balance between immune response and regulation can be upset by both a reduced number of Tregs as well as a decline in their functionality, potentially leading to autoimmune related pathology.[32] The most extreme example is the IPEX syndrome, caused by mutations in the FoxP3 gene.[33] IPEX is short for immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, which is entirely caused by the complete dysfunction of regulatory T cells. On the other hand, Treg mediated immunosuppression is a key mechanism used by tumors in evading our body's defenses, possibly causing cancer progression.[34] In melanoma for example, numerous studies have shown an excess of Tregs in peripheral blood of patients with metastatic melanoma.[35] These Tregs have been shown to be functionally suppressing the immune response.

1.5 Regulatory T cells in peripheral tissues

Regulatory T cells not only are found in circulation but also reside in secondary lymphoid tissues and in the majority of the non-lymphoid tissues, both in the presence or absence of inflammation.[36] The 21st century has seen many studies published describing the various Treg homing receptors used for effective tissue distribution. These include but are not limited to CCR7 for migration to lymph nodes and the spleen, α4β7 integrin and CCR9 for migration to the intestines and the associated lymph nodes, and CCR4 and CLA, for migration towards the skin.[36] The skin, being the first and foremost line of defense against external harmful agents, is especially vulnerable to defects in regulatory T cells. Because of its role in immune homeostasis the skin features a large amount of resident Tregs and

most of the circulating Tregs carry functional skin-homing receptors.[37,38] In short, regulatory T cells are highly important, truly versatile and omnipresent in the immune system throughout our entire body, leading to numerous pathologies and diseases when they fail.

1.6 Allogeneic hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) is a curative method for treating benign as well as malignant hematologic diseases. Among them are several types of leukemia, lymphoma and hereditary immunodeficiencies. Every year over fifty thousand patients receive HSCT using either their own stem cells, called autologous HSCT, or stem cells from a donor, termed allogeneic HSCT (alloHSCT).[39] Fifty-three percent of all stem cell transplantations are autologous, the remaining forty-seven percent are allogeneic. A key outcome of leukemia treatment with allogeneic HSCT is the graft-versus-leukemia effect, in which the immune response arising from the transplanted stem cells attacks leukemic cells.[40]

Since only half of HSCT can be achieved with cells by autologous transplantation, the need for transplantation of cells and tissues from a foreign individual through allogeneic transplantation can lead to immunological rejection. The success of alloHSCT treatment depends on the engraftment of donor hematopoietic stem cells and the complete reconstitution of the immune system deriving from those cells.

1.7 Immune reconstitution after allogeneic hematopoietic stem cell transplantation

The reconstitution of the array of immune cell subsets does not progress synchronously.[41] After the conditioning regimen preceding HSCT, which may include chemotherapy, monoclonal antibody therapy and entire body radiation, an aplastic or neutropenic phase follows, lasting between 14-30 days depending on the type of transplantation. This phase carries a lot of risk for bacterial infection. Cellular immune deficiencies cause the main issues until around the 100th day post-transplant with reduced numbers of cytotoxic

lymphocytes, natural killer cells and T cells, often giving rise to viral and fungal infections.

The first steps in the recovery of the T-cell compartment depends on expansion of memory T cells in the periphery, which is powered by cytokines and alloreactive antigens, until thymic production of naïve T cells restarts. CD4 T cells, including Tregs, reconstitute at a later stage than CD8 T cells since they rely more on production of naïve cells in the thymus, inversing the CD4/CD8. Reacting to this $CD4^+$ lymphopenia, Tregs undergo higher levels of proliferation than conventional $CD4^+$ T cells (Tcons), but in doing so become more susceptible to Fas-mediated apoptosis.[42] In the first 9 months after HSCT, Tregs expand at a fast rate and achieve normal levels. However, after the initial Treg population expansion from the memory Tregs, a slight decline is observed due to the lack of naïve thymic cells.

The immune system reconstitution must include critical regulatory elements as well as highly diverse populations of effector cells. Tregs have the ability to mediate transplantation tolerance, as has been shown in animal models.[43] Animals that received host or donor Treg in combination with bone marrow transplantation were less likely to suffer from rejection in the short and long term.

When this careful balance is upset, as is the case all too often unfortunately, the newly engrafted immune system can turn against its host and target the recipient's own tissues. This affliction is named graft-versus-host disease (GVHD), a severe immunopathological disease. Hence, GVHD remains the main barrier to use HSCT more widely to cure many nonmalignant conditions.

1.8 Graft-versus-Host disease: an imbalance between effector and regulatory T cells

GVHD is a leading cause of morbidity and mortality in patients after HSCT. It occurs in roughly half of patients that receive alloHSCT and kills one tenth of the people suffering from it. There are two types of GVHD: acute and chronic graft-versus-host disease. These diseases are two distinct entities, chronic GVHD does not develop from acute GVHD and both show different symptoms and have separate pathophysiological profiles.[44,45]

The recognition of regulatory T cells as potent immunosuppressant cells brought a new era of studies exploring the potential impact of allogeneic Tregs in GVHD experimental models. The group of dr. Bruce Bazar was a pioneer in demonstrating both in vitro and in vivo the capacity of Treg cells to regulate tolerance to alloantigens.[46] Firstly, in 2001 this group showed that $CD4^+$ $CD25^+$ Treg cells can induce tolerance for alloantigens. They used in vitro tolerance induction assays, whereas co-culturing $CD4^+$ T cells obtained from lymph nodes from B6 mice with non-lymphocytic allogeneic splenic cells from a Bm12 mice, it led to a prominent proliferative response. Those cells had at least three amino acids different within their MHC class II region.[46] They noticed by depletion attempts, that removing $CD4^+$ $CD25^+$ T cells (Tregs) from the lymphocytic cell pool, that these cells were crucial to obtain tolerance towards alloantigens. The same team followed with in vivo studies, where $CD4^+$ $CD25^+$ Tregs protected mice against induced GVHD.[47] T-cell deficient bone marrow of B6 mice together with purified CD4 T cells were injected in irradiated bm12 mice to induce GVHD. GVHD development was greatly delayed when $CD4^+$ $CD25^+$ Tregs were simultaneously injected in equivalent numbers with remaining $CD4^+$ T cells. Furthermore, when $CD4^+$ $CD25^+$ Tregs were expanded ex vivo and then injected in high cell numbers to the allogeneic transplantation mouse model, it resulted in greater inhibition of GVHD and lead to prolonged survival. It started a new era of studies referring to Tregs as promising solving therapeutic for transplant rejection. Follow-up studies from other groups established that it was crucial to have the $CD4^+$ $CD25^+$ Tregs from the same donor in order for these to be functional and protect from otherwise lethal GVHD.[48,49] It became also more evident that IL-10 was important for Tregs to maintain their inhibitory capacity.

Although Treg cells had a very promising immune tolerance role after transplantation, it was equally crucial that these cells would not jeopardize the anti-tumor activity of conventional T cells (Tcon, $CD4^+$ $CD25^-$ and $CD8^+$ $CD25^-$ T cells). Edinger et al. were capable to demonstrate in a major MHC (class I and class II) mismatched transplant model (B6BALB/c) how indeed Tcon, when transplanted together with Treg, maintained their capacity to generate cytokines and comparable activation levels, relative to Tcon transplanted without Tregs.[50] In vitro experiments confirmed how Tcons cultured together with Tregs do not lose their cytotoxic function against leukemia cells, while Treg simultaneously suppressed alloantigen proliferation of Tcons. It showed how regulatory T

cells do not inhibit the Tcon activation and still can suppress their proliferation. Furthermore, using a haploidentical murine transplant model, it became clear that Tregs must be unique to the recipient-type alloantigens to prevent GVHD.[51] Interestingly, Tregs were also shown to, in some cases, diminishing the GVT development, depending on multiple factors such as tumor strain, rate of growth or location. Recently a newer study reconfirmed how GVT for leukemia is still preserved even when Treg cell expansion signals are strongly activated. In this study mice was treated with injections of IL-2 and TL1A-iG, which respectively stimulate the CD25 and tumor necrosis factor superfamily receptor 25 (TNFRSF25) pathways, inducing in vivo expansion of the Tregs, that were then transferred to a major MHC mismatched mouse (transplant mouse model, B6 -> BALB/c).[52]

These series of experiments showing how Tregs can positively impact GVHD without affecting the GVT effect, encourage continuous studies for therapeutic use if Treg cells for treatment and prevention of GVHD.

1.9 Overview of acute Graft-versus-Host disease

Acute GVHD (aGVHD) occurs in 35-80% of patients after alloHSCT and even though it can affect all organs, it affects mostly the skin, gastrointestinal tract and liver. It generally happens within the first 100 days after receiving a transplant. The myeloablative conditioning regimens used prior to transplantation cause tissue damage releasing proinflammatory cytokines, which can activate host antigen-presenting cells and increase their capacity to express donor-recipient polymorphic tissue antigens to the donor alloreactive T cells.[53] However, there are multiple factors that make this time-frame classification more abstract, since we should consider phenotypes of overlap with chronic GVHD. Those factors are for example the vast heterogeneous population of patients and medical conditions, the conditioning regimens, type of transplant, donor and donor source, GVHD prophylaxis regimens and adjuvant immunotherapy.[54] Despite terrific advances in novel therapies and prophylactic regimens, aGVHD persists as the second cause of death after disease relapse.[55]

Until recently, it was assumed that aGVHD was primarily mediated by donor effector T cells. However, in a publication from last year, we show that host tissue-resident T cells may play a previously unappreciated pathogenic role in acute GVHD.[56] While blood of patients with aGVHD contained primarily donor-derived T cells, most T cells in the skin were host derived. Host T cells were present in all skin and colon acute GVHD specimens studied, yet were largely absent in blood. We observed acute skin GVHD in the presence of 100% host T cells. Analysis demonstrated that a subset of host T cells in peripheral tissues were proliferating ($Ki67^{+}$) and producing the proinflammatory cytokines IFN-γ and IL-17 in situ. Comparatively, the majority of antigen-presenting cells (APCs) in tissue in acute GVHD were donor derived, and donor-derived APCs were observed directly adjacent to host T cells. Furthermore, we demonstrated in a humanized mouse model that host skin-resident T cells could be activated by donor monocytes to generate a GVHD-like dermatitis.[56]

Historically, based on peripheral blood studies, the pathophysiology of aGVHD has been summarized in three phases: 1) an afferent phase, 2) an efferent phase and 3) an effector phase.[57] As mentioned previously, the tissue damage caused by the pre-conditioning regimens and the immune reaction to the allograft can lead to an exaggerated inflammatory response activating the host antigen presenting cells, such as dendritic cells and Langerhans cells. The excessive release of cytokines like TNF alpha, IL6 and IL1, increases the cell surface adhesions molecules on the recipient cells and up regulates the expression of MHC antigens. The APCs will present co-stimulatory alloantigens to which the donor T cells will react to.[58,59] Damage of solid tissues has been notice, most prominently the intestinal epithelium with alteration of the microbiome, which releases cytokines and causes injury of gut paneth cells and immune stem cells. Although studies continue supporting the tissue damage as a crucial role in the afferent phase, mostly based simply on the observation of gut damage prior to aGVHD, this idea has been contested. Consider the fact that donor lymphocyte infusion does not involve a conditioning regimen and still can lead to aGVHD. Furthermore, reduced-intensity conditioning regimens do not have less incidence of severity of aGVHD.[60]

The efferent phase will follow with expansion and trafficking of T host T cells.[61,62] CD8 T cells seem to be mostly activated by host hematopoietic APCs, while grafted CD4 T cells can also be activated by non-hematopoietic APC, for example within the gastrointestinal

tract.[58] At last, the effector phase initiates cytolytic donor effector T cells generating a complex cascade of multiple cellular and inflammatory effectors causing further tissue damage and end-organ damage.[63] Many effector cells are implicated in this phase, such as natural killer cells (NK), NK T cells, neutrophils and macrophages.[64,65]

1.10 Overview of chronic Graft-versus-Host disease

Chronic GVHD (cGVHD) arises usually after 100 days and can affect a vast array of organs and tissues, including the mucosa, lungs, heart, muscles, liver, gastrointestinal tract and the skin. Depending on the tissue affected by GVHD the symptoms range from ulcerations and severe diarrhea to jaundice and pericarditis. Chronic graft-versus-host disease is the main cause of late mortality and morbidity after allogeneic hematopoietic stem cell transplantation. Compared with the advances in our knowledge of aGVHD, our understanding of cGVHD has not expanded as prolific. Mild forms can be managed with local or low-dose systemic immunosuppression and have no negative affect on long-term survival. More severe forms of the disease require a high amount of care and decrease survival.

For a long time now, it has been shown that the loss of balance between T-lymphocyte subsets, the overexpansion of pathological groups and lack of regulation causes cGVHD. Succinctly, cGVHD occurs when donor B cells and effector T cells fail to create immune tolerance to auto- and alloantigens in the recipient. The contributing role of allogeneic T cells to chronic GVHD is supported by the observation that T-cell depletion leads to less cGVHD after HLA-matched sibling marrow transplantation than after peripheral blood stem cell transplantation and donor lymphocyte infusions.[66]

Recently there have been several developments in uncovering the role of regulatory T cells in cGVHD. In patients with cGVHD, a profound decrease in Treg numbers after alloHSCT has been observed and is said to be critical for immunological tolerance of cGVHD after alloHSCT.[67] The inverse relationship between Tregs and cytolytic T cells (CTLs) in the post-transplant period is key to the development of chronic graft-versus-host disease. Hence, a decrease of Tregs and higher quantities of CTLs result in cGVHD. On the other hand, CTLs are necessary to secure the graft-versus-tumor (GVT) effect, preventing the cancer recurrence. The balance between Tregs and CTLs is a complex, precise but crucial

balance, influenced by multiple factors. Its' disruption can either lead to cGVHD or cancer recurrence. The optimal strategy to achieve this fine balance between GVT and avoid GVHD has not yet been determined. Currently there are various heterogeneous pharmacological protocols to induce immunosuppression, using for example methotrexate, calcineurin inhibitors or cyclophosphamide. The immune modulation using Treg cells could provide a partial beneficial alternative, avoiding GVHD without impairing GVT.

Altering the relative and absolute numbers and effect of regulatory T cells after alloHSCT has been at the frontier of the development of new treatment strategies against cGVHD. For example, taking advantage of the sensitivity of Tregs to IL-2, it has been shown that low-dose IL-2 administered to patients suffering from chronic GVHD results in the sustained expansion of Tregs.[68] This is achieved without significantly augmenting the Tcon or $CD8^+$ T cell compartments and clinically improved more than 50% of cGVHD patients. In order to develop possible novel therapies to prevent or treat chronic graft-versus-host disease after allogeneic stem cell transplant it is paramount to obtain further evidence on characterization and diversity of regulatory T cells in patients after alloHSCT and during cGVHD.

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CHAPTER 2 – AIMS AND OUTLINE OF THE THESIS

2.1 Aims of the Thesis

The core aim of this thesis was to study and characterize in depth regulatory T cells in different settings and through different and novel research techniques. It intended to supply the thesis's author with (1) a vast knowledge of translational research techniques and clinical trials, and (2) a detailed knowledge of the immune mechanisms related to Tregs.

Different clinical settings:

We started by studying Tregs in umbilical cord blood (CB) and then in healthy adults. Tregs were analyzed through a 2-year period post-alloHSCT and both during acute- and chronic-GVHD. We studied Tregs in vitro and in vivo, subjected to two different novel biological therapies for GVHD (low-dose of IL2 and CD3/CD7-IT). Lastly, we study the impact of idelalisib on the Treg compartment in patients with chronic lymphocytic leukemia (CLL).

Different translational research techniques:

Sample preparation and storage. Mononuclear cells isolation from peripheral blood and CB. Cells isolation by Sorting Flow Cytometry or Magnetic Cells Separation. In vitro culture and stimulation of Treg and mononuclear cells.

A revolutionary technique called mass cytometry by time of flight (CyTOF) has been fundamental to discoveries in the field of medicine in recent years.[1] It allows investigation of a single cell in unprecedented detail through its ability to detect and quantify over 40 markers.[2] Resulting data were analyzed with software available through Cytobank (www.cytobank.org).

To quantify Treg heterogeneity we intend to use viSNE and ACCENSE:
viSNE is a data visualization tool that creates a two-dimensional view of high-parameter biological information, making it easier to not only visually identify interesting and rare biological subsets, but also to gate single-cell events across different samples.[3]
ACCENSE is a tool for exploratory analysis of high-dimensional single-cell data such as that generated by mass cytometry.[4] By combining a nonlinear dimensionality reduction algorithm (t-SNE) with a k-means clustering algorithm both visualization for exploratory analysis and automated cell classification into subpopulations is performed.
The high definition of the mass cytometry data combined with these analysis tools allows us to quantify human Treg heterogeneity based on expression of a large set of activation, proliferation, tissue homing and functional markers in conjunction with stages of Treg maturation and differentiation.

Flow Cytometry: A panel of directly conjugated monoclonal antibodies was used to define functionally distinct T-cell subsets and homeostatic characteristics of each subset.

High-throughput sequencing (HTS) of the T-cell receptor (TCR) is a rapidly advancing technique that allows sensitive and accurate identification and quantification of every distinct T-cell clone present within any biological sample. The relative frequency of each individual clone within the full T-cell repertoire can also be studied.[5]

2.2 Outline of the Thesis

This is chapter 1, the general introduction and outline of the thesis. We start by defining Treg cells and explaining how these cells are key layers to maintain tolerance and crucial to preserve the immune system homeostasis. We then analyze the relationship between Treg cells and allogeneic hematopoietic stem cell transplantation. Tregs have the ability to mediate transplantation tolerance, and when this careful balance is disturbed, it can lead to graft-versus-host disease (GVHD). The imbalance between effector and regulatory T cells that occurs during cGVHD is in detail explored. Furthermore, we highlight the relevance of low-dose IL-2 therapy for these patients and how it positively recuperates the Treg population. Lastly, we briefly describe the main aims of the current thesis. In chapter 2 we specify the aims and outline of this thesis.

In chapter 3, we investigate the Treg cell compartment diversity at birth, in healthy adults and throughout the period of 2 years after alloHSCT and in patients suffering from chronic GVHD. We show that not only adults had significant higher percentages of Treg than those present in umbilical cord blood (CB), as adults also have additional maturation and functional Tregs subpopulations. The quantity of both maturation and functional Treg subpopulations expand from birth to adulthood. As for patients after alloHSCT, the full repertoire of mature Treg subsets is already present after alloHSCT, while the diversity of Treg cells still expands within the 2 years period of post-transplant. cGVHD patients had significantly lower volume of functionally distinct Treg subpopulations, proposing a correlation between a disrupted Treg heterogeneity and cGVHD.

Low-dose IL-2 therapy has been shown to selectively induce the expansion of CD4 Tregs and improve clinical manifestations of chronic GVHD. Using mass cytometry, we demonstrate in chapter 4 that low concentrations of IL-2 selectively induce STAT5 phosphorylation in Helios^{+} CD4Tregs and CD56bright CD16^{-} NK cells in vitro and in vivo. The effects of low-dose IL-2 therapy on conventional CD4^{+} T cells and CD8^{+} T cells were limited to increased expression of PD-1 on effector memory T cells. It revealed the selective effects of low-dose IL-2 therapy on Helios^{+} CD4 Tregs and CD56^{bright} NK cells that constitutively express high-affinity IL-2 receptors as well as the indirect effects of prolonged exposure to low concentrations of IL-2 in vivo.

Acknowledging that we urgently need further effective therapies for treating patients with steroid-refractory acute graft-versus-host-disease (SR-aGVHD), we conducted in chapter 5, a phase I/II clinical trial to examine the safety and efficacy a new biological therapy for SR-aGVHD. An immunotoxin combination of a mixture of anti-CD3 and anti-CD7 antibodies separately conjugated to recombinant ricin A (CD3/CD7-IT), which induces in vivo depletion of T cells and natural killer (NK) cells and suppresses cell receptor activation. On day 28 after the start of CD3/CD7-IT therapy, the overall response rate was 60%, with 50% achieving a complete response. The 6-month overall survival rate was 60%. The 1-week course of treatment with CD3/CD7-IT caused profound but transient depletion of T cells and NK cells, followed by rapid recovery of the immune system with a diverse TCR Vβ repertoire, and preservation of Epstein-Barr virus and cytomegalovirus-specific T cell clones. CD3/CD7-IT was safe and well tolerated, with a relatively low prevalence of manageable and reversible adverse events, primarily worsening of hypoalbuminemia, microangiopathy, and thrombocytopenia.

Lastly, we were interested to test the same mass-cytometry panel used in chapter 3, to study Treg cells in other immune-disrupted circumstances, not HSCT-related. In chapter 6 we evaluate idelalisib as front-line therapy for the treatment of relapsed/refractory chronic lymphocytic leukemia (CLL). After a median follow-up period of 14.7 months, hepatotoxicity was found to be a frequent and often severe adverse event. Multiple lines of evidence suggest that this hepatotoxicity was immune mediated. A lymphocytic infiltrate was seen on liver biopsy specimens taken from 2 subjects with transaminitis, and levels of the proinflammatory cytokines CCL-3 and CCL-4 were higher in subjects experiencing hepatotoxicity. A decrease in peripheral blood regulatory T cells was seen in patients experiencing toxicity on therapy, which is consistent with an immune-mediated mechanism.

With chapter 7 we conclude this thesis summarizing and discussing the results.

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CHAPTER 3

Maturation and phenotypic heterogeneity of human $CD4^+$ regulatory T cells from birth to adulthood and after allogeneic stem cell transplantation

Tiago R. Matos[1,2,3,4], Masahiro Hirakawa[1,2], Ana C. Alho[1,2,3], Lars Neleman[4], Luis Graca[3], Jerome Ritz[1,2]

Thesis' author contribution: T.R.M. designed the research studies, conducted the experiments, acquired and analyzed the data, and wrote the manuscript.

Affiliations:

[1]Division of Hematologic Malignancies and Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.

[2]Harvard Medical School, Boston, Massachusetts, USA.

[3]Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal.

[4]Amsterdam University Medical Centers, Department of Dermatology, University of Amsterdam, Amsterdam, Netherlands.

ABSTRACT

$CD4^+$ Regulatory T cells (Treg) play a critical role in maintaining immune homeostasis. Various Treg subsets have been identified, however the heterogeneity of Treg subpopulations during development remains uncharacterized. Using mass cytometry, we obtained single cell data on expression of 35 functional markers to examine the heterogeneity of Treg cells at birth and in adults. Unsupervised clustering algorithms FlowSOM and ACCENSE were used to quantify Treg heterogeneity. As expected, Treg in umbilical cord blood were predominately naïve while Treg in adult blood were predominately central memory and effector memory cells. Although umbilical cord blood Treg are mostly naïve cells, we observed multiple phenotypic Treg subsets in cord blood. Nevertheless, peripheral blood in adults contained higher percentages of Treg and the heterogeneity of Treg was significantly increased in adults. We also studied Treg heterogeneity throughout a 2-year period after allogeneic hematopoietic stem cell transplantation (alloHSCT) and in patients with chronic graft-versus-host disease (cGVHD). Treg heterogeneity recovered rapidly after alloHSCT and gradually increased in the first two years post-transplant. However, patients with cGVHD had significantly fewer distinct Treg subpopulations, proposing a correlation between a disrupted Treg heterogeneity and cGVHD. Our study is the first to compare human Treg heterogeneity at birth, in healthy adults and in patients after alloHSCT with and without cGVHD. This approach to characterize Treg heterogeneity based on expression of a large panel of functional markers may enable future studies to identify specific Treg defects that contribute to immune dysfunction.

INTRODUCTION

Regulatory T cells (Tregs) are essential elements of a healthy immune system. They comprise between 5-10% of the peripheral blood $CD4^+$ T cell compartment in healthy individuals and play a critical role protecting their host against immunopathological damage following inflammatory or immunological challenges.[1,2] Tregs are able to suppress a range of effector cell types through several mechanisms, both direct and indirect, ensuring peripheral tolerance and immune homeostasis.[3,4] The intricate balance between immune response and suppression can be disturbed by both a reduced number of Tregs as well as a decline in their functionality, potentially leading to autoimmune pathology.[5]

Tregs originate in the thymus and can be identified by expression of CD3, CD4, high levels of surface CD25, low levels of CD127 and intracellular FoxP3.[6] Tregs can be further divided into three main maturation categories: naïve, central memory (CM) and effector memory (EM).[7] Naïve Tregs express high levels of CD45RA and low levels of CD45R0 and FoxP3, whereas memory Tregs are $FoxP3^{hi}$ and $CD45RA^-$.[8,9] In healthy individuals up to 30% of the regulatory T cells are naïve.[10] As individuals age, the relative fraction of naïve Tregs decreases due to thymic involution, while the proportion of memory Tregs increases.[11] Nevertheless, recent thymic emigrants (RTE), which co-express CD31 and CD45RA, are still present in adults constituting up to 11% of all naive Tregs.

It has been reported that the Treg population in adult peripheral blood contains up to 22 phenotypically distinct subpopulations, thus offering new insights into the heterogeneity of these cells.[12] Nevertheless, extensive comparisons between Treg in adult peripheral blood and umbilical cord blood (CB) have not previously been undertaken. Presumably, CB Treg are mostly comprised of a homogeneous population of naïve cells.[10,13,14] However, even though the majority of CB Tregs are naïve, CB also contains small numbers of memory Tregs, possibly due to prenatal antigen activation.[15] It has also been proposed that maternal cells pass the placenta and remain in fetal lymph nodes, where these cells induce the development of fetal Tregs that suppress anti-maternal immunity.[16] The expansion potential of CB Treg has been shown to be higher than adult Treg, and expanded CB Treg are functionally active.[17] This has led to the use of expanded CB Tregs for treatment of GVHD after allo-HSCT.[17] However, the extent to which CB Tregs are a homogenous

population has not been studied or whether the extensive heterogeneity present in adult Treg is also present in CB Treg.

Mass cytometry by time of flight (CyTOF) allowed us to investigate CB and adult Treg in unprecedented detail by simultaneously detecting and quantifying 35 markers in individual cells.[18-20] To quantify heterogeneity and provide more insight into the phenotype of CB Treg we used FlowSOM and ACCENSE for high dimensional analysis of mass cytometry data. These analytic tools allow us to quantify human Treg heterogeneity based on expression of a large set of activation, proliferation, tissue-homing and functional markers in conjunction with stages of Treg maturation and differentiation.

These tools revealed heterogeneous populations of Treg in both CB and adult blood but CB Treg were less heterogeneous with respect to maturity and functional markers. After allo-HSCT the number of distinct Treg subpopulations gradually increased during a two-year follow-up period. Patients with cGVHD had significantly fewer distinct Treg subpopulations based on functional markers, proposing a correlation between a disrupted Treg heterogeneity and cGVHD.

METHODS

Donor and patient characteristics. Peripheral blood samples were obtained from 14 healthy individuals (8 males and 6 females) with a median age of 44 years (range, 20-69 years), 2 children (male of 2 years old and female of 10 years old) and from 5 discarded umbilical cord blood collections (from 2 males and 3 females). We also studied peripheral blood from 10 adult patients who underwent allogeneic HSCT at the Dana-Farber Cancer Institute and Brigham and Women's Hospital, Boston Massachusetts. All transplant patients received reduced intensity conditioning with fludarabine plus busulfan followed by infusion of unmodified G-CSF mobilized peripheral stem cell grafts. No patients received anti-thymocyte globulin for GVHD prophylaxis or low-dose interleukin-2 (IL-2) for treatment of chronic GVHD. Fresh blood samples were obtained at 6 different time points (0, 1, 3, 6, 12, 24 months) after transplant or during cGVHD (6 months). Patients with relapse were not included. 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.

Sample preparation. CB mononuclear cells (CBMCs) and PBMCs were isolated from freshly drawn samples by density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare). Freshly isolated CBMCs and PBMCs from healthy donors were immediately used for antibody staining. PBMCs from patients were washed and cryopreserved in BAMBANKER (Lymphotech) before being analyzed.

Metal-tagged monoclonal antibodies. A panel of 35 metal-tagged monoclonal antibodies was used for analysis of CBMCs and PBMCs. A list of all antibodies and corresponding metal tags is provided in Supplemental Table 1. All pre-conjugated antibodies were purchased from Fluidigm. All other antibodies were purchased in carrier-protein-free PBS and conjugated with the respective metal isotope using the MaxPAR antibody conjugation kit (Fluidigm) according to the manufacturer's recommended protocol. Metal-labeled antibodies were diluted to 0.5 mg/ml in Candor PBS Antibody Stabilization solution (Candor Bioscience GmbH) for long-term storage at 4°C.

Antibody staining for mass cytometry. CBMCs and PBMCs were washed with MaxPar Cell Staining Buffer (Fluidigm) and blocked with Human FcR Blocking Reagent (Miltenyi Biotec) for 10 minutes at room temperature. Cells were then incubated with all antibodies targeting cell surface markers for 30 minutes at room temperature and then washed twice with Cell Staining Buffer. After washing, cells were fixed with Cytofix Fixation Buffer (BD Biosciences) and permeabilized with Phosflow Perm Buffer III (BD Biosciences) following the manufacturer's instructions. Fixed/permeabilized cells were washed twice with Cell Staining Buffer and incubated with all antibodies targeting intracellular antigens for 30 minutes at room temperature. After staining with intracellular antibodies, cells were washed twice with a Cell Staining Buffer and incubated with 191/193Ir DNA intercalator (Fluidigm) following the manufacturer's instructions. Prior to mass cytometry analysis, cells were washed twice with Cell Staining Buffer and twice with MaxPar Water (Fluidigm).

Mass cytometry. Cells were analyzed on a CyTOF 2 mass cytometer (Fluidigm) at an event rate of approximately 500 cells/second. To normalize CyTOF data over different days, EQ Four Element Calibration Beads (Fluidigm) were added in all samples. Resulting

data were analyzed with software available through Cytobank (www.cytobank.org). To remove debris and doublets, single cells were gated based on cell length and DNA content as described by Bendall et al.[21] To interpret high dimensional single-cell data produced by mass cytometry, we used a visualization tool based on the viSNE algorithm that creates a two-dimensional view of high-dimensional cytometry data at single-cell resolution, making it possible to not only visually identify interesting and rare subsets while preserving nonlinearity, but also to gate single-cell events across different samples.[22]

Gating of populations. Treg were defined by $CD25^{+}FOXP3^{+}$ co-expression (Fig. 1A). Naive cells were gated from the Treg population with the expression of $CD45RA^{+}CD62L^{+}$, CM as $CD45RA^{-}CD62L^{+}$, and EM as $CD45RA^{-}CD62L^{-}$. Recent thymic emigration (RTE) cells were gated by co-expression of CD45RA and CD31.[23] Each sample was gated manually via Cytobank.

Clustering analysis. FlowSOM (R package accessible within the www.bioconductor.org platform) was used for automated clustering. FlowSOM was run in R Studio and allows for unsupervised clustering and dimensionality reduction of data obtained from mass cytometry. Subsequent subpopulation analysis was repeated in ACCENSE (standalone application accessible from http://www.cellaccense.com/) for reliability purposes. ACCENSE is a tool for exploratory analysis of high-dimensional single-cell data such as that generated by mass cytometry.[24] By combining a nonlinear dimensionality reduction algorithm (t-SNE) with a k-means clustering algorithm both visualization for exploratory analysis and automated cell classification into subpopulations is performed. Subpopulation quantification was completed twice based on 26 functional markers and 6 maturity markers from the cytometry panel. ACCENSE was also used to make subsequent visual figure maps. Supplemental Table 2 shows which markers were used.

Statistical analysis. Graphpad Prism 7.04 was used for data analysis. Mann-Whitney test was used to compare unpaired populations. The Wilcoxon signed-rank test was used to compare paired samples for continuous variables and expression levels of proteins between subpopulations and between different time points. All tests were 2-sided at the significance level of 0.05 and multiple comparisons were not considered.

RESULTS

Prevalence of Treg maturation subsets at birth and adulthood

To compare the maturity of the Treg cell compartment between cord blood and adult blood we gated the single cell data biaxially in Cytobank to distinguish Tregs in all samples (Figure 1A). Treg were classified by the co-expression of intracellular FoxP3 and high-expression of surface CD25.[6] For complete visualization of the gating strategy see Supplemental Figure 1. The median percentage of regulatory T cells in adult blood was a 4.6-fold higher than cord blood (6.0% vs 1.3%; Figure 1B) ($p<0.0001$). Specific maturation subsets were defined as follows: naïve ($CD45RA^{+}CD62L^{+}$), CM ($CD45RA^{-}CD62L^{+}$), and EM ($CD45RA^{-}CD62L^{-}$).[7–9] Figure 1C identifies these three distinct populations within the Treg compartment and visually represents the difference between CBMC and adult PBMC. Naïve Treg cells make up the largest subset in CB at 85.2%, followed by CM cells at 5.6% and EM at 4.5%. In adult PBMC, CM Treg form the major subset at 56.5%, followed by EM (25.2%) and naïve cells (15.2%) (Figure 1D). We also quantified the fraction of recent thymic emigrants (RTE) within the naïve Treg subset. RTE can be gated from the naïve population via the expression of CD31, seen in Figure 1E. Within CB Treg, a median of 70.7% of gated $CD45RA^{+}$ cells expressed CD31. In adult PB Treg, a median of 10.19% of gated $CD45RA^{+}$ cells expressed CD31 ($p=0.0002$)

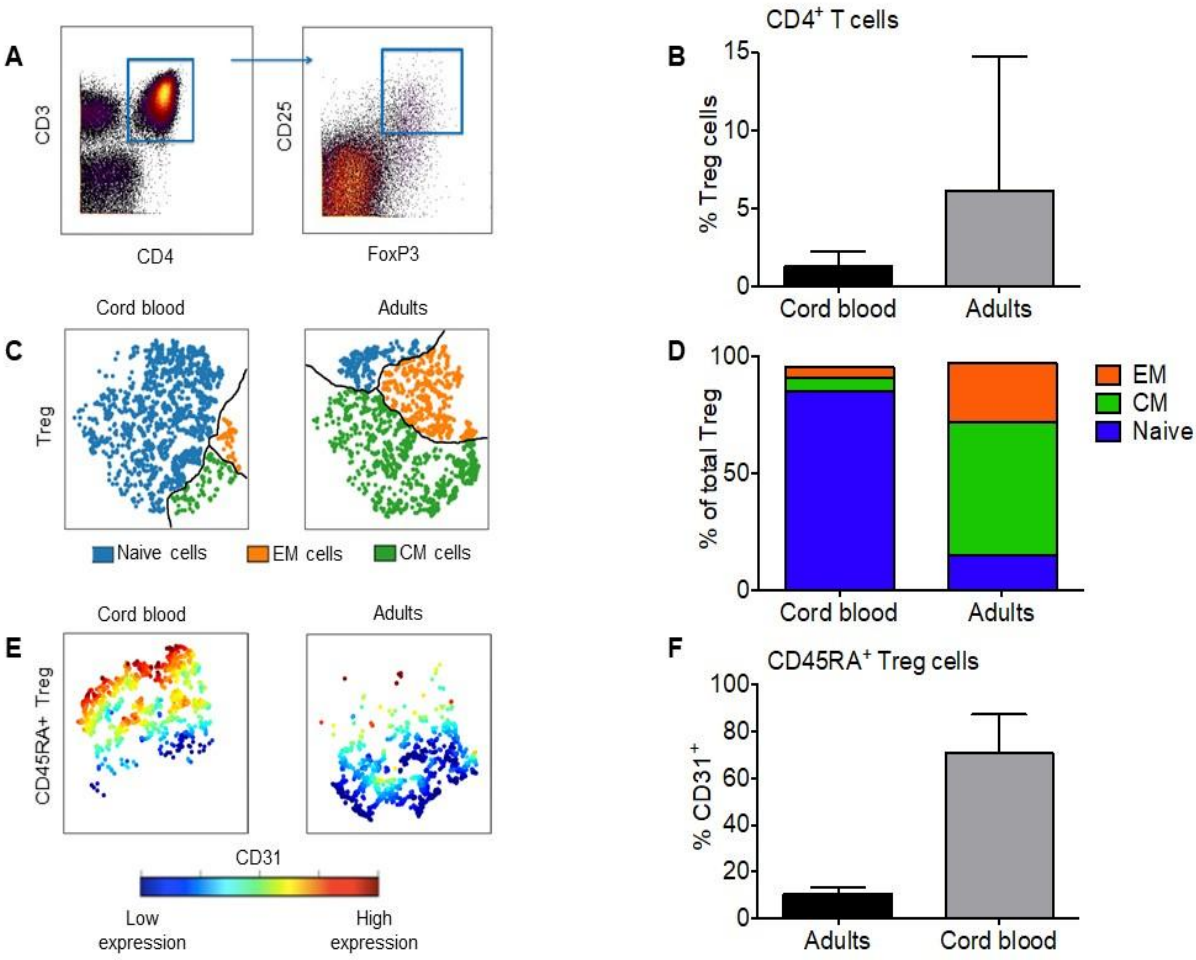

Figure 1. Regulatory T cells from cord blood are mostly CD31$^+$ naive cells. (A) Manual gating of Treg based on expression of CD4 and CD25$^+$FOXP3$^+$ co-expression. (B) Median percentage of Tregs in the CD4$^+$ compartment of cord blood and adult PBMC; the error bars show the range. (C) Representative visual composition of Treg maturation subsets in viSNE maps showing single cell relationships; subsets are divided by the black line: blue = naïve subset; green = Central Memory (CM) subset; orange = Effector Memory (EM) subset. (D) Relative composition of Naïve, CM and EM subsets in cord blood and adult Treg. Median values are shown for 5 CB and 14 adult Treg samples. (E) Expression of CD31 visualized with viSNE maps within naïve fraction of CB and adult Treg. Cells are colored according to intensity of CD31 expression. (F) Median percentage of naïve Treg cells expressing CD31$^+$, the error bars show the range. *represents statistical significance (p-value < 0.05). In ViSNE maps, each point represents a single cell.

CD4 Treg cell heterogeneity is established in umbilical cord blood and increases with age

We then examined the phenotypic heterogeneity of Treg in CB and adult peripheral blood by clustering Tregs based on the expression of 26 functional markers. To quantify heterogeneity within Treg, we used unsupervised cluster analysis with FlowSOM. Weber et al, 2016. previously compared 13 flow and mass cytometry clustering tools, recommending that FlowSOM (with optimal meta-clustering but without automatic selection of number of clusters) be used as a first choice for analyzing new data sets[25]. We replicated this analysis with our 26-marker panel and results were further validated by a separate analysis tool, ACCENSE. Figure 2A shows Treg cell clustering on functional markers included in our panel for 4 CB and 4 adult PB samples. Unsupervised clustering based on all 26 marker parameters revealed a median of 16 distinct clusters in adult Treg (range 10-22) and 12 Treg clusters in CB Treg (range 6-13) (p = 0.008) (Figure 2B). Although heterogeneity of CB Treg is substantial this heterogeneity increases significantly in adults. There was no variation of number of clusters within healthy controls regarding their age. In fact, we analyzed Treg heterogeneity in two children (2 and 10 years old), and both had 16 and 18 clusters, respectively (Supplemental Figure 2). This finding suggests that Treg heterogeneity is acquired very early in life.

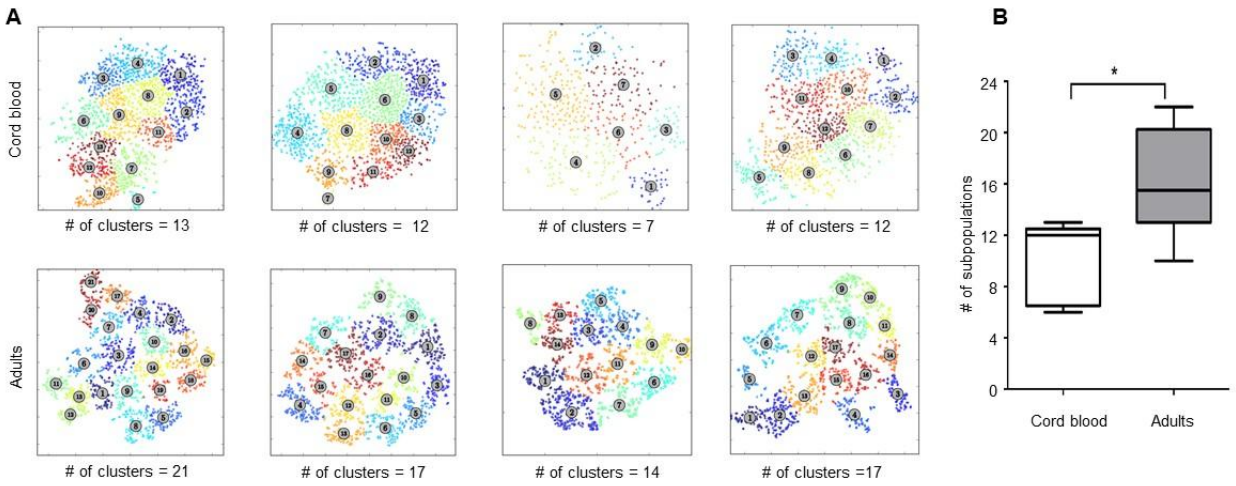

Figure 2. Analysis of Treg heterogeneity by unsupervised clustering based on expression of 26 functional markers. (A) Treg metaclusters identified by ACCENSE in 4 representative samples of cord blood (CB) and 4 adult Treg samples. Each point represents one cell, the color of the cells denotes a specific cluster. The number of metaclusters identified in each sample is shown below each box. (B) Phenotypic heterogeneity of Treg in CB and adult PB. 5 CB and 14 adult PB samples were studied. The center bar in the box is the median; Whiskers illustrate the minimum and maximum values obtained. * represents statistical significance (p-value < 0.05).

CD4 Treg cell maturation heterogeneity also increases between birth and adulthood

After establishing that heterogeneity of Treg in umbilical cord blood increases in adulthood, we further analyzed phenotypic heterogeneity related to levels of Treg maturity within naïve, CM and EM Treg subpopulations. Figures 3A and 3B show the gating strategy using CD45RA and CD62L to identify the naïve/CM/EM subsets in a viSNE map. This allowed us to visualize and quantify the following phenotypic and functional markers in each subset: CD31 (recent thymic emigrant marker), Ki-67 (marker for proliferation), CD95 (marker of extrinsic pathway apoptosis), and HLA-DR (activation marker) (Figure 3C/3D).

The percentage of $CD31^+$ Tregs within the naïve subset of CB was significantly higher than in naïve adult Tregs (p=0.0002). These results are consistent with the previously established higher expression of CD31 in CB cells compared to adult Tregs. In contrast, all

3 maturation subsets of adult Tregs express significantly more CD95 than corresponding CB Treg subpopulations. Although few naïve Tregs express CD95, the expression of this marker was also significantly different (p=0.01) between CB an adult Treg. Larger differences were seen when comparing CD95 expression in CM and EM Treg populations in CB and adults (p-value=0.005 and 0.0002, respectively). This suggests that more memory Tregs are susceptible to apoptosis in adult than in CB Tregs. With regard to the activation marker HLA-DR, naïve CB Tregs expressed higher levels of HLA-DR than naïve adult Tregs (p=0.0002). This suggests that naïve Tregs are the most activated subset within CB. There was little difference in expression of HLA-DR in CM and EM Tregs of newborns and adults, perhaps indicating that these subsets have similar activity in both age groups. Ki-67 is expressed at higher levels in all CB Treg subsets, indicating that all CB Treg cells proliferate at a higher rate than their adult counterparts. For each of the subgroups, significant differences were found, with p=0.0003 for naïve, p=0.0002 for CM and p=0.0002 for EM Tregs.

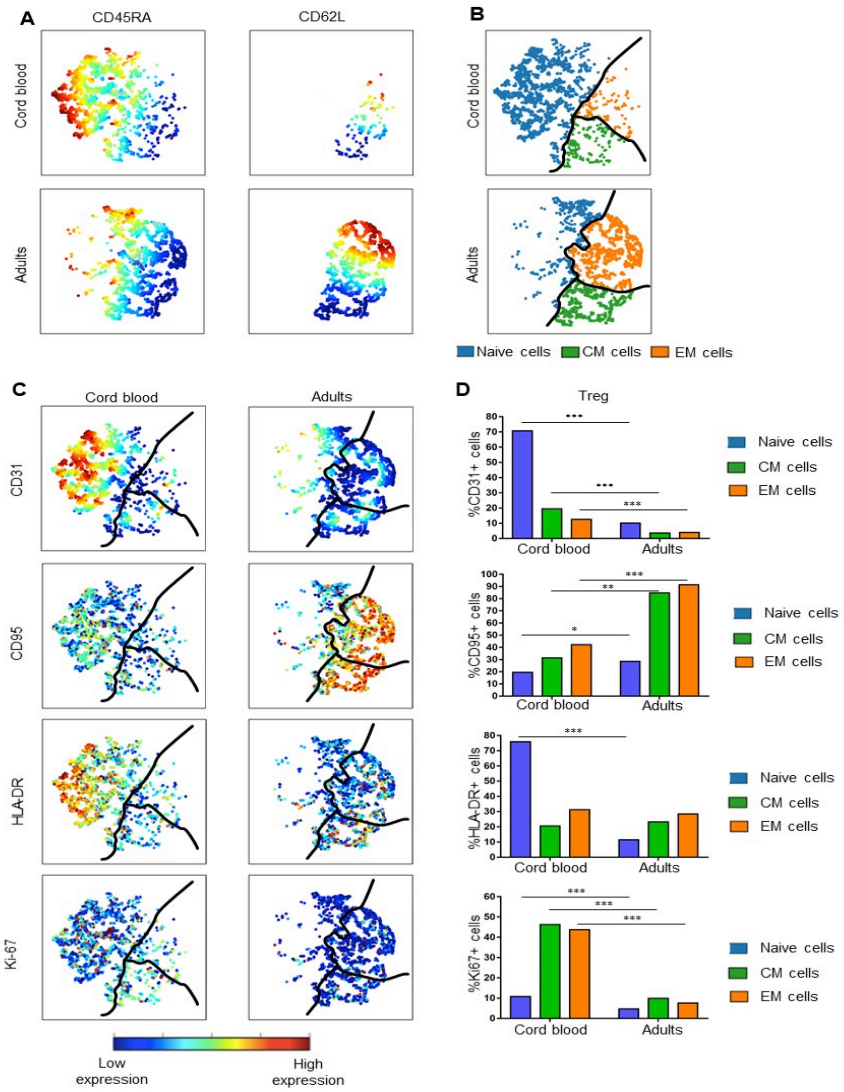

Figure 3. Treg maturation in cord blood and adult peripheral blood. (A) Expression of CD45RA and CD62L in cord blood and adult Treg. Color indicates the level of expression of the labeled marker in viSNE maps from 2 representative samples. (B) Visual representation of Treg maturation in viSNE maps based on expression of CD45RA and CD62L. Subsets are divided by the black line: blue = naïve Treg; green = Central Memory (CM) Treg; orange = Effector Memory (EM) Treg. (C) Expression of specific functional markers in representative examples of cord blood and adult Treg. (D) Median percentage of marker positive cells within naïve, CM and EM Treg subsets. Results are compared for 5 cord blood Treg samples and 14 adult Treg samples. *represents statistical significance (*p-value < 0.05, **< 0.005, ***< 0.0005). In viSNE maps, each point represents a single cell. Cells are colored according to intensity of expression of the indicated marker (excluding B).

Differential expression of markers in distinct populations reveal functional characteristics

Even though our method of unsupervised clustering did not allow us to directly compare expressions of markers in different metaclusters, we analyzed the expression of all functional markers in CB versus adult PBMC. From the 26 functional markers (Supplemental Figure 3), 8 showed significantly different levels of expression between umbilical cord blood and adult peripheral blood. CB Treg cells expressed higher levels of Ki-67 (p=0.0093) (a nuclear proliferation marker), PD-1 (p=0.0485) (a marker for reduced apoptosis and exhaustion), CCR9 (p=0.0196) (a chemokine receptor that regulates lymphocyte trafficking to the small intestine), and CCR7 (p=0.0036) (a chemokine receptor that regulates lymphocyte trafficking to lymph nodes). On the other hand, adults showed higher expression levels of CCR4 (p=0.0196) (a chemokine receptor that regulates lymphocyte trafficking to skin), CCR5 (p=0.0021) and CXCR3 (p=0.0010) (chemokine receptors that regulates lymphocyte trafficking to inflamed tissues), and CD95 (p=0.0273) (a marker of extrinsic pathway apoptosis) (Figure 4). Other functional marker differences between CB and adult PBMC that were of interest included CTLA-4 (p=0.0624), CLA (p=0.0800), Tbet (p=0.0800), ICOS (p=0.0800) and PDL-1 (p=0.0800), which were slightly lower in adult Tregs.

Treg cell heterogeneity increases after alloHSCT

Acknowledging the important role of regulatory T cells in allogeneic hematopoietic stem cell transplantation (alloHSCT) and following our previous studies describing the reconstitution of Treg after alloHSCT[23], we examined the heterogeneity of Treg subsets at

various times after alloHSCT. Using the same mass cytometry panel, we analyzed Tregs from 5 patients that received unmodified peripheral blood stem cell grafts at 6 time points: Day 0 and 1, 3, 6, 12 and 24 months after transplant. None of these patients developed acute or chronic GVHD during this time period. The percentage of $CD4^+$ Treg cells increased gradually in the first 6 months after alloHSCT and subsequently remained relatively stable throughout the 2 year follow up period (Figure 5A).

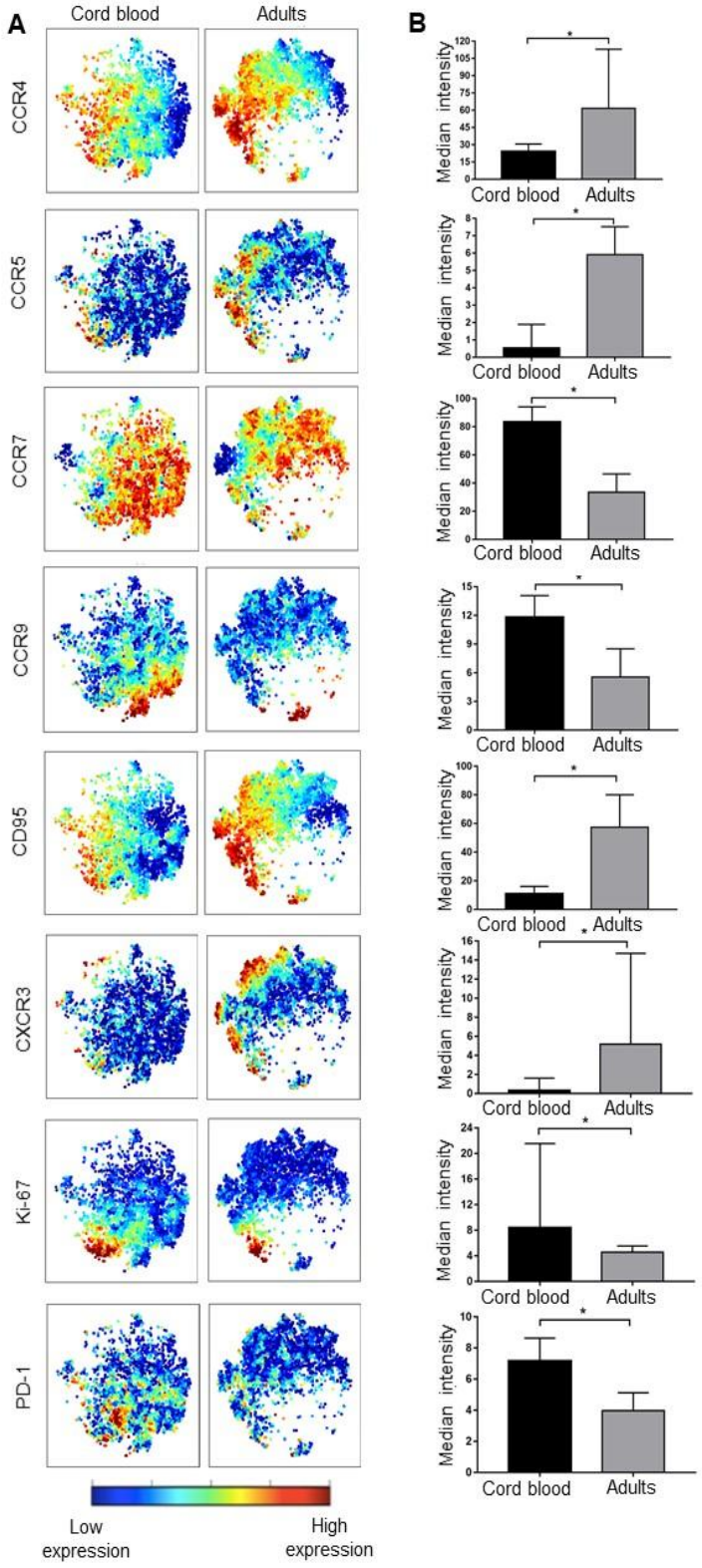

Figure 4. Cord blood and adult peripheral T regulatory cells differ in expression of various markers. (a) viSNE maps show the expression of functional Treg markers in cord blood and adult PBMC, color indicates the level of expression of the labeled marker. (b) Bar graphs matching the viSNE representation to their left showing median expression intensity for each marker, the error bars show the IQ range. * represents statistical significance (p-value < 0.05). In ViSNE maps, each point represents a single cell. Cells are colored according to intensity of expression of the indicated marker.

We subsequently examined Treg heterogeneity during this period (Figure 5B, C). Compared to Treg pre transplant, there was a decrease in the number of Treg subsets 1 month after transplant. We then observed an increase from a median of 9.5 subpopulations to 14 subpopulations 3 months after transplant. This level of Treg heterogeneity remained relatively stable from 3 to 24 months after transplant in this group of patients who did not develop acute or chronic GVHD. Interestingly, only a few markers vary their expression noticeably since the transplant. D49a, GITR, CTLA-4, CXCR3, Tim-3, CCR7, CD28 and CCR4 are 2-fold more expressed at day 0 compared to after month 1. From month 3 onwards the expression of the markers remains constant among alloHSCT patients and similar to adult Treg cells.

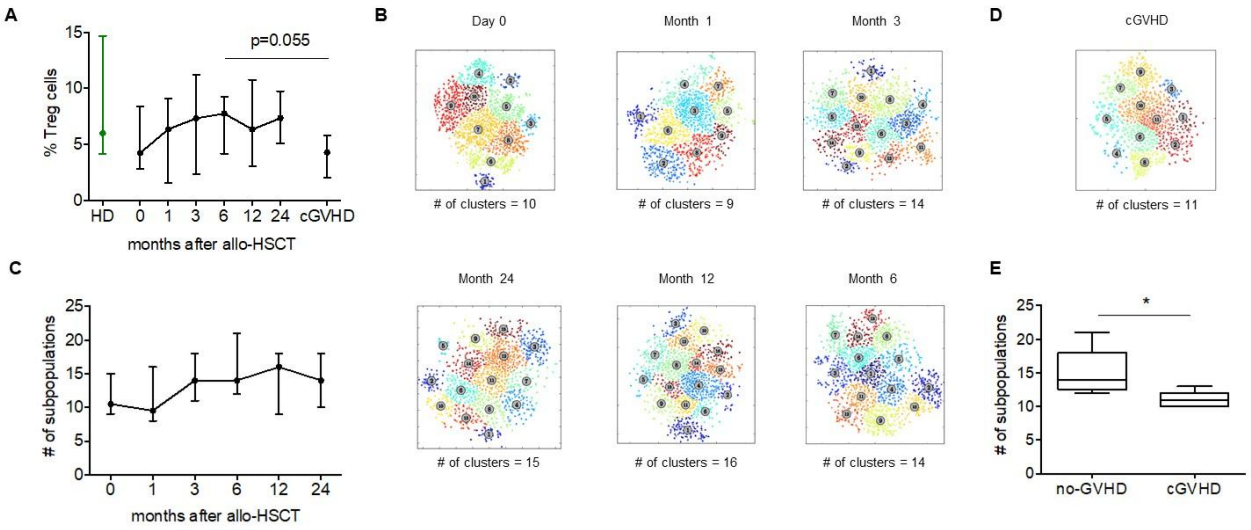

Figure 5. Regulatory T cell heterogeneity after alloHSCT and during cGVHD. (A) The median Treg percentage within the $CD4^+$ compartment in healthy donors, before and after alloHSCT and in patients with chronic GVHD 6 months after alloHSCT. Error bars show the range of values. (B) Treg metaclusters after alloHSCT identified by ACCENSE, based on expression of 26 functional markers. Each point represents one cell and colors denote individual clusters. Results shown are for a representative patient and the number of metaclusers at each time point is shown below each figure. (C) The number of Treg metaclusters identified before and after alloHSCT. Results shown are median values for 5 patients without acute or chronic GVHD after transplant. Error bars show range for values for each time point. (D) Treg metaclusters in a representative patient with chronic GVHD 6 months after alloHSCT. Colors denote individual clusters identified by ACCENSE. (E) Treg heterogeneity in patients with and without chronic GVHD 6 months after alloHSCT. Box and whisker plots show the number of Treg metaclusters based on expression of 26 functional markers. The center bar in the box is the median. Whiskers illustrate the minimum and maximum values.

Treg cell heterogeneity is reduced during cGVHD

Previous studies have shown that patients with active chronic GVHD have a lower frequency of Treg cells.[22,26] To determine whether this quantitative Treg deficiency was also associated with abnormal Treg heterogeneity we examined peripheral blood from 5 patients with chronic GVHD and compared results to samples obtained 6 months after alloHSCT from 5 patients without GVHD that had been previously analyzed. Gating on CD4+FoxP3+ Treg, we first compared Treg percentages in patients with and without cGVHD. Median %Treg was 4.28 (range 2.03-6.77) in patients with cGVHD compared to 7.74 (range 4.18-9.27) in patients without cGVHD ($p=0.055$). Using FlowSOM, we compared Treg heterogeneity in samples from patients with and without cGVHD. Treg from patients with cGVHD were found to have 11 Treg metaclusters (range 10-13) compared to 14 metaclusters (range 12-21) in patients without cGVHD ($p=0.02$) (Figure 5D, E). Although our method of unsupervised clustering did not identify specific characteristics of missing metaclusters in cGVHD patients, comparison of marker expression found a significant difference in expression of Helios and CLA between people with and without cGVHD (supplemental figure 4). Helios was expressed at higher levels in patients without cGVHD while the expression of CLA was higher in cGVHD patients. PD-1, CCR4 and HLA-DR also were highly expressed (non-significant) in non-cGVHD patients. For CD49a, CCR5, CD62L, BCL-2 and CD39 the opposite was observed, with a non-significant but higher expression in cGVHD patients.

DISCUSSION

Using mass cytometry and advanced computational algorithms to simultaneously measure expression of 35 phenotypic and functional markers in individual cells, we undertook a detailed analysis of Treg heterogeneity in humans. Our study initially focused on Treg in umbilical cord blood and peripheral blood in healthy adults and subsequently was expanded to include Tregs reconstituting after allogeneic HSCT and in patients with cGVHD. Unsupervised clustering algorithms based on 26 functional markers were used to establish the number of Treg metaclusters in individual samples and thereby quantify heterogeneity within the Treg population in each sample at each time point. This resulted in a unique examination of Treg heterogeneity throughout life and through a critical period where Treg are known to play an important role in immune reconstitution and the establishment of immune tolerance.

At birth, CB Tregs are predominantly comprised of naïve cells.[13,14] Genomic diversity and proliferative capacity of CB Treg is very high,[17] and this has facilitated the use of CB-derived in vitro expanded Treg for treatment or prevention of GVHD after alloHSCT.[28] Our studies confirmed that CB Treg are predominately naïve cells, with a high fraction of recent thymic emigrants. In contrast, adult Treg are more mature, being predominantly CM and EM cells with a much smaller fraction of naïve cells and recent thymic emigrants. Nevertheless, CB Treg were found to be relatively heterogeneous reflecting variable states of differentiation, maturation and activation, despite being predominantly naïve cells. Treg heterogeneity increased significantly in healthy adults reflecting past exposures and additional levels of differentiation, maturation and activation in vivo.

To assess heterogeneity within defined stages of Treg maturation we compared the expression of CD31 (recent thymic emigrant), Ki-67 (proliferation), CD95 (apoptosis) and HLA-DR (functional activation) in naïve, CM and EM Treg. The frequency of $CD31^+$ Tregs in the naïve CB Treg was significantly higher than in naïve adult Treg. This likely reflects decreased thymic function and increased homeostatic expansion of naïve T cells in adults. We also observed increased expression of CD95 in naïve, CM and EM subpopulations in adult Tregs compared to CB Treg. This likely reflects higher levels of exhaustion and terminal differentiation in adult Treg. In contrast, HLA-DR was more highly expressed naïve CB Treg and Ki-67 was more highly expressed at all levels of

differentiation in CB Treg, indicating that all subsets of CB Treg are activated and highly proliferative.

In patients who undergo alloHSCT, recipient T cells are rapidly replaced as donor cells engraft and reconstitute a fully functional immune system in the recipient. Proper functioning of the donor immune system requires balanced recovery of regulatory elements as well as effector cells and the development of cGVHD can be predicted by impaired recovery of Treg leading to an abnormally low ratio of Treg to conventional effector T cells.[27] Enhancement of Treg recovery through Treg infusions or administration of low dose IL-2 to selectively induce expansion of Treg in vivo can prevent or treat cGVHD progression.[28-33] The ability to manipulate Treg after alloHSCT and in patients with autoimmune diseases has sparked interest in the development of Treg-directed therapies. However, there has been relatively little consideration of the potential importance of functional Treg heterogeneity in addition to the ability to simply increase Treg counts in vivo. In our analysis of Treg heterogeneity in adult patients after transplant we found that Treg heterogeneity recovered rapidly in patients without acute or chronic GVHD. By 3-6 months after alloHSCT, levels of Treg heterogeneity were similar to healthy adults. However, the number of Treg metaclusters in patients with cGVHD at 6 months was significantly decreased compared to patients without cGVHD. These findings suggest that lack of functional Treg subsets may contribute to the development of cGVHD in addition to a simple numerical deficiency in this setting. High T cell receptor (TCR) diversity has been correlated with establishment and maintenance of self-tolerance,[34] and required for optimal suppressive function of Treg cells in murine models of GVHD.[35] Hence, future studies should correlate the TCR repertoire diversity to Treg subpopulations in order to determine whether subpopulations share a clonal origin.

In summary, our findings reveal considerable heterogeneity of Treg subsets that is not detected in routine characterization of Treg by flow cytometry with a limited set of markers. We also show that Treg heterogeneity varies considerably among individuals and in patients after alloHSCT. Although this heterogeneity is based on the variable expression of functional markers, further studies are needed to establish the extent to which this phenotypic heterogeneity reflects actual functional differences between different Treg metaclusters and the ability of different Treg metaclusters to regulate different immune

cells and immune networks in vivo. The main limitation of our study was the inability to define each individual cluster and compare them between samples. Since Treg is known to be capable of functional plasticity it will also be important to examine the stability of distinct metaclusters in vitro and in vivo. As Treg directed therapies are evaluated in patients with autoimmune diseases as well as GVHD, it may be important to examine the effects of these interventions on Treg heterogeneity as well as the number of circulating Treg.

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SUPPLEMENTAL MATERIALS

Supplemental Table 1. Mass cytometry antibody-conjugate panel. *Antibody was kindly provided by Prof. Eugene Butcher, Butcher Laboratory, Stanford University.

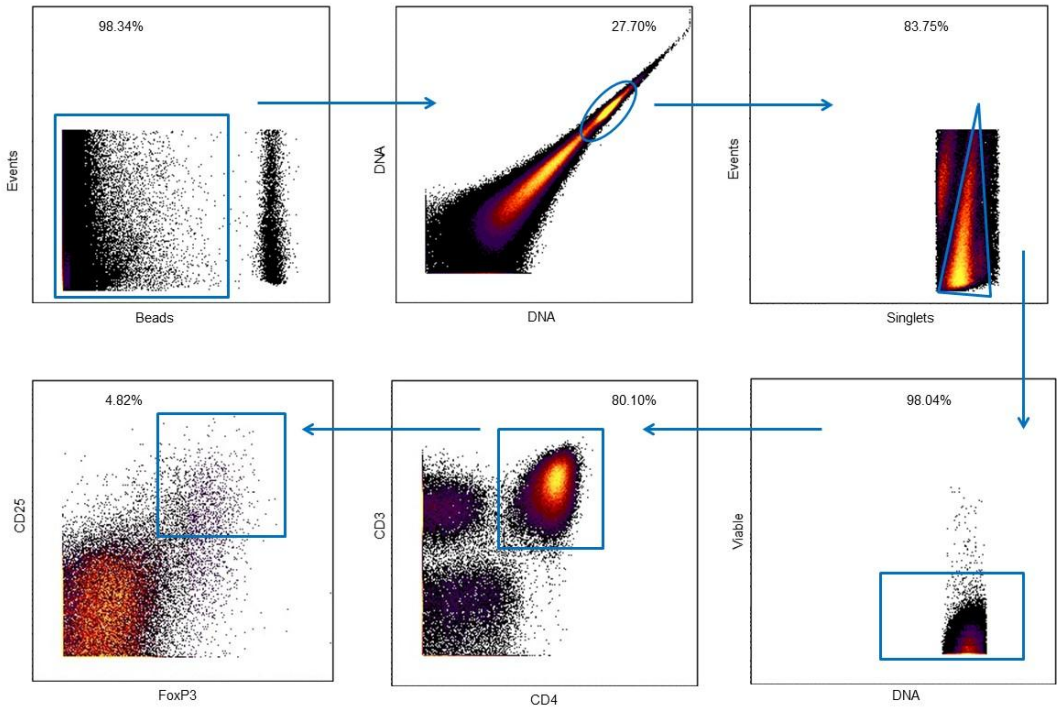
	TARGET	CLONE	ISOTOPE	MANUFACTURER
1	CD3	UCHT1	170Er	Fluidigm
2	CD4	SK3	174Yb	Fluidigm
3	CD25	2A3	149Sm	Fluidigm
4	CD127	A019D5	176Yb	Fluidigm
5	CD8	RPA T8	146Nd	Biolegend
6	CD45RA	HI100	169Tm	Fluidigm
7	CD31	WM59	145Nd	Fluidigm
8	CD28	CD28.2	148Nd	Biolegend
9	HLA-DR	L243	141Pr	BioLegend
10	CD62L	DREG-56	153Eu	Fluidigm
11	CD197 (CCR7)	G043H7	159Tb	Fluidigm
12	CD194 (CCR4)	205410	158Gd	Fluidigm
13	ACT-1 (α4β7)	-	163Dy	*
14	PD-1	EH12.2H7	167Er	Biolegend
15	CD95	DX2	164Dy	Fluidigm
16	CD195 (CCR5)	NP-6G4	144Nd	Fluidigm
17	CD39	A1	160Gd	Fluidigm
18	CD278 (ICOS)	C398.4A	154Sm	Biolegend
19	CD183 (CXCR3)	G025H7	142Nd	Biolegend
20	CD49a (VLA1)	TS2/7	162Dy	BioLegend
21	Tim-3	F38-2E2	150Nd	Biolegend
22	CD152 (CTLA-4)	14D3	152Sm	eBioscience
23	CD274 (PDL-1)	29E.2A3	172Yb	Biolegend
24	GITR	621	166Er	Biolegend
25	CD199 (CCR9)	L053E8	168Er	BioLegend
26	CLA	HECA-452	143Nd	Biolegend
27	FOXP3	PCH101	165Ho	eBioscience

28	Helios	22F6	156Gd	BioLegend
29	Tbet	4B10	175Lu	Biolegend
30	Granzyme B	GB11	147Sm	Thermo Fisher Scientific
31	BCL-2	Bcl-2/100	171Yb	BD Biosciences
32	Ki-67	B56	151Eu	BD Biosciences
33	DNA	UCHT1	103Rh	Fluidigm
34	DNA	SK3	191Ir	Fluidigm
35	DNA	2A3	193Ir	Fluidigm

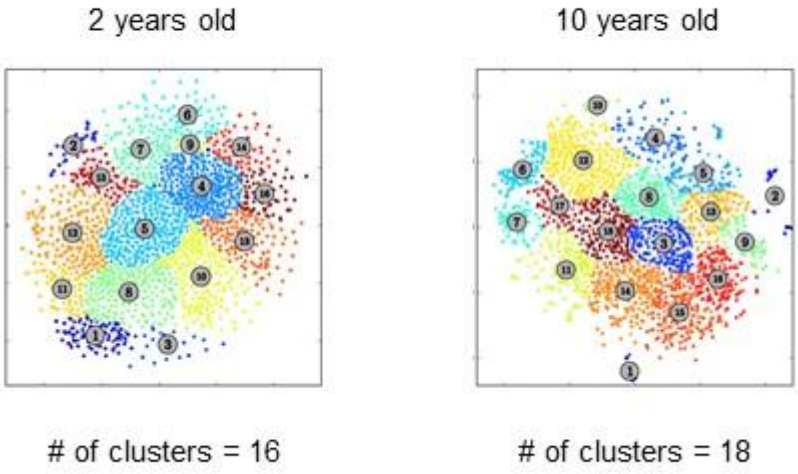
Supplemental Table 2. FlowSOM and ACCENSE markers. List of markers used for unsupervised clustering.

	TARGET	CLONE	ISOTOPE	MANUFACTURER
1	CD45RA	HI100	169Tm	Fluidigm
2	CD31	WM59	145Nd	Fluidigm
3	CD28	CD28.2	148Nd	Biolegend
4	HLA-DR	L243	141Pr	BioLegend
5	CD62L	DREG-56	153Eu	Fluidigm
6	CD197 (CCR7)	G043H7	159Tb	Fluidigm
7	CD194 (CCR4)	205410	158Gd	Fluidigm
8	ACT-1 (α4β7)	-	163Dy	*
9	PD-1	EH12.2H7	167Er	Biolegend
10	CD95	DX2	164Dy	Fluidigm
11	CD195 (CCR5)	NP-6G4	144Nd	Fluidigm
12	CD39	A1	160Gd	Fluidigm
13	CD278 (ICOS)	C398.4A	154Sm	Biolegend
14	CD183 (CXCR3)	G025H7	142Nd	Biolegend
15	CD49a (VLA1)	TS2/7	162Dy	BioLegend
16	Tim-3	F38-2E2	150Nd	Biolegend
17	CD152 (CTLA-4)	14D3	152Sm	eBioscience
18	CD274 (PDL-1)	29E.2A3	172Yb	Biolegend
19	GITR	621	166Er	Biolegend
20	CD199 (CCR9)	L053E8	168Er	BioLegend
21	CLA	HECA-452	143Nd	Biolegend
22	Helios	22F6	156Gd	BioLegend
23	Tbet	4B10	175Lu	Biolegend
24	BCL-2	Bcl-2/100	171Yb	BD Biosciences
25	Ki-67	B56	151Eu	BD Biosciences
26	Granzyme B	GB11	147Sm	Thermo Fisher Scientific

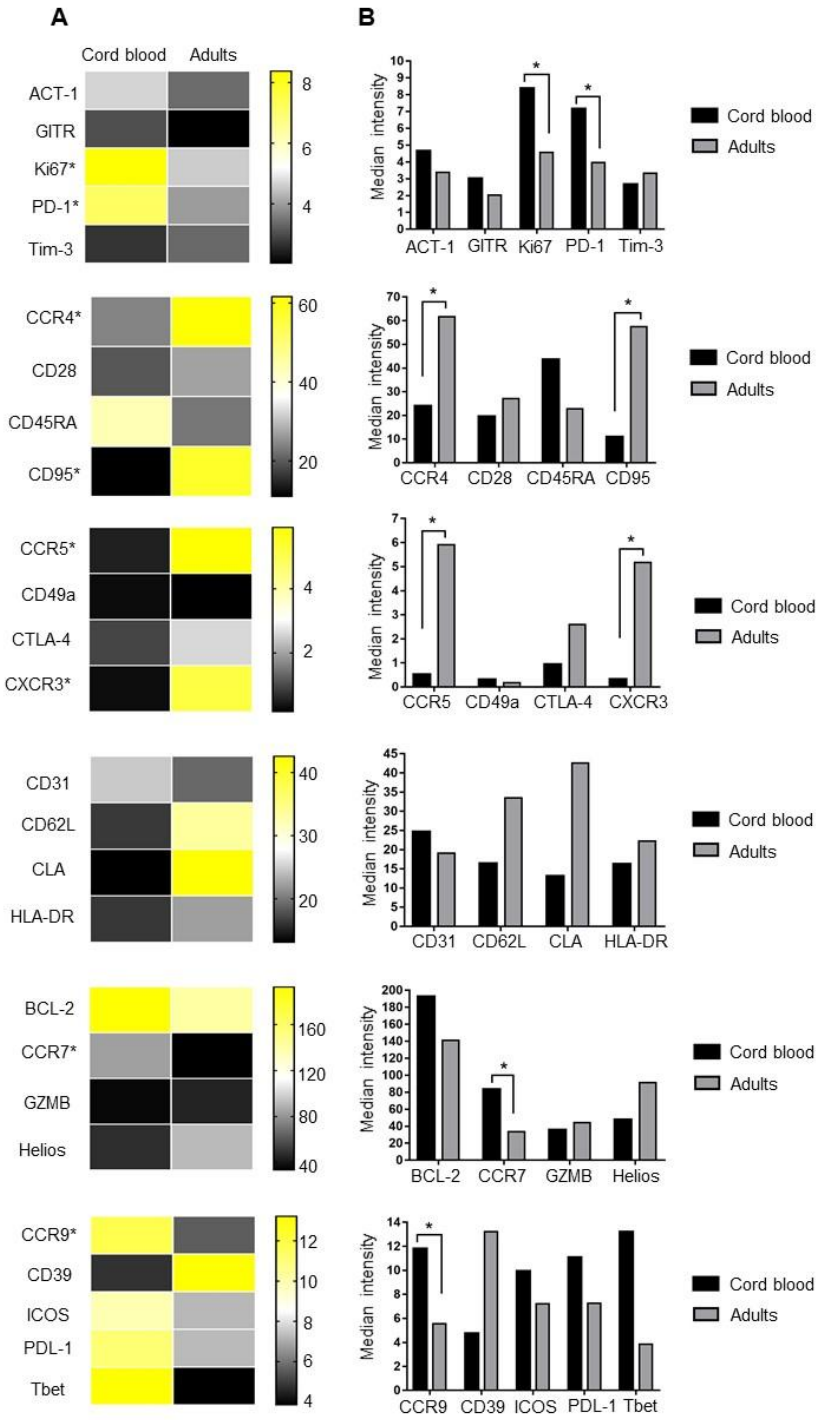
Supplemental Figure 1. CyTOF gating strategy. (a) The gating strategy shown was used on all data prior to downstream analysis, manual gating was performed in Cytobank.



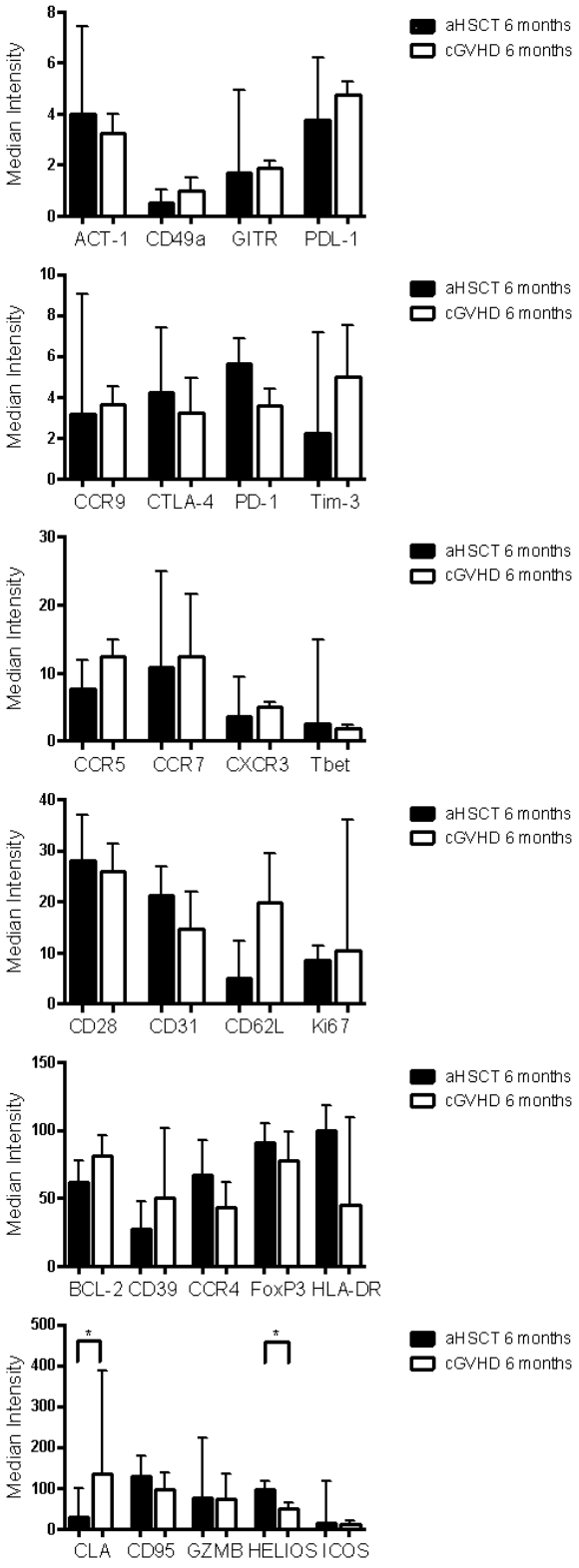
Supplemental Figure 2. Children have distinct regulatory T cells subsets based on all 26 functional markers. Treg subpopulation clusters made with ACCENSE showing 2 representative samples of children (2 years old and 10 years old) peripheral blood samples. Each point represents one cell, the color of the cells denotes a specific cluster.



Supplemental Figure 3. Functional marker expression comparison of cord blood and adult PBMC. (a) Heatmaps with marker expression from low (black) to high (yellow). (b) Bar graphs showing the median intensity range. * represents statistical significance (p-value < 0.05).



Supplemental Figure 4. Functional marker discrepancies between AlloHSCT and cGVHD. Samples arranged by median intensity and comparing alloHSCT 6 months after transplants and cGVHD samples 6 months post-transplant, the error bars show the IQ range. * represents statistical significance (p-value < 0.05).



CHAPTER 4

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

Masahiro Hirakawa,[1,2] Tiago Matos,[1,2,3] Hongye Liu,[1,2] John Koreth,[1,2] Haesook T. Kim,[4,5] Nicole E. Paul,[1] Kazuyuki Murase,[1,2] Jennifer Whangbo,[1,2,6] Ana C. Alho,[1,2,3] Sarah Nikiforow,[1,2] Corey Cutler,[1,2] Vincent T. Ho,[1,2] Philippe Armand,[1,2] Edwin P. Alyea,[1,2] Joseph H. Antin,[1,2] Bruce R. Blazar,[7] Joao F. Lacerda,[3] Robert J. Soiffer,[1,2] and Jerome Ritz[1,2]

Thesis' author contribution: T.R.M. conducted experiments, acquired and analyzed data, and edited the manuscript.

Affiliations:

[1]Division of Hematologic Malignancies and Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.

[2]Harvard Medical School, Boston, Massachusetts, USA.

[3]Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal.

[4]Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.

[5]Harvard T. H. Chan School of Public Health, Boston, Massachusetts, USA.

[6]Division of Hematology/Oncology, Boston Children's Hospital, Boston, Massachusetts, USA.

[7]Division of Blood and Marrow Transplantation, University of Minnesota, Minneapolis, Minnesota, USA.

ABSTRACT

CD4+ regulatory T cells (CD4Tregs) play a critical role in the maintenance of immune tolerance and prevention of chronic graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation. IL-2 supports the proliferation and survival of CD4 Tregs and previous studies have demonstrated that IL-2 induces selective expansion of CD4 Tregs and improves clinical manifestations of chronic GVHD. However, mechanisms for selective activation of CD4 Tregs and the effects of low-dose IL-2 on other immune cells are not well understood. Using mass cytometry, we demonstrate that low concentrations of IL-2 selectively induce STAT5 phosphorylation in Helios^{+} CD4 Tregs and CD56^{bright}CD16^{-} NK cells in vitro. Preferential activation and expansion of Helios^{+} CD4 Tregs and CD56^{bright}CD16^{-} NK cells were also demonstrated in patients with chronic GVHD receiving low-dose IL-2. With prolonged IL-2 treatment for 48 weeks, phenotypic changes were also observed in Helios^{-} CD4 Tregs. The effects of low-dose IL-2 therapy on conventional CD4^{+} T cells and CD8^{+} T cells were limited to increased expression of PD-1 on effector memory T cells. These studies reveal the selective effects of low-dose IL-2 therapy on Helios^{+} CD4 Tregs and CD56^{bright} NK cells that constitutively express high-affinity IL-2 receptors as well as the indirect effects of prolonged exposure to low concentrations of IL-2 in vivo.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative therapy for patients with various hematologic malignancies, immune deficiencies, and bone marrow failure syndromes. However, despite improved conditioning regimens, immunosuppressive therapies, and supportive care, chronic graft-versus-host disease (GVHD) remains a major complication of allogeneic HSCT and is the leading cause of long-term morbidity and mortality (1, 2). Advances in our understanding of chronic GVHD have established that both T and B cells, interacting in a highly complex network, contribute to tissue injury and the varied clinical manifestations of chronic GVHD (3, 4). Therapeutic approaches have relied primarily on broadly immunosuppressive agents, with corticosteroids being the most effective standard therapy. B cell–directed therapies can also be effective but these also result in prolonged B cell depletion (5, 6).

$CD4^+$ regulatory T cells (CD4Tregs), defined by expression of CD25 and FoxP3, play an indispensable role in the maintenance of self-tolerance and immune homeostasis (7, 8). Following allogeneic HSCT, thymic generation of CD4 Tregs is markedly impaired and reconstitution of this critical T cell population is primarily driven by proliferation and expansion of mature memory CD4 Tregs (9). Following transplant, rapidly proliferating CD4Tregs also exhibit increased susceptibility to Fas-mediated apoptosis (10) and increased mitochondrial apoptotic priming (11). Short telomeres and low levels of telomerase activity also contribute to reduced survival of CD4 Tregs in vivo (12). Since these factors do not affect other T cell populations to the same extent, these factors all contribute to a relative deficiency of CD4 Tregs compared with effector T cells and the subsequent development of chronic GVHD (9, 10).

To understand the functional heterogeneity of CD4 Tregs and better define the differentiation of these cells in vivo, previous studies have examined expression of various cell surface and intracellular markers including CD45RA, HLA-DR, CD62L, FoxP3, RUNX, and Helios (13–18). Helios is an Ikaros-family transcription factor that was initially thought to be a marker of thymus-derived or natural CD4 Tregs (18).

However, other studies have shown that Helios is also expressed by induced CD4Tregs and that Helios expression is associated with activation, proliferation, and suppressive capacity of CD4 Tregs (19–23).

Taken together, these studies have established the considerable phenotypic and functional heterogeneity of CD4 Tregs and provide a framework in which to further characterize the functional role of distinct CD4 Treg subsets in disease settings and in response to therapeutic interventions.

IL-2 plays a critical role in the development, proliferation, functional activity, and survival of CD4 Tregs (24–27). In contrast with effector T cells, CD4 Tregs constitutively express high levels of CD25, forming a high-affinity receptor for IL-2. Since CD4 Tregs cannot produce IL-2, these cells are inherently dependent on exogenous sources, predominantly activated effector T cells, for this critical homeostatic factor (28). However, because CD4 Tregs express a high-affinity receptor, these cells respond to low concentrations of IL-2. Taking advantage of the sensitivity of CD4 Tregs to IL-2, we have shown that daily administration of low-dose IL-2 in patients with active chronic GVHD results in sustained expansion of CD4 Tregs without a significant increase in conventional $CD4^+$ T cells (CD4Tcons) or $CD8^+$ T cells and clinical improvement in more than 50% of patients with chronic GVHD (29, 30). Clinical trials at other centers have shown the selective effect of low-dose IL-2 therapy on CD4 Tregs in healthy individuals, patients with hepatitis C virus–induced vasculitis, type 1 diabetes, acute GVHD, alopecia areata, and systemic lupus erythematosus (31–36). Laboratory studies have examined the mechanisms underlying the effects of IL-2 on CD4 Tregs (37–39), but the effects of low-dose IL-2 on heterogeneous CD4 Treg subsets and other lymphocyte populations remain poorly defined.

In the present study, we applied single-cell mass cytometry to dissect the effects of IL-2 on CD4 Tregs, CD4Tcons, CD8+ T cells, NK cells, and B cells in vitro. Mass cytometry allowed us to simultaneously measure expression of 33 proteins with single-cell resolution in multiple phenotypically and functionally defined subsets within each major lymphocyte population (40–42). Since our panel included markers of different cell signaling pathways as well as functional proteins and cell surface proteins, we were able to track intracellular signaling in response to IL-2 in each cellular subset. Finally, by comparing results of IL-2 stimulation in vitro with analysis of samples obtained from patients receiving low-dose IL-2 therapy, we were able to demonstrate that the therapeutic administration of exogenous low-dose IL-2 closely mirrored the effects of short-term stimulation with low concentrations of IL-2 in vitro. Thus, daily IL-2 therapy resulted in the selective activation and expansion of distinct subsets of $Helios^+$ CD4 Tregs and $CD56^{bright}CD16^-$ NK cells

without activation of CD4 Tcons or $CD8^+$ T cells. With continued daily IL-2 treatment, these functional effects were maintained for prolonged periods up to 48 weeks.

METHODS

Patient characteristics. Laboratory studies were undertaken with blood samples from 14 adult patients with active chronic GVHD at the Dana-Farber Cancer Institute and the Brigham and Women's Hospital, Boston, Massachusetts, USA. Each patient was enrolled in a clinical protocol to define the efficacy of daily low-dose IL-2 for treatment of glucocorticoid-refractory chronic GVHD (30). Recombinant IL-2 (aldesleukin, provided by Prometheus Laboratories) was administered subcutaneously once daily at a dose of 1.0×10^6 IU/m2 for 12 weeks, followed by a 4-week hiatus. Previous immune-suppressive medications were continued during IL-2 therapy. All study patients completed 12 weeks of treatment. After a 4-week treatment hiatus, 8 patients who achieved a clinical response received extended IL-2 therapy at the same daily dose of IL-2. Clinical characteristics of these patients are summarized in Table 1. The median age of the patients was 55 years (range 29–70). All patients had previously received peripheral blood stem cells (PBSCs) with standard immunosuppressive regimens for GVHD prophylaxis. Fifty percent of patients had a prior history of acute GVHD (grade 2–4). The median time from HSCT to initiation of IL-2 therapy was 636.5 days (range 271–1,950), and the median time from the onset of chronic GVHD to initiation of IL-2 therapy was 236.5 days (range 47–1,651). Ten patients (71.4%) were receiving more than 10 mg of prednisone when IL-2 therapy was started. Similar studies were also undertaken with blood samples from 5 healthy donors (median age 37 years; range 30–55; male/female, 3:2).

Sample preparation. PBMCs were isolated from freshly drawn blood samples by density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare). Freshly isolated PBMCs from healthy donors were immediately used for in vitro stimulation and antibody staining. PBMCs from patients were washed and cryopreserved in BAMBANKER (Lymphotech) before being analyzed.

Metal-tagged monoclonal antibodies. A panel of 33 metal-tagged monoclonal antibodies was used for analysis of healthy donor and patient PBMCs. A detailed listing of antibodies and corresponding metal tags is provided in Supplemental Table 1. All pre-conjugated antibodies were purchased from Fluidigm. All other antibodies were purchased in carrier-

protein-free PBS and conjugated with the respective metal isotope using the MaxPAR antibody conjugation kit (Fluidigm) according to the manufacturer's recommended protocol. Metal-labeled antibodies were diluted to 0.5 mg/ml in Candor PBS Antibody Stabilization solution (Candor Bioscience GmbH) for long-term storage at 4°C.

Table 1. Clinical characteristics of patients receiving low dose IL-2 (*n* = 14)

	n	(%)		*n*	(%)
Age, median (range)	55	(29–70)	Acute GVHD		
			Grade 2–4	7	(50%)
Patient sex					
Male	8	(57.1)	Time since HSCT		
Female	6	(42.9)	Median days (range)	636.5	(271–1,950)
Diagnosis			Time since onset chronic GVHD		
AML	3	(21.4)	Median days (range)	236.5	(47–1,651)
CLL	3	(21.4)			
ALL	1	(7.1)	Immunosuppressive therapy[A]		
MDS	3	(21.4)	Prednisone		
NHL	4	(28.6)	0 mg	0	(0)
			0 to ≤ 10 mg	4	(28.6)
Conditioning regimen			> 10 mg	10	(71.4)
Myeloablative	7	(50.0)	Tacrolimus		
Nonmyeloablative	7	(50.0)	0 mg	6	(42.9)
			0 to ≤ 1 mg	5	(35.7)
Stem cell source			> 1 mg	3	(21.4)
PBSC	14	(100)	Sirolimus		
Bone marrow	0	(0)	0 mg	12	(85.8)
			0 to ≤ 1 mg	1	(7.1)
Donor type			> 1 mg	1	(7.1)
Matched-related	6	(42.9)	MMF		
Matched-unrelated	7	(50.0)	0 mg	12	(85.8)
Mismatched-unrelated	1	(7.1)	0 to ≤ 500 mg	1	(7.1)
			> 500 mg	1	(7.1)
Acute GVHD prophylaxis					
Sirolimus containing	7	(50.0)			
No sirolimus containing	7	(50.0)			

[A]Immunosuppressive therapy at baseline. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; MDS, myelodysplastic syndromes; MMF, mycophenolate mofetil; NHL, non-Hodgkin lymphoma; PBSC, peripheral blood stem cell.

Table 1. Clinical characteristics of patients receiving low dose IL-2 (*n* = 14)

Antibody staining for mass cytometry. PBMCs were washed with MaxPar Cell Staining Buffer (Fluidigm) and blocked with Human FcR Blocking Reagent (Miltenyi Biotec) for 10 minutes at room temperature. Cells were then incubated with all antibodies targeting cell surface markers for 30 minutes at room temperature and then washed twice with Cell Staining Buffer. After washing, cells were fixed with Cytofix Fixation Buffer (BD Biosciences) and permeabilized with Phosflow Perm Buffer III (BD Biosciences) following the manufacturer's instructions. Fixed/permeabilized cells were washed twice with Cell Staining Buffer and incubated with all antibodies targeting intracellular antigens for 30 minutes at room temperature. After staining with intracellular antibodies, cells were washed twice with a Cell Staining Buffer and incubated with 191/193Ir DNA intercalator (Fluidigm) following the manufacturer's instructions. Prior to mass cytometry analysis, cells were washed twice with Cell Staining Buffer and twice with MaxPar Water (Fluidigm).

In experiments where PBMCs from healthy donors were stimulated in vitro, fresh samples were processed and stained simultaneously. Patient samples were obtained at different time points after starting IL-2 therapy: baseline pretreatment; 1, 2, 4, 8, and 12 weeks during treatment; 4 weeks after discontinuation of IL-2 (16 weeks); and at 48 weeks in patients receiving extended IL-2 therapy. All samples from an individual patient were thawed and stained simultaneously. Therefore, all samples from a single in vitro stimulation experiment and all samples from an individual patient were processed identically to facilitate comparison across multiple samples.

In vitro stimulation of normal PBMCs with IL-2. To examine IL-2 signaling, freshly isolated PBMCs from healthy donors were stained with surface antibodies prior to in vitro stimulation with IL-2. After washing, cells were incubated in prewarmed PBS supplemented with 2% FBS for 15 minutes at 37°C with 5% CO2, and then stimulated with different concentrations of IL-2 for 15 minutes at 37°C. To halt signal transduction after stimulation, cells were immediately fixed with Cytofix Fixation Buffer and stained for intracellular markers as described above.

Mass cytometry analysis. Cells were analyzed on a CyTOF 2 mass cytometer (Fluidigm) at an event rate of approximately 500 cells/second. To normalize CyTOF data over different days, EQ Four Element Calibration Beads (Fluidigm) were added in all samples. Resulting data were analyzed with software available through Cytobank (www.cytobank.org). To remove debris and doublets, single cells were gated based on cell length and DNA content as described by Bendall et al. (41). To interpret high dimensional single-cell data that were produced by mass cytometry, we used a visualization tool based on the viSNE algorithm, which allows visualization of high-dimensional cytometry data on a 2-dimensional map at single-cell resolution and preserve the nonlinearity (43).

Statistics. Data was analyzed with GraphPad Prism version 6.01. The Wilcoxon signed-rank test was used to compare paired samples for continuous variables and expression levels of proteins between subpopulations and between 2 different time points. All tests were 2-sided at the significance level of 0.05 and multiple comparisons were not considered.

Study approval. Patients were enrolled in a phase 2 clinical trial designed to assess the efficacy, safety, and immunologic effects of daily low-dose IL-2 therapy in patients with active chronic GVHD (30). Healthy stem cell donors were enrolled in a tissue-banking protocol for patients with hematologic malignancies and stem cell donors. Protocols were approved by the human subject's protection committee of the Dana-Farber/Harvard Cancer Center. Written informed consent was obtained from each patient and healthy donor before sample collection.

RESULTS

Definition of lymphocyte subsets in peripheral blood.

Mass cytometry allowed us to simultaneously quantify expression of a large number of key functional markers in well-defined functional subsets of T, B, and NK cells in normal peripheral blood (Supplemental Table 1). Major peripheral blood mononuclear cell (PBMC) subsets were first identified by 2-dimensional gating (Figure 1A). Lymphocytes were identified by expression of CD45 and within this gate, major PBMC subsets were identified as follows: CD4Tcon, $CD3^{+}CD4^{+}CD25^{neg\text{-}low}FoxP3^{-}$; CD4Treg, $CD3^{+}CD4^{+}CD25^{med\text{-}high}FoxP3^{+}$; $CD8^{+}$ T cell; $CD3^{+}CD8^{+}$; B cell, $CD3^{-}CD19^{+}$; NK cell, $CD3^{-}$ $CD19^{-}CD56^{+}$. We then applied the t-distributed stochastic neighbor embedding

algorithm (viSNE) to visualize high-dimensional cytometry data on a 2-dimensional map at single-cell resolution (43) and characterized each of the major PBMC subsets. Figure 1B shows a representative viSNE map of CD4 Tregs. Based on expression of CD45RA, CD4Tregs were subdivided into naive ($CD45RA^+$) and memory ($CD45RA^-$) subsets. Helios, a marker of CD4 Treg functional activity, was used to further characterize naive and memory CD4 Tregs (21, 22). Figure 1, C and D show representative viSNE maps of CD4Tcons and $CD8^+$ T cells, respectively. Based on expression of CD45RA and CD62L, CD4Tcons were clearly divided into 3 subsets (naive Tcon, central memory Tcon, and effector memory Tcon) and $CD8^+$ T cells into 4 subsets (naive, central memory, effector memory, and terminally differentiated effector [TEMRA] $CD8^+$ T cells). Figure 1E shows a representative viSNE map of NK cells. Based on expression of CD56 and CD16, NK cells were divided into 3 subsets ($CD56^{dim}CD16^+$, $CD56^{dim}CD16^-$, and $CD56^{bright}CD16^-$ NK cells) (44–46). Lastly, the viSNE map of B cells is shown in Figure 1F. Based on expression of IgD and CD38, B cells were divided into 4 subsets (IgD^+CD38^{lo} naive, IgD^+CD38^{++} transitional, $IgD^{lo}CD38^{lo}$ memory B cells, and $IgD^{lo}CD38^{++}$ plasmablasts) (5, 47, 48).

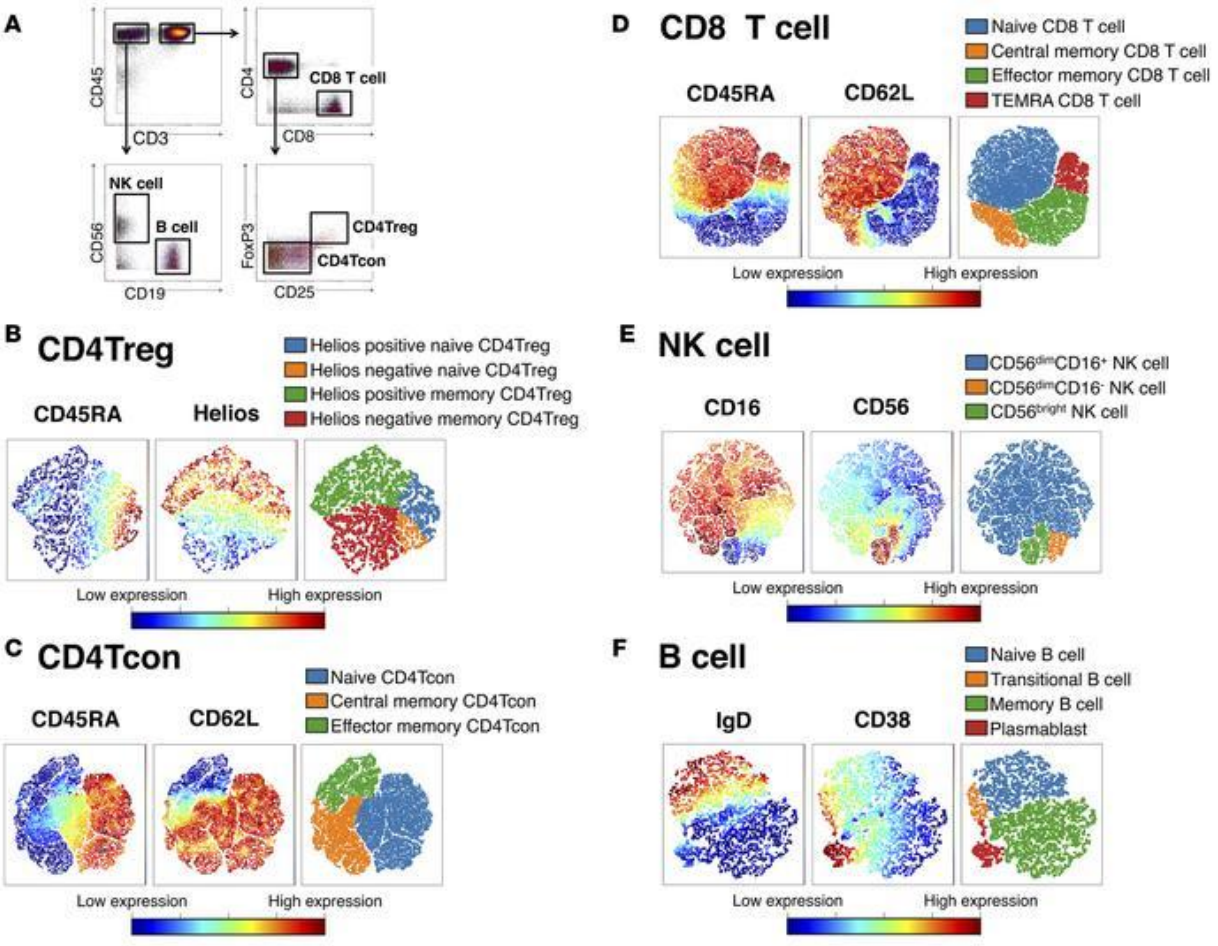


Figure 1. Representative gating and identification of phenotypic lymphocyte subsets in peripheral blood. (**A**) Representative 2-dimensional gating for identification of major lymphocyte subsets in peripheral blood from a healthy donor. (**B**–**F**) viSNE maps of each major lymphocyte subset identified in **A** are shown. Each point in the viSNE map represents an individual cell, and cells are colored according to intensity of expression of the indicated markers. The far-right map in each figure identifies phenotypic subsets based on expression of the indicated markers and the map is color coded for each subset as shown in the color legend.

Restricted expression of functional proteins in lymphocyte subsets.

To further characterize the lymphocyte subsets identified in viSNE, we analyzed expression of several important functional proteins in each major lymphocyte population in normal PBMCs. Figure 2A shows representative expression patterns of these proteins in each T cell population. Notably, these proteins were not uniformly expressed within the major T cell populations. Instead, expression was often segregated within specific subsets. For example, FoxP3 and CD25 expression was highest in Helios$^+$ memory CD4 Tregs and low-level CD25 expression was restricted to central and effector memory CD4 Tcons. CD127 expression was highest in effector memory CD4 Tcons, moderate in central memory CD4 Tcons, low in naive CD4 Tcons and naive CD8$^+$ T cells, and very low in TEMRA CD8$^+$ T cells. Expression of CD95 was primarily limited to memory subsets but was highest in Helios$^+$ memory CD4 Tregs. BCL2 was expressed at relatively high levels in most CD4 Tregs and CD4 Tcons, but within CD8$^+$ T cells, BCL2 was only expressed at high levels in naive cells. The MHC class II protein HLA-DR is a known marker of T cell activation. Within the CD4Treg population, HLA-DR$^+$ cells were found within the Helios$^+$ memory subset. This same cluster of cells expressed the highest levels of FoxP3, CD25, and CD95 and lowest levels of CD127 and BCL2. Similarly, small clusters of HLA-DR$^+$ cells were evident within CD4Tcons and CD8$^+$ T cells. These activated cells were present primarily in memory subsets and also expressed higher levels of CD95 and lower levels of BCL2 and CD127. Although FoxP3 is primarily expressed in CD4 Tregs, small clusters of FoxP3$^+$ cells were also present within CD4Tcons and naive CD8$^+$ T cells.

CD56^{dim}CD16$^-$ and CD56^{bright}CD16$^-$ NK cells are relatively minor NK cell subsets in healthy donors (Figure 2B). CD56^{bright} NK cells express higher levels of CD25, HLA-DR, and BIM than CD56^{dim}CD16$^+$ NK cells (49). CD56^{dim}CD16$^-$ NK cells express low levels of BCL2 and high levels of HLA-DR compared with CD56^{dim}CD16$^+$ NK cells. Figure 2C shows representative expression of key proteins in B cell subsets. As expected, CD27 was expressed at high levels in memory B cells and plasmablasts. B cell–activating factor receptor (BAFF-R) was expressed at high levels in all B cell subsets except for

plasmablasts. Expression of CD95 was relatively low in B cells compared with T cells, but its low-level expression in memory B cells and plasmablasts appears to mirror expression of CD27. BCL2 expression was high in memory B cells and low in transitional B cells and plasmablasts. BIM was expressed at higher levels in memory B cells and plasmablasts compared with naive and transitional B cells. As shown by viSNE, each B cell subset expressed a different pattern of these functional molecules, with the most distinctive pattern being in the plasmablast subset that expressed high levels of CD27, BIM, and CD95, with low levels of BCL2 and BAFF-R, suggesting that plasmablasts are more highly susceptible to apoptosis than other B cell subsets. Taken together, this comprehensive single-cell platform provides an unprecedented analysis of all major lymphocyte populations and allows the comparison of functional markers within well-defined subsets within each major population.

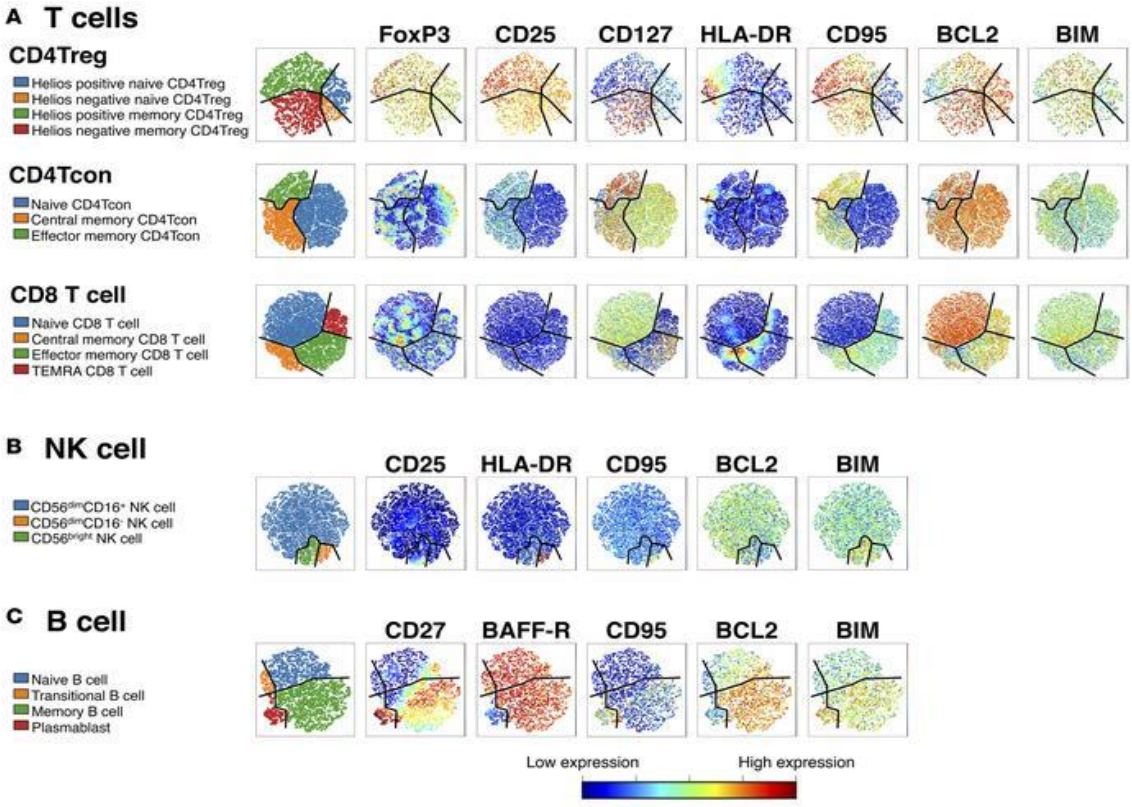


Figure 2. Protein expression in lymphocyte subsets. The viSNE maps for each lymphocyte population are the same as for Figure 1. Each viSNE map shows intensity of expression of the marker indicated for each column. Expression levels of each protein are normalized across all lymphocyte subsets. The farleft map in each row identifies the same phenotypic cell subsets as shown in the color legend and in Figure 1. Expression levels of proteins are shown in (**A**) T cells, (**B**) NK cells, and (**C**) B cells. Data shown are from a single individual and are representative of 5 healthy donor samples.

Constitutive activation of signaling proteins in resting lymphocytes.

Our mass cytometry marker panel included 5 antibodies specific for phosphorylated intracellular proteins involved in different cell signaling pathways and viSNE allowed us to assess the baseline levels of each of these phosphoproteins as a measure of constitutive signaling pathway activity in each lymphocyte subset. Figure 3 shows viSNE maps from the same healthy donor shown in Figures 1 and 2. These results are representative of 5 healthy donors. Levels of phospho-STAT5 (p-STAT5) were higher in CD4 Tregs than in other T cells at baseline. In CD4Tcons and $CD8^+$ T cells, naive subsets expressed higher levels of p-STAT3 compared with memory subsets. In contrast, p-AKT was expressed at higher levels in memory CD4 Tcon and $CD8^+$ T cell subsets compared with naive subsets. Expression of p-S6 and p-ERK was not detected in any resting T cell populations. Within resting NK cells, low levels of p-STAT5 and p-AKT were detected in the major $CD56^{dim}CD16^+$ subset. When compared with T and NK cells, resting B cells exhibited relatively high levels of several signaling proteins including p-STAT3, p-AKT, p-S6, and p-ERK. Expression of p-AKT, p-S6, and p-ERK was highest in naive and transitional B cells and lowest in plasmablasts.

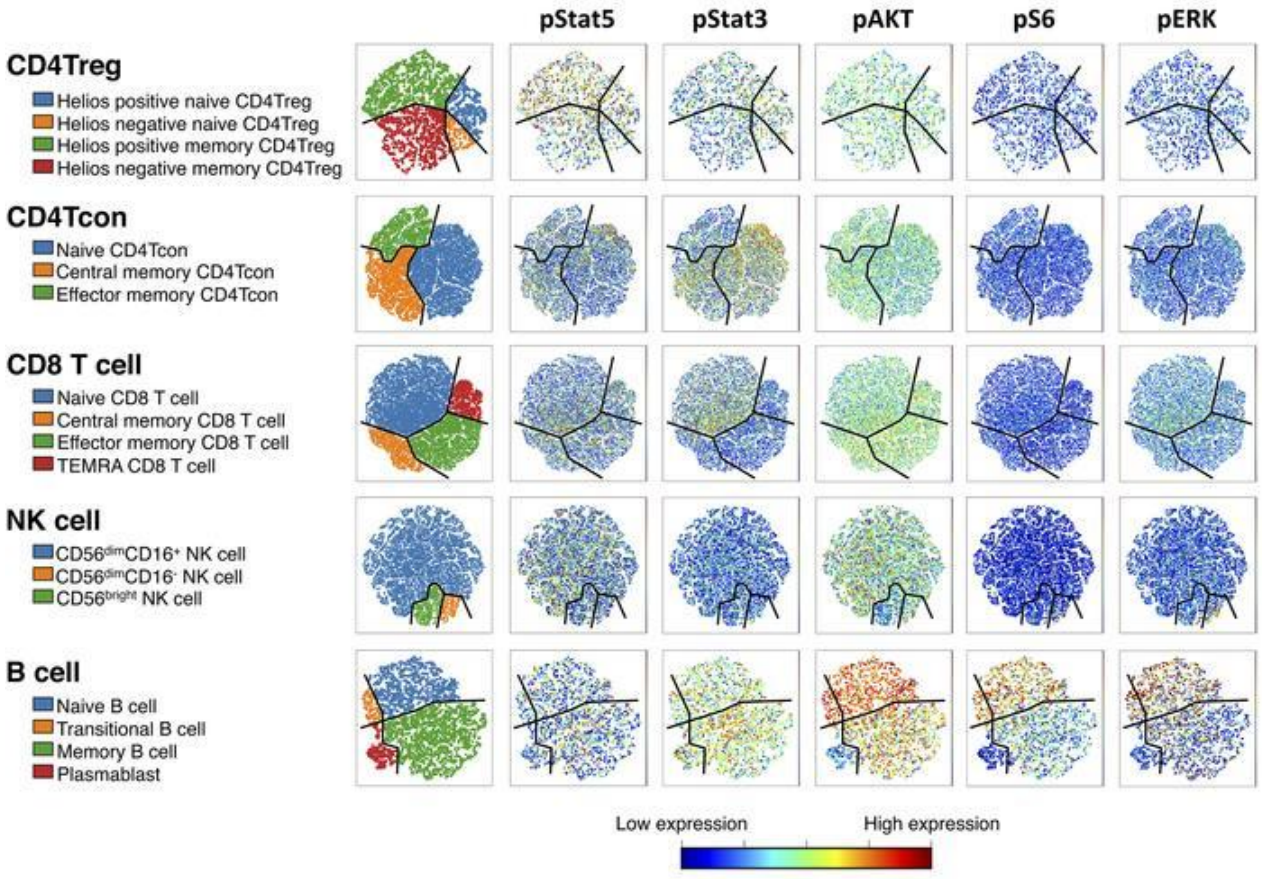


Figure 3. Basal expression of phosphorylated signaling proteins in resting lymphocyte subsets. The viSNE maps for each lymphocyte population are the same as for Figures 1 and 2. Each viSNE map shows intensity of expression of the marker indicated in each column. Expression levels of each phosphoprotein (column) are normalized across lymphocyte subsets. The far-left map in each row identifies the phenotypic cell subsets shown in the color legend. Data shown are from a single individual and are representative of 5 healthy donor samples.

IL-2 concentration-dependent activation of T and NK cell subsets in vitro.

Ligand activation of the IL-2 receptor is known to induce phosphorylation of JAK1 and JAK3, leading to phosphorylation of STAT5 (50–52). We previously reported that low concentrations of IL-2 preferentially activate STAT5 in CD4 Tregs compared with CD4Tcons (37). However, to the best of our knowledge the differential effects of IL-2 on various lymphocyte subsets or other signaling pathways have not been examined. To compare the response of each lymphocyte population to IL-2, we stimulated PBMCs from healthy donors with various concentrations of IL-2 for 15 minutes in vitro and simultaneously evaluated the expression of signaling proteins by mass cytometry (Figure 4). As expected, low IL-2 concentrations (1 to 10 IU/ml), preferentially induced p-STAT5 in CD4 Tregs. At these low concentrations, p-STAT5 was activated more strongly in Helios^{+} memory and Helios^{+} naive CD4 Tregs, compared with Helios^{-} CD4 Treg subsets. At high IL-2 concentrations (100 to 1,000 IU/ml), p-STAT5 was activated in all CD4 Treg subsets. Within CD4Tcons, high IL-2 concentrations (100 to 1,000 IU/ml) were required to induce p-STAT5 activation and this preferentially occurred in central memory and effector memory subsets. Within CD8+ T cells, very high IL-2 concentrations (≥1,000 IU/ml) were required to induce p-STAT5 and both naive and memory subsets were strongly activated.

Within NK cells, p-STAT5 was detected in the CD56^{bright}CD16^{-} subset after stimulation with a relatively low IL-2 concentration (10 IU/ml), but the level of activation was lower than in CD4Tregs. At high IL-2 concentrations (100 to 1,000 IU/ml), p-STAT5 was also activated in the major CD56^{dim}CD16^{+} NK subset, but the level of activation remained higher in the CD56^{bright}CD16^{-} subset. Very little p-STAT5 activation was detected in the CD56^{dim}CD16^{-} NK subset. Within B cells, p-STAT5 was not activated at any concentration of IL-2.

Stimulation with IL-2 induced relatively little activation of other signaling pathways (Supplemental Figures 1–4). IL-2–induced p-STAT3 expression was not detected in any lymphocyte population except CD56^{bright}CD16^{-} NK cells (Supplemental Figure 1). In control experiments, IL-6 induced p-STAT3 expression in naive T cell subsets (data not shown). IL-2 induced p-AKT and p-ERK expression in CD56^{bright}CD16^{-} NK cells at a relatively high concentration (100 IU/ml) (Supplemental Figures 2 and 3). At a very high IL-2 concentration (1,000 IU/ml), p-AKT was activated in all NK cell subsets (Supplemental Figure 2). Minimal p-S6 activation was detected in CD56^{bright}CD16^{-} NK cells at a very high IL-2 concentration (1,000 IU/ml), but IL-2 did not induce p-S6 in any T cell population (Supplemental Figure 4). Activated p-S6 was present at high levels in naive and transitional B cells, but expression was not altered by IL-2 stimulation (Supplemental Figure 4).

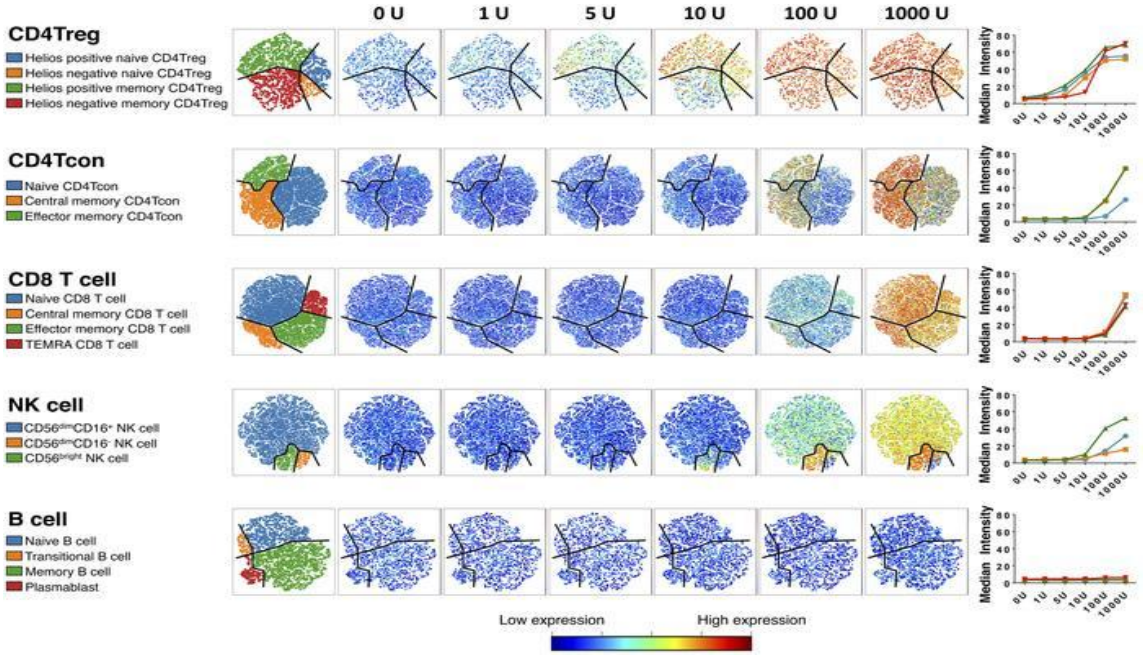


Figure 4. Visualization of p-STAT5 activation after stimulation with varying concentrations of IL-2 in vitro. Peripheral blood mononuclear cells (PBMCs) from healthy donors were stimulated for 15 minutes with varying concentrations of IL-2. Dose-dependent phosphorylation of STAT5 in each lymphocyte subset was monitored by mass cytometry. Each viSNE map shows intensity of expression of p-STAT5 with each indicated concentration of IL-2 (0 to 1,000 IU/ml). Expression levels of p-STAT5 are normalized across all lymphocyte subsets and all concentrations of IL-2. The far-left map in each row identifies the phenotypic cell subsets shown in the color legend. Results in each viSNE map show data from a single representative experiment. Results shown in the far-right graph in each row summarize results (median p-STAT5 expression values) of 5 independent experiments. Median values and ranges for each data point in these graphs are provided in Supplemental Table 2.

Preferential expansion of Helios$^+$ CD4 Tregs and CD56^{bright}CD16$^-$ NK cells during low-dose IL-2 therapy in vivo.

To examine immunological effects of IL-2 in vivo, we examined PBMCs from 14 patients with refractory chronic GVHD who received daily subcutaneous injections of low-dose IL-2 (1 × 106 IU/m2/day) for 12 weeks, followed by a 4-week hiatus. Eight of these patients noted clinical improvement during the initial 12-week treatment period and elected to restart daily IL-2 therapy at the same dose at 16 weeks. Extended daily IL-2 therapy was continued for at least 48 weeks. Clinical characteristics of these patients are summarized in Table 1. We first analyzed changes in the proportion of each lymphocyte population during the first 12 weeks of low-dose IL-2 therapy. Contour plots of viSNE maps focusing on CD4$^+$ T cells from 1 representative patient are shown in Figure 5A. Prior to IL-2 therapy, patients with active chronic GVHD have characteristically low numbers of CD4 Tregs. Selective expansion of CD4 Tregs was evident after 1 week of treatment. CD4 Treg expansion stabilized at 4 weeks and remained elevated at the end of therapy (12 weeks). Four weeks after stopping IL-2 (16 weeks), CD4 Treg levels returned to baseline levels. Changes in the proportion of CD4 Tregs and CD4 Tcons for all 14 patients during low-dose IL-2 are summarized in Figure 5B. The proportion of CD4 Tregs peaked 2 weeks after starting low-dose IL-2 and remained elevated during the entire 12-week course of therapy. CD4 Treg levels returned to baseline 4 weeks after stopping IL-2.

Applying viSNE to characterize CD4 Treg subsets, the expansion of CD4 Tregs during low-dose IL-2 therapy was primarily evident in Helios$^+$ subsets (Figure 5C). Changes in CD4 Treg subsets from all patients are summarized in Figure 5D. Within both naive and memory CD4 Tregs, the proportion of Helios$^+$ cells increased during low-dose IL-2 therapy, while the overall proportions of naive and memory CD4 Tregs remained stable. As shown in Figure 5, E and F, selective expansion of CD56^{bright}CD16$^-$ NK cells also occurred during low-dose IL-2 therapy and returned to baseline after IL-2 therapy was stopped. In contrast, no changes were noted in CD4Tcon, CD8$^+$ T cell, or B cell subsets during the 12-week period of low-dose IL-2 therapy (Supplemental Figure 5, A–C).

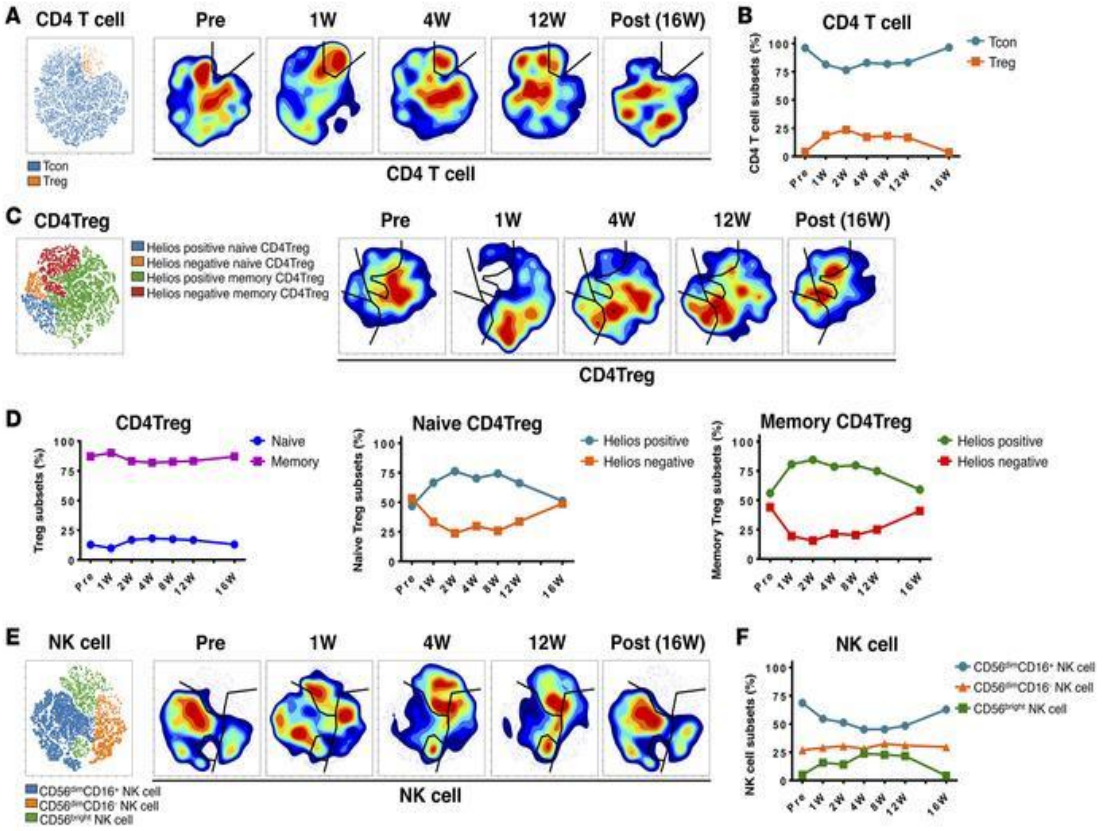


Figure 5. Proportional representation of each lymphocyte subset during low-dose IL-2 therapy in vivo. (**A**) Contour viSNE plots of $CD4^+$ T cells in a representative patient with chronic graft-versus-host disease (GVHD) receiving low-dose IL-2 therapy. The far-left color map identifies $CD4^+$ T cell subsets based on expression of CD25 and FoxP3, and the map is colored to identify conventional $CD4^+$ T cells (CD4Tcons) and $CD4^+$ Tregs (CD4Tregs). The contour maps represent cell density in each region of the map at the indicated time point (in weeks [W]) during IL-2 therapy. (**B**) Summary of $CD4^+$ T cell subset distributions in 14 patients with chronic GVHD receiving low-dose IL-2 therapy, expressed as percentages of total CD4+ T cells, at the indicated time point during therapy. (**C**) Contour viSNE plots of Tregs in a representative patient at the indicated time points during IL-2 therapy. The far-left color map identifies CD4 Treg subpopulations that were defined in Figure 1B, and the color for each subpopulation is indicated. (**D**) Summary of CD4 Treg subsets in 14 patients receiving daily IL-2 expressed as a percentage of total CD4 Tregs (left), naive CD4 Tregs (middle), and memory CD4 Tregs (right) at the indicated times during therapy. (**E**) Contour viSNE plots of NK cells in a representative patient receiving daily IL-2. The far-left color map next to the contour plots identifies NK subsets that were defined in Figure 1E, and the color for each NK subset is indicated. (**F**) Summary of changes in NK cell subsets in 14 patients receiving daily IL-2 expressed as a percentage of total $CD56^+$ NK cells. Median values and interquartile ranges for each data point in each of these graphs are provided in Supplemental Table 3. Statistical comparisons with pretreatment values are also provided in Supplemental Table 3. Pre, before IL-2 therapy; Post, 4 weeks after stopping IL-2 therapy.

Effects of low-dose IL-2 therapy on expression of functional proteins.

Mass cytometry allowed us to track expression of several important functional proteins in peripheral lymphocytes during low-dose IL-2 therapy. As shown in Figure 6, most changes during low-dose IL-2 therapy occurred in CD4 Tregs and $CD56^{bright}CD16^{-}$ NK cells. Consistent with in vitro studies, p-STAT5 was selectively activated in CD4 Tregs, but we did not detect increased expression of p-STAT5 in NK cells. The expression of p-STAT5 in CD4 Tregs peaked 1 week after IL-2 therapy began (Figure 6A). CD4Treg p-STAT5 expression decreased after 2 weeks but remained higher than baseline for the duration of therapy (P = 0.0001 at 12 weeks). Expression of FoxP3, CD95, CTLA4, and HLA-DR also increased in CD4 Tregs, with peak levels occurring 1 week after starting IL-2 (Figure 6, B–E). Although expression of these proteins gradually declined as IL-2 therapy continued, expression levels remained higher than baseline after 12 weeks of daily treatment ($P \leq 0.0002$). As shown in Figure 6F, a dramatic increase in proliferation of CD4 Tregs and $CD56^{bright}CD16^{-}$ NK cells were also observed 1 week after starting IL-2. Proliferation of these cells rapidly returned to baseline levels after 4 weeks despite continued daily IL-2 therapy. Changes in expression of antiapoptotic BCL2 and proapoptotic BIM proteins were also restricted to CD4 Tregs (Figure 6, G and H). Expression of both BCL2 and BIM decreased 1 week after starting IL-2 but recovered to baseline levels at 2 weeks. Subsequently, expression of BCL2 and BIM gradually increased with peak levels occurring 8 weeks after starting IL-2. Expression of PD1 also increased in CD4 Tregs during IL-2 therapy, with peak expression occurring 2 weeks after starting treatment (Figure 6I). PD1 was also the only marker in our panel whose expression increased in CD4Tcons and $CD8^{+}$ T cells during IL-2 therapy. Finally, IL-2 therapy also appeared to induce expression of CTLA4 in the NK cell population.

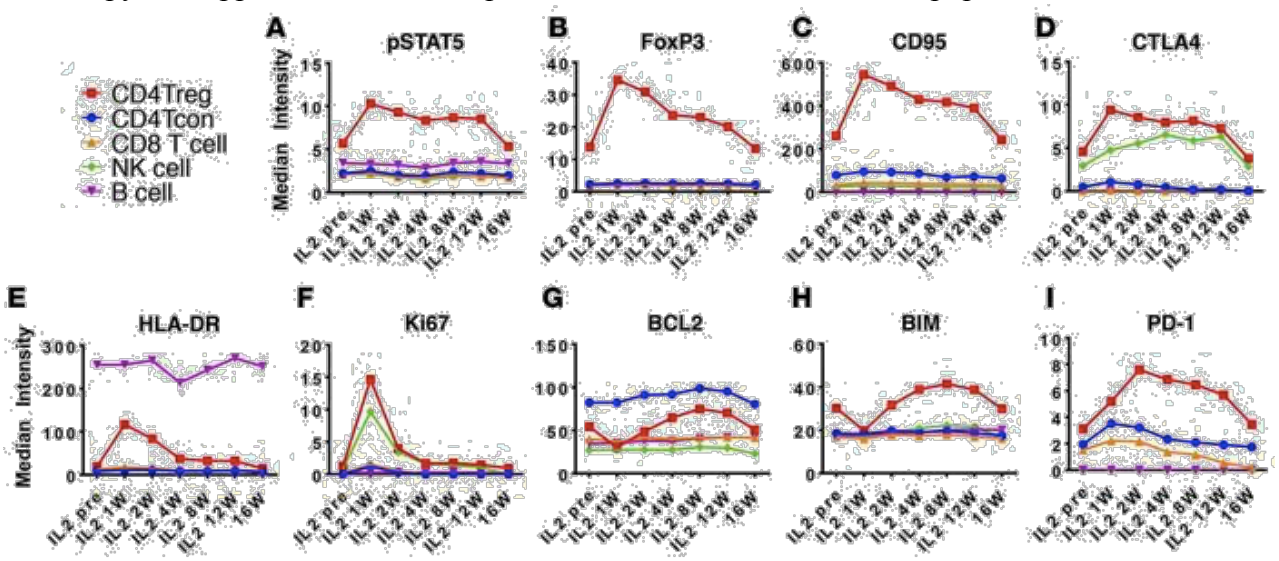


Figure 6. Effect of daily low-dose IL-2 therapy on each major lymphocyte population in vivo. (**A**–**I**) Each graph shows changes in expression of the indicated marker in each lymphocyte population during 12 weeks (12W) of daily low-dose IL-2 therapy and 4 weeks after stopping therapy (16W). Data points represent median values for each indicated marker for 14 patients tested at every time point during therapy. Median values and interquartile ranges for each data point in each of these graphs are provided in Supplemental Table 4. Statistical comparisons with pretreatment values are also provided in Supplemental Table 4.

Helios$^+$ CD4Treg subsets preferentially respond to low-dose IL-2 in vivo.

Using viSNE, we were able to further define the effects of IL-2 therapy on CD4 Treg subsets in vivo. Figure 7 shows serial viSNE maps of CD4 Tregs from a representative patient as well as a summary of changes in protein expression in each CD4Treg subset from all 14 patients. Consistent with results of in vitro stimulation, p-STAT5 was preferentially activated in Helios$^+$ naive and memory CD4 Treg subsets during low-dose IL-2 (Helios$^+$ versus Helios$^-$ naive Tregs at 1 week, $P = 0.0002$; Helios$^+$ versus Helios$^-$ memory Tregs at 1 week, $P = 0.0001$) (Figure 7A). Similarly, expression of FoxP3, HLA-DR, Ki67, CD25, and BIM increased primarily in Helios$^+$ CD4 Treg subsets (Figure 7, B, E–G, and I). Expression of CD95, CTLA4, and PD-1 increased in Helios$^-$ memory as well as Helios$^+$ CD4 Treg subsets. In contrast, BCL2 expression increased preferentially in naive CD4 Tregs, including both Helios$^+$ and Helios$^-$ subsets (Figure 7J). The only marker in our panel with decreased expression in CD4 Tregs during IL-2 therapy was CD127 (Figure 7K).

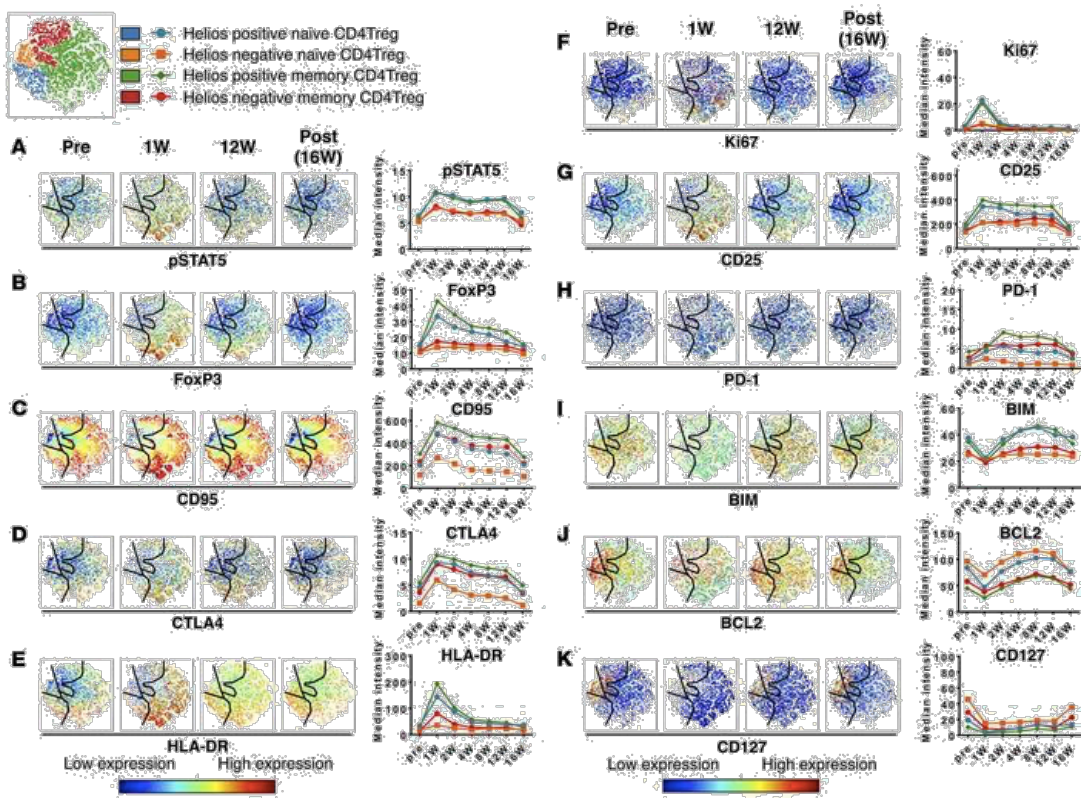


Figure 7. Effects of daily low-dose IL-2 therapy on CD4$^+$ Tregs in vivo. (**A**–**K**) Each row shows the viSNE maps of CD4$^+$ Tregs (CD4 Tregs) in a single representative patient at different time points (in weeks [W]) during daily IL-2 therapy. Different proteins are tracked in each row. In each viSNE map, cells are colored to show intensity of expression of each marker. The location of CD4 Treg subsets is color coded at the top of this figure. Median values for expression of each marker in samples from 14 patients at the indicated time points are shown in the graphs to the far right of each row. Median values and interquartile ranges for each data point in each of these graphs are provided in Supplemental Table 5. Statistical comparisons with pretreatment values are also provided in Supplemental Table 5. Pre, before IL-2 therapy; Post, 4 weeks after stopping IL-2 therapy.

Effects of low-dose IL-2 therapy on NK cell, CD4Tcon, and CD8$^+$ T cell subsets.

Our analyses of major lymphocyte populations during low-dose IL-2 therapy demonstrated upregulation of Ki67 and CTLA4 in NK cells and increased expression of PD-1 in CD4Tcons and CD8$^+$ T cells. More detailed analysis shown in Figure 8A demonstrated that increased proliferation primarily occurred in CD56^{bright}CD16$^-$ NK cells, peaking at 1 week after starting IL-2 therapy and then declining. A similar pattern of increased proliferation was also detected in the major population of CD56^{dim}CD16$^-$ NK cells, albeit at lower levels. While analysis of the entire NK cell population demonstrated increased expression of CTLA4, more detailed analysis in Figure 8B showed that the CD56brightCD16– NK cell subset constitutively expressed high levels of CTLA4. High-level expression of CTLA4 in this subset did not change during IL-2 therapy, but the selective expansion of CD56^{bright}CD16$^-$ NK cells led to the measured increase in CTLA4 expression when the entire NK cell population was considered. Detailed analysis of PD-1 expression in CD4Tcons and CD8$^+$ T cell subsets revealed that increased expression of PD-1 was most evident in effector memory CD4 Tcons and effector memory CD8$^+$ T cells (baseline versus 1 week, $P = 0.0023$ and $P = 0.018$, respectively) (Figure 8, C and D). Although small numbers of FoxP3$^+$ cells were detected within CD4Tcon and CD8$^+$ T cells, there was no increase in FoxP3 expression in these populations during IL-2 therapy.

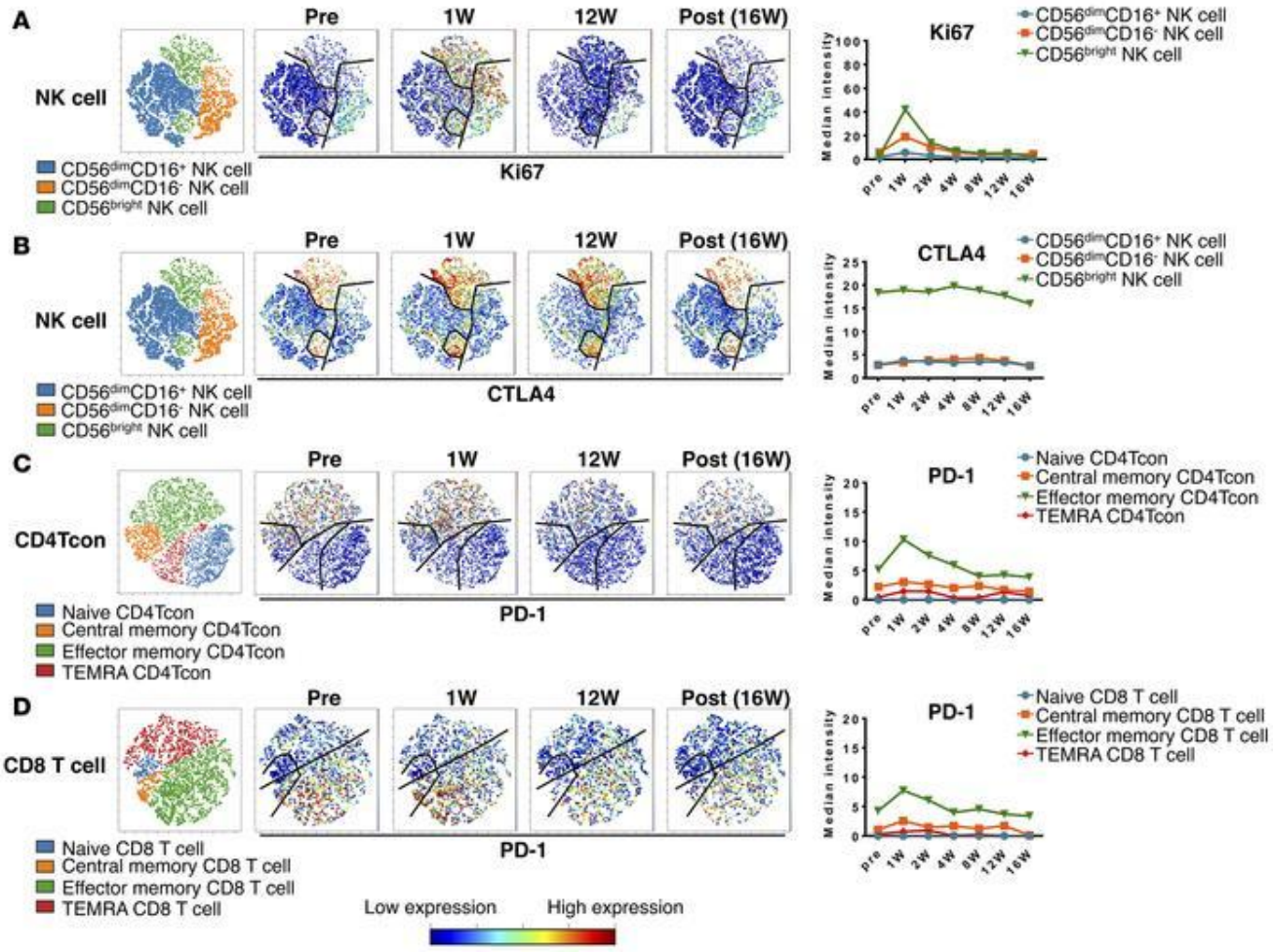


Figure 8. Effects of daily low-dose IL-2 therapy on conventional CD4+ T cells, CD8$^+$ T cells, and NK cells in vivo. After gating on (**A** and **B**) NK cells, (**C**) conventional CD4$^+$ T cells (CD4Tcons), or (**D**) CD8$^+$ T cells, each row shows the viSNE maps for a single representative patient at different time points (in weeks [W]) during therapy. Expression of different proteins is tracked in each row. In viSNE maps, cells are colored according to intensity of expression of each indicated marker. The viSNE maps for NK cells (**A** and **B**) are the same as Figure 5E and the maps for CD4Tcons (**C**) and CD8$^+$ T cells (**D**) were defined as in Figure 1, C and D, respectively. Median values for expression of each marker in samples from 14 patients at the indicated time points are shown in the graphs to the far right of each row. Median values and interquartile ranges for each data point in each of these graphs are provided in Supplemental Table 6. Statistical comparisons with pretreatment values are also provided in Supplemental Table 6. Pre, before IL-2 therapy; Post, 4 weeks after stopping IL-2 therapy.

Sustained effects of extended low-dose IL-2 therapy on CD4 Tregs in vivo.

To examine the immunologic effects of extended IL-2 therapy on CD4 Tregs, we examined PBMCs from 8 patients who received continuous treatment for at least 48 weeks. The clinical characteristics of these patients are summarized in Supplemental Table 1. In Figure 9, protein expression in CD4 Treg subsets measured after 48 weeks of daily low-dose IL-2 is compared with mass cytometry measurements obtained at the end of the initial 12-week IL-2 regimen and 4 weeks after discontinuation of IL-2 (16 weeks). p-STAT5 levels decreased 4 weeks after stopping IL-2, but p-STAT5 levels increased when IL-2 was restarted and remained elevated with extended therapy 48 weeks later (Figure 9A). Similar to the initial course of IL-2 therapy, p-STAT5 was preferentially increased in $Helios^+$ CD4 Treg subsets during extended therapy. As shown in Figure 9, B–F, expression of other proteins that increased during the initial 12-week treatment period (FoxP3, CD95, CTLA4, CD25, and BCL2) decreased when IL-2 was stopped and increased when IL-2 was restarted. For each of these functionally important proteins, increased expression levels were sustained after 48 weeks of daily IL-2 therapy and their relative expression within different CD4 Treg subsets remained stable. For example, expression of FoxP3 and CD25 was preferentially increased in $Helios^+$ CD4 Treg subsets. Expression of CD95 and CTLA4 increased more in $Helios^-$ memory CD4 Treg as well as $Helios^+$ naive and memory CD4 Treg subsets compared with $Helios^-$ naive CD4 Tregs. In contrast, BCL2 expression increased preferentially in naive CD4 Tregs and changes in BIM expression were not significant (Figure 9G). Although Ki67 expression increased 1 week after starting IL-2, expression was not increased relative to baseline at 12 weeks and there was no significant increase at 48 weeks (data not shown). In contrast with other proteins shown in Figure 9, CD127 (IL-7 receptor) expression in CD4 Tregs increased 4 weeks after discontinuation of IL-2 (Figure 9H). CD127 expression decreased during extended therapy, although this change was only statistically significant in $Helios^+$ CD4 Treg subsets. PD-1 expression did not change significantly during extended IL-2 therapy except for a significant increase in $Helios^+$ naive CD4 Tregs (Figure 9I).

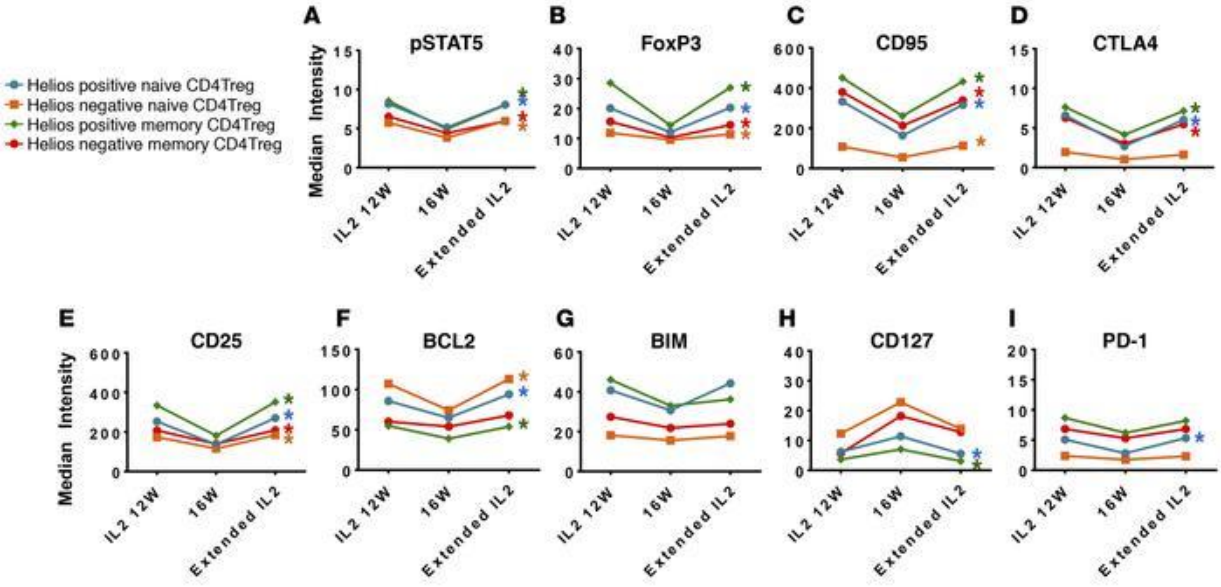


Figure 9. Effect of extended low-dose IL-2 therapy on CD4$^+$ Tregs. (**A**–**I**) Individual graphs show changes in expression of each indicated marker in CD4$^+$ Treg (CD4 Treg) subsets during extended IL-2 therapy. Data points represent median values for each indicated marker from 14 patients at 3 time points (in weeks [W]) during IL-2 therapy: 12W (at completion of initial therapy), 16W (4 weeks after stopping therapy), and 48 weeks after resuming extended therapy. Median values and interquartile ranges for each data point in each of these graphs are provided in Supplemental Table 7. *$P < 0.05$, 16W versus extended period (48W), 2-sided Wilcoxon signed-rank test. All statistical comparisons of 16W and 48W values are provided in Supplemental Table 7.

DISCUSSION

While previous studies have described expression of various functional proteins in different lymphocyte populations, the use of mass cytometry combined with machine learning techniques such as viSNE allowed us to undertake a comprehensive and simultaneous assessment of expression of 33 phenotypic and functional markers in all major lymphocyte subsets at single-cell resolution (43, 53). Application of this marker panel following in vitro stimulation with IL-2 for 15 minutes was used to monitor direct activation of different signaling pathways in each well-defined lymphocyte subset and to correlate signaling activity with expression of functional intracellular proteins as well as surface cytokine receptors. Comparison of a wide range of IL-2 concentrations subsequently identified specific subsets of T cells and NK cells that respond to either physiologic or supraphysiologic concentrations of this important immune cytokine. Finally, examination of serial samples obtained from patients receiving daily low-dose IL-2 therapy for almost 1 year allowed us to compare the results of prolonged administration

of exogenous cytokine at physiologic doses with the effects observed following short-term activation in vitro.

The IL-2 receptor is composed of 3 distinct components, each encoded by different genes and subject to different regulatory mechanisms. IL-2Rβ (CD122) and IL-2Rγ (CD132) are constitutively expressed on all T cells and NK cells and the intracellular domains of these components are responsible for all receptor mediated signaling activity. However, when expressed individually, IL-2Rβ and IL-2Rγ exhibit low affinity for IL-2 and when expressed together exhibit moderate binding affinity for IL-2. Constitutive expression of IL-2Rα (CD25) is limited to CD4Tregs and $CD56^{bright}$ NK cells, but expression of IL-2Rα by CD4Tcons and $CD8^+$ T cells is rapidly induced following T cell receptor (TCR) activation. IL-2Rα has no signaling function, but expression of IL-2Rα in conjunction with IL-2Rβ and IL-2Rγ creates a high-affinity trimeric receptor that is capable of responding to low concentrations of IL-2 (54). Our studies demonstrate that constitutive expression of CD25 is restricted to CD4Tregs and CD56bright NK cells and only these populations exhibit signaling activity in response to low concentrations of IL-2. Moreover, within the CD4Treg population, CD25 is expressed at relatively high levels in those cells that express the Helios transcription factor. Both memory and naive CD4 Tregs that express Helios can be activated by very low concentrations of IL-2 (1–10 IU/ml). Within CD4 Tregs, Helios expression correlates with high expression of FoxP3 and appears to be a marker of the most functionally active or primed subset of CD4 Tregs (20, 23). Nevertheless, as IL-2 concentrations increase (10–100 IU/ml), all CD4 Tregs are activated. This is consistent with the constitutive expression of high-affinity IL-2Rαβγ on all CD4 Tregs. Lacking constitutive expression of CD25, CD4Tcons and $CD8^+$ T cells are not activated at low concentrations of IL-2. However, CD4Tcons and $CD8^+$ T cells express both IL-2Rβ and IL-2Rγ, and IL-2–mediated signaling can be achieved with higher ligand concentrations (100–1,000 IU/ml). Within CD4Tcons, naive cells appear to be relatively resistant to activation, even at very high ligand concentrations. In contrast, all $CD8^+$ T cell subsets are equally activated in response to supraphysiologic concentrations of IL-2. Within all T cell populations, IL-2 signaling appears to be primarily mediated through phosphorylation of STAT5 and we found no activation of STAT3, ERK, AKT, or S6 kinase, even at very high concentrations of IL-2.

Within the NK cell population, cells that express the highest levels of CD56 ($CD56^{bright}CD16^{-}$) represent a relatively small but discrete subset that exhibits high levels of immune regulatory activity and relatively low levels of cytolytic activity (55). These cells also express CD25 and respond to low concentrations of IL-2 (10 IU/ml). In contrast, the majority of NK cells ($CD56^{dim}CD16^{+}$) express only IL-2Rβγ and therefore higher concentrations of IL-2 (100–1,000 IU/ml) are required to induce activation of these cells. The small NK cell subset that expresses low levels of CD56 without co-expression of CD16 ($CD56^{dim}CD16^{-}$) are relatively resistant to high concentrations of IL-2, suggesting that these cells do not express IL-2Rβγ. Like T cells, signaling in NK cells was restricted to STAT5 at low IL-2 concentrations but other pathways (AKT and ERK) were activated at high concentrations of IL-2.

The effects of daily administration of low-dose IL-2 in patients with chronic GVHD closely mirrored the effects of short-term in vitro stimulation of PBMCs with low concentrations of IL-2. Increased proliferation of CD4 Tregs was noted 1 week after starting IL-2 treatment and this was associated with selective activation of p-STAT5 in these cells in vivo. STAT3, ERK, AKT, and S6 kinase were not activated in vivo during IL-2 therapy. Activated CD4Tregs rapidly expressed higher levels of FoxP3, CD95, CTLA4, and HLA-DR. CD4 Treg expression of PD-1, BCL2, and BIM increased more slowly, with peak expression occurring 2–8 weeks after starting IL-2. Detailed analysis of CD4 Treg subsets revealed that these phenotypic changes occurred primarily in $Helios^{+}$ CD4 Tregs. Increased expression of CD95, CTLA4, and PD-1 was also detected in $Helios^{-}$ memory CD4 Tregs, but the expansion of CD4 Tregs during 12 weeks of IL-2 therapy was primarily due to selective expansion of $Helios^{+}$ CD4 Tregs, including naive as well as memory CD4 Tregs. Proliferation of CD4 Tregs and increased expression of HLA-DR returned to baseline levels 2–3 weeks after starting IL-2, but increased expression of other functional markers persisted for the entire 12-week period of daily IL-2 therapy. When IL-2 therapy was stopped, the population of $Helios^{+}$ CD4 Tregs contracted and expression of p-STAT5, FoxP3, CD95, CTLA4, BCL2, BIM, and PD-1 reverted to baseline levels. Taken together, these findings are consistent with murine studies demonstrating that Helios, an Ikaros-family transcription factor, acts to support the functional activity of $FoxP3^{+}$ Tregs (23). In our studies, expression of Helios specifically identifies human CD4 Tregs most capable of responding to low physiological concentrations of IL-2, regardless of whether these cells have a naive or memory phenotype (20). These cells are also highly

dependent on IL-2 and the marked expansion of Helios^{+} CD4 Tregs in vivo was rapidly reversed when IL-2 therapy was stopped. Notably, Helios expression in CD4 Tregs did not increase with IL-2 therapy and very few CD4Tcons or CD8^{+} T cells were found to express Helios.

Consistent with the results of short-term in vitro stimulation, expansion of NK cells during low-dose IL-2 therapy was restricted to the relatively small subset of CD56^{bright}CD16^{-} NK cells. Although this subset normally represents a small fraction of NK cells in peripheral blood, CD56^{bright}CD16^{-} NK cells represent the major NK cell population in lymph nodes and tonsils where they reside primarily in parafollicular T cell zones (55). Lacking expression of CD16, these cells do not mediate antibody-dependent cellular cytotoxicity (ADCC). Expression of cytolytic molecules such as perforin and granzyme is reduced and these cells contain fewer cytolytic granules than CD56^{dim}CD16^{+} NK cells. CD56^{bright}CD16^{-} NK cells typically do not express killer inhibitory receptors (KIRs) but express high levels of inflammatory and immune regulatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-10, and IL-13. Consistent with these phenotypic characteristics and tissue distribution, CD56^{bright}CD16^{-} NK cells exhibit less cytolytic activity and greater immune regulatory functions than CD56^{dim}CD16^{+} NK cells. However, the cytolytic functions of CD56^{bright}CD16^{-} NK cells increase when these cells are activated (56). CD56^{bright}CD16^{-} NK cells can also differentiate into CD56^{dim}CD16^{+} NK cells under appropriate conditions (55, 57). Our studies demonstrate that the proliferation and expansion of these cells in vivo is very responsive to low concentrations of IL-2, but these conditions in vivo are not sufficient to induce widespread activation and release of regulatory cytokines. Thus, the marked expansion of CD56^{bright}CD16^{-} NK cells in vivo was well tolerated and not associated with toxicities typically associated with cytokine release. Importantly, the expansion of these cells did not lead to exacerbation of chronic GVHD, which improved in more than 50% of patients (30). Although the goal of our clinical studies was to enhance tolerance induction through expansion of CD4 Tregs, the ability to selectively enhance the immune regulatory functions of NK cells in vivo with low-dose IL-2 may also have clinical utility. In fact, a recent study found that low levels of CD56^{bright} NK cells were associated with chronic GVHD (58), and the expansion of these cells in response to IL-2 therapy may well have beneficial consequences in patients with chronic GVHD.

While the most dramatic effects of IL-2 therapy were observed within CD4 Treg and NK cell populations, we were particularly interested in whether mass cytometry would allow us to detect activated CD4 Tcons or $CD8^+$ T cells that were responsible for mediating alloimmune responses in patients with active chronic GVHD. Since TCR-activated effector T cells express CD25, these cells would also be expected to respond to low concentrations of IL-2 in vivo. Using activation of STAT5 as a sensitive marker of IL-2 activation, we did not observe expression of p-STAT5 in any subset of CD4Tcons or $CD8^+$ T cells during IL-2 therapy. Instead, we found that expression of PD-1 increased in both CD4Tcons and $CD8^+$ T cells. This was most evident 1 week after starting IL-2 and coincided with the peak activation of CD4 Tregs. Notably, PD-1 expression in CD4 Tcons and $CD8^+$ T cells was restricted to cells within the effector memory subset. Since this was not associated with increased proliferation or activation of p-STAT5, this likely represents an indirect effect demonstrating the suppressive activity of CD4 Tregs.

In our phase 2 clinical trial, patients with active chronic GVHD received daily injections of low-dose IL-2 for 12 weeks followed by a mandatory 4-week period without IL-2 treatment. Patients with clinical improvement of chronic GVHD were eligible to restart IL-2 after the 4-week off-treatment period and could continue daily treatment indefinitely. Although CD4Treg levels reverted to baseline 4 weeks after stopping IL-2, CD4 Treg levels increased again when IL-2 was restarted and CD4 Treg expansion was maintained for the entire duration of extended IL-2 therapy (30). In the present study, we examined PBMCs from 8 patients who had been on extended IL-2 therapy for 48 weeks. At this late time point, expression of all functional markers of CD4 Tregs in our panel was similar to levels that were present at the end of the first 12 weeks of IL-2 therapy. Detailed analysis of CD4 Treg subsets after 48 weeks of continuous daily IL-2 treatment revealed that expression of p-STAT5, FoxP3, and CD25 increased most in $Helios^+$ CD4 Treg subsets, while CD95 and CTLA4 expression increased in $Helios^-$ memory CD4 Tregs as well as $Helios^+$ CD4 Tregs. Both CD25 and CTLA4 expressions by CD4 Tregs are closely linked to the immune regulatory functions of these cells. Through expression of CD25, creating high-affinity IL-2R, CD4 Tregs are able to sequester IL-2 from effector T cells and thereby limit the expansion of effector T cells in vivo. Similarly, CTLA4 expressed by CD4Tregs binds to CD80 and CD86 to antagonize co-stimulation of effector T cells

through CD28 (59–61). With prolonged treatment, increased expression of these functional molecules was detected in Helios$^-$ as well as Helios$^+$ CD4 Tregs. Taken together, these changes suggest that prolonged low-dose IL-2 therapy results in significant changes in CD4 Treg homeostasis that include all CD4 Treg subsets. Although not shown, there was no evidence of activation of CD4 Tcons or CD8$^+$ T cells with extended IL-2 therapy. Nevertheless, prolonged low-dose IL-2 therapy also resulted in the gradual increase and normalization of CD4Tcon and CD8$^+$ T cell counts and restoration of the normal balance between regulatory and effector T cell populations (30).

Recent reports have suggested that low-dose IL-2 may promote immune tolerance in a wide variety of clinical settings in addition to GVHD (32–35, 38, 39, 62, 63). The profound but highly selective immunologic effects of prolonged low-dose IL-2 therapy demonstrated in our studies support further clinical evaluation of this approach in various settings where selective enhancement of CD4 Tregs may be needed to restore or maintain immune tolerance.

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SUPPLEMENTAL MATERIALS

Supplemental Table 1. Mass cytometry antibody-conjugate panel

	TARGET	SPECIES	CLONE	ISOTOPE	Manufacturer
	Surface				
1	CD45	Human	HI30	154Sm	Fluidigm
2	HLA-DR	Cross	L243	141Pr	Biolegend
3	CD95	Human	DX2	164Dy	Fluidigm
4	CD3	Human	UCHT1	170Er	Fluidigm
5	CD4	Human	SK3	174Yb	Fluidigm
6	CD8	Human	SK1	168Er	Fluidigm
7	CD25	Human	2A3	149Sm	Fluidigm
8	CD127	Human	A019D5	176Yb	Fluidigm
9	CD45RA	Human	HI100	169Tm	Fluidigm
10	CD62L	Human	DREG-56	153Eu	Fluidigm
11	CD31	Human	WM59	145Nd	Fluidigm
12	CD279(PD1)	Human	EH12.2H7	175Lu	Fluidigm
13	CD152(CTLA4)	Human	14D3	163Dy	eBioscience
14	CD19	Human	HIB19	142Nd	Fluidigm
15	IgD	Human	IA6-2	146Nd	Fluidigm
16	CD38	Human	HIT2	144Nd	Fluidigm
17	CD27	Human	L128	155Gd	Fluidigm
18	CD5	Human	UCHT2	143Nd	Fluidigm
19	BAFF-R(CD268)	Human	11C1	160Gd	Biolegend
20	CD56	Human	HCD56	162Dy	Biolegend
21	CD16	Human	3G8	148Nd	Fluidigm
22	NKG2D（CD314）	Human	1D11	161Dy	Fluidigm
	Intracellular				
23	Foxp3	Human	PCH101	165Ho	eBioscience
24	BCL-2	Human	Bcl-2/100	171Yb	BD Bioscience
25	Ki67	Cross	B56	151Eu	BD Bioscience
26	BIM	Cross	Polyclonal	159Tb	BD Bioscience
27	Helios	Cross	22F6	156Gd	Biolegend
	Signaling				
28	pStat5 [Y694]	Cross	47	150Nd	Fluidigm
29	pStat3 [Y705]	Cross	4/P-Stat3	158Gd	Fluidigm
30	pERK 1/2 [T202/Y204]	Cross	D13.14.4E	167Er	Fluidigm
31	pS6 Ribo(S235/236)	Cross	N7-548	172Yb	Fluidigm
32	p-AKT	Cross	S473	152Sm	Fluidigm
	Negative control				
33	HIV1-p24	Human	39/5.4A	166Er	Abcam

Figure Legends S1-S4.
Data are representative of three independent experiments and the viSNE maps are the same as described in Figure 4. Expression of each signaling protein in all cell subsets are shown after stimulation with increasing concentrations of IL-2 (0-1,000 IU/ml) and results were analyzed as in Figure 4.

Figure S1: Effect of IL-2 stimulation in vitro on pSTAT3 activation in lymphocyte subsets

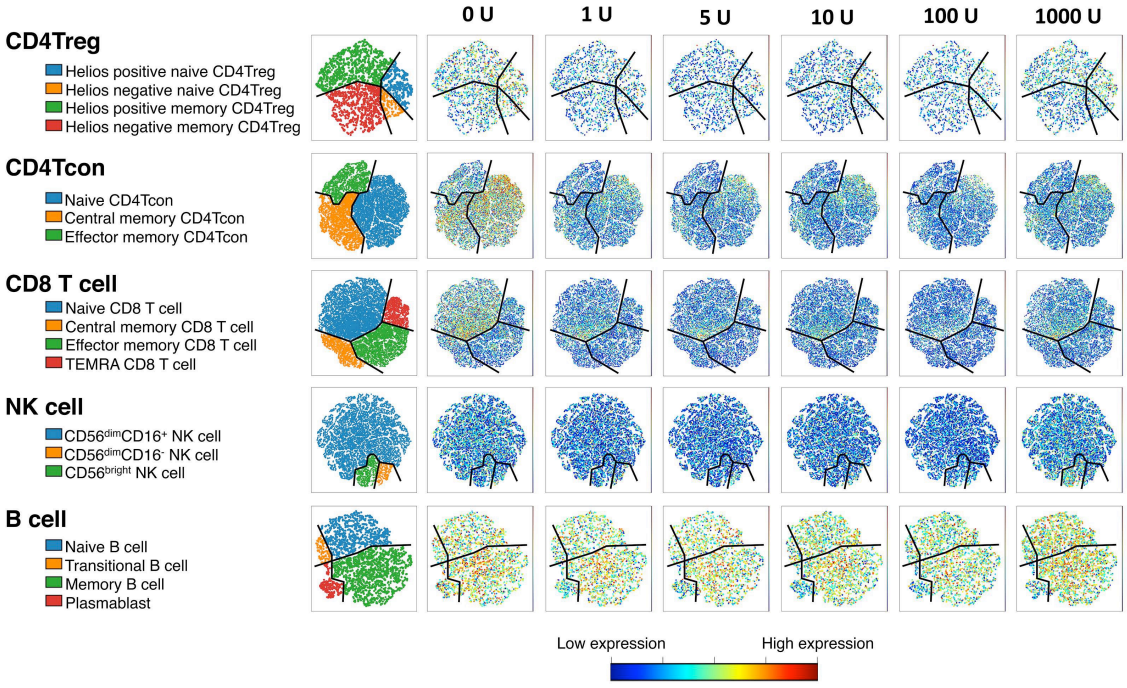


Figure S2: Effect of IL-2 stimulation in vitro on pAKT activation in lymphocyte subsets

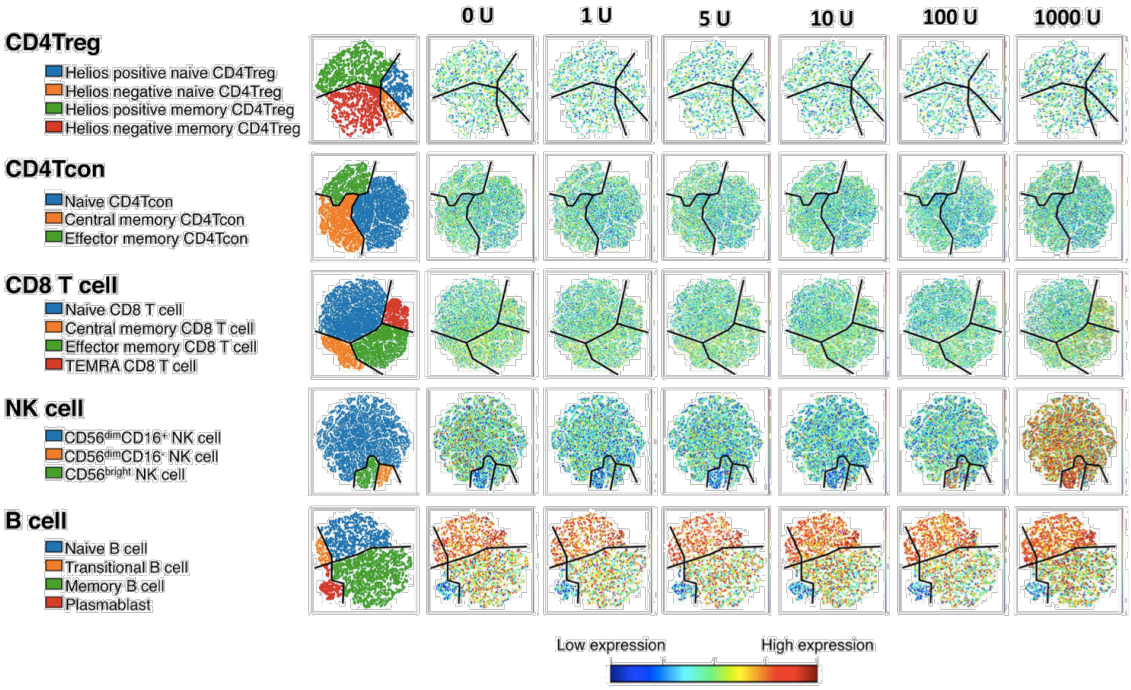


Figure S3: Effect of IL-2 stimulation in vitro on pERK activation in lymphocyte subsets

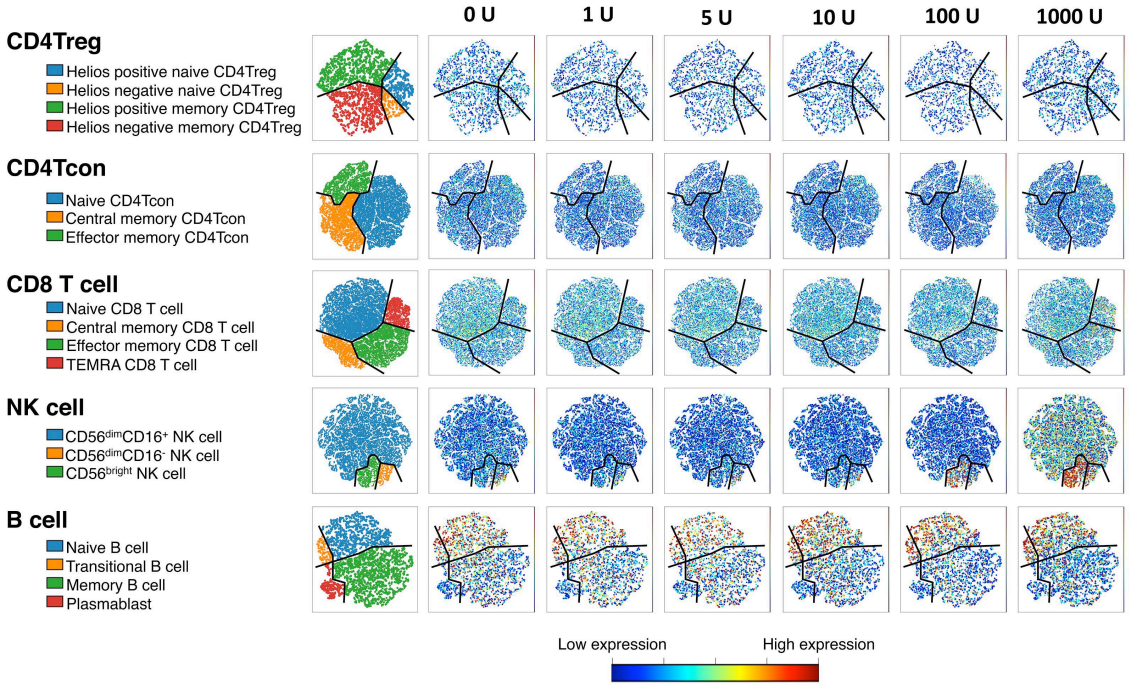


Figure S4: Effect of IL-2 stimulation in vitro on pS6 activation in lymphocyte subsets

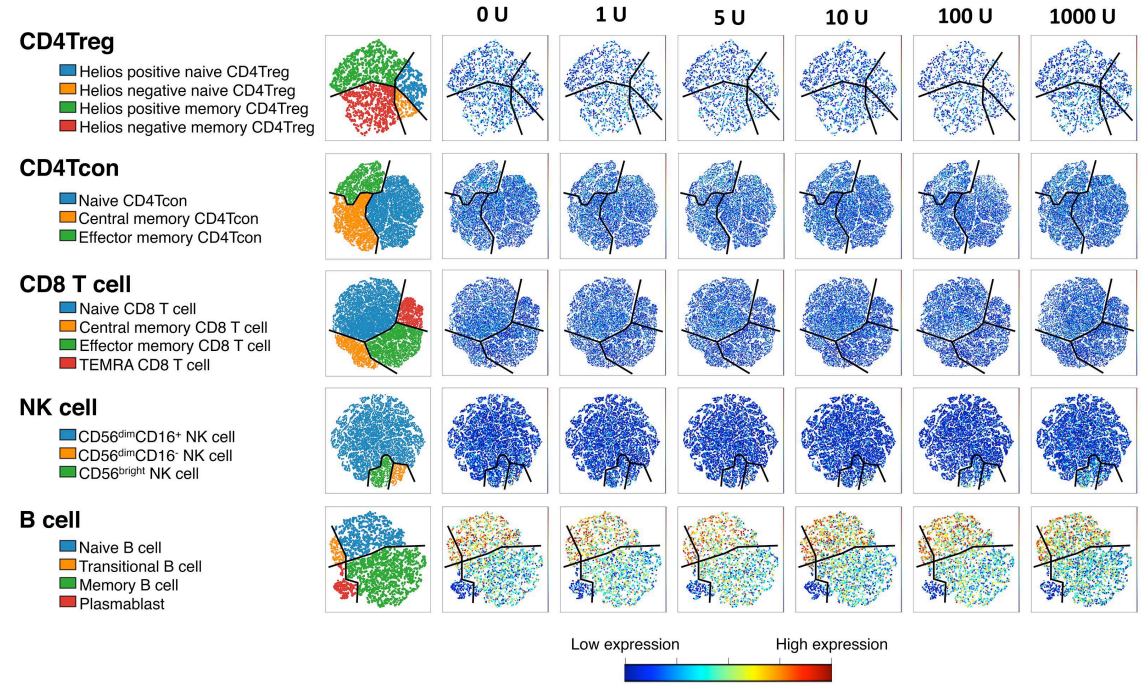
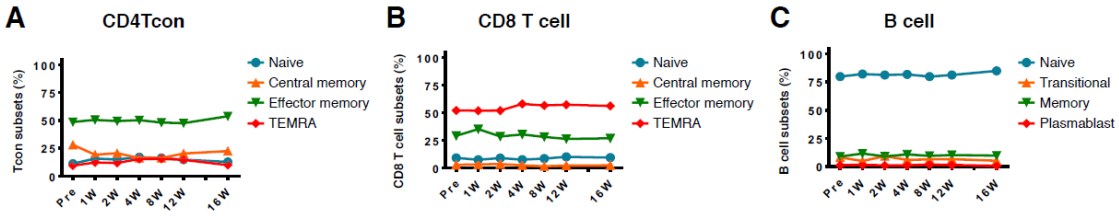


Figure S5. Effect of low-dose IL-2 therapy on CD4Tcon, CD8 and B cell subsets. (**A-C**) Summary of CD4Tcon, CD8 T cell and B cell subset distribution from the 14 patients receiving IL-2 shown in Figure 5. Results at each time point are expressed as percentages of (A) total CD4Tcon, (B) total CD8 T cells and (C) total B cells.



CHAPTER 5

Phase I/II Trial of a Combination of Anti-CD3/CD7 Immunotoxins for Steroid-Refractory Acute Graft-versus-Host Disease

Christoph Groth,[1#] Lenneke F.J. van Groningen,[2,3#] Tiago R. Matos,[4] Manita E. Bremmers,[2] Frank W.M.B. Preijers,[5] Harry Dolstra,[5] Christian Reicherts,[1] Nicolaas P.M. Schaap,[2,3] Eric H.G. van Hooren,[6] Joanna IntHout,[3,7] Rosalinde Masereeuw,[8] Mihai G. Netea,[9] John E. Levine,[10] George Morales,[10] James L. Ferrara,[10] Nicole M.A. Blijlevens,[2,3] Ypke V.J.M. van Oosterhout,[6] Matthias Stelljes,[1] and Walter J.F.M. van der Velden[2,3]

[#] contributed equally to this work.

Thesis' author contribution: T.R.M. performed the research, analyzed and interpreted the data, performed statistical analyses and wrote the manuscript.

Affiliations:

[1]Department of Medicine A/Hematology and Oncology, University Hospital of Muenster, Muenster, Germany

[2]Department of Hematology, Radboud University Medical Center, Nijmegen, The Netherlands

[3]Radboud Institute of Health Sciences, Radboud University Medical Center, Nijmegen, The Netherlands

[4]Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

[5]Department of Laboratory Medicine, Laboratory for Hematology, Radboud University Medical Center, Nijmegen, The Netherlands

[6]Xenikos B.V., Nijmegen, The Netherlands

[7]Section of Biostatistics, Department for Health Evidence, Radboud University Medical Center, Nijmegen, The Netherlands

[8]Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands

[9]Department of Internal Medicine, Radboud University Medical Center, Nijmegen, The Netherlands

[10]Tisch Cancer Institute, The Icahn School of Medicine at Mount Sinai Hospital, New York NY

ABSTRACT

Effective therapies for treating patients with steroid-refractory acute graft-versus-host-disease (SR-aGVHD), particularly strategies that reduce the duration of immunosuppression following remission, are urgently needed. The investigated immunotoxin combination consists of a mixture of anti-CD3 and anti-CD7 antibodies separately conjugated to recombinant ricin A (CD3/CD7–IT), which induces in vivo depletion of T cells and natural killer (NK) cells and suppresses T cell receptor activation. We conducted a phase I/II trial to examine the safety and efficacy of CD3/CD7-IT in 20 patients with SR-aGVHD; 17 of these patients (85%) had severe SR-aGVHD, and all 20 patients had visceral organ involvement, including 18 (90%) with gastrointestinal (GI) involvement and 5 (25%) with liver involvement. A validated 2-biomarker algorithm classified the majority of patients (11 of 20) as high risk. On day 28 after the start of CD3/CD7-IT therapy, the overall response rate was 60% (12 of 20), with 10 patients (50%) achieving a complete response. The 6-month overall survival rate was 60% (12 of 20), including 64% (7 of 11) classified as high risk by biomarkers. The 1-week course of treatment with CD3/CD7-IT caused profound but transient depletion of T cells and NK cells, followed by rapid recovery of the immune system with a diverse TCR Vβ repertoire, and preservation of Epstein-Barr virus- and cytomegalovirus-specific T cell clones. Furthermore, our results indicate that CD3/CD7-IT appeared to be safe and well tolerated, with a relatively low prevalence of manageable and reversible adverse events, primarily worsening of hypoalbuminemia, microangiopathy, and thrombocytopenia. These encouraging results suggest that CD3/CD7-IT may improve patient outcomes in patients with SR-aGVHD.

INTRODUCTION

Acute graft-versus-host disease (aGVHD) is a major complication that can occur following allogeneic hematopoietic stem cell transplantation (HSCT). The prognosis for patients who develop aGVHD is poor, particularly in cases of severe steroid-refractory aGVHD (SR-aGVHD) with gastrointestinal (GI) and/or liver involvement [1,2]. At present, no standard second-line therapy is approved for SR-aGVHD, and none of the available treatment options seems to provide convincingly superior results with on average only 30% complete responders [1,3,4]. Six-month survival approximates 50%, but long-term survival is achieved in only 1 out of 5 patients [2].

The underlying core of a graft-versus-host immune reaction is the proliferation and differentiation of alloreactive donor T cells in response to the host's antigen-presenting cells, which induce tissue damage and the propagation of inflammation during the effector phase [5–7]. Therefore, many of the currently used therapies consist of antibodies that cause the depletion of T cells or either biologicals or small-molecule inhibitors designed to suppress T cell function [1,3,6,8]. The obvious challenge of such approaches is that the induced immunosuppression should be as selective and as brief as possible, to avoid infectious complications and relapse of the underlying hematologic malignancy, which otherwise could counterbalance the immediate benefit of controlling the aGVHD reaction [9–12].

As a novel approach to achieving this goal, we developed a combination of 2 anti-T cell immunotoxins designed to induce a synergistic in vivo depletion and suppression of T cells while allowing for rapid post-treatment reconstitution of the immune system [13,14]. This combination product consists of a 1:1 mixture of 2 murine monoclonal antibodies against CD3 and CD7, each of which is separately conjugated to a recombinant ricin toxin A chain [15] (T-Guard, designated CD3/CD7-IT hereinafter) [16,17]. Preclinical studies have shown that CD3/CD7-IT induces apoptosis of both T cells—particularly activated T cells—and natural killer (NK) cells by inhibiting protein synthesis, and also reduces T cell activation by blocking and modulating the TCR/CD3 complex (supplementary 1 etcetera; Figure 1) [17]. In a dose-escalation study, 5 out of 7 patients with SR-aGVHD responded to CD3/CD7-IT as third-line therapy [17]. The promising outcome of that study led to the phase I/II study of CD3/CD7-IT for the treatment of SR-aGVHD reported here.

METHODS

This prospective single-arm phase I/II study was approved by the Ethics Committees and Institutional Review Boards at the Radboud University Medical Center Nijmegen and the University Medical Center Muenster. Informed consent was obtained from all patients. This trial has been registered at www.ClinicalTrials.gov (NCT02027805).

The 4 mg/m2 T-Guard starting dose of the phase I/II study was selected on the basis of the outcome of the dose escalation study [17]. A Bryant-Day 2-stage design was applied [18], with a pre-scheduled interim analysis after 8 patients, to protect patients from unnecessary exposure to an ineffective or toxic treatment. If after the first 8 patients, 2 or fewer (≤25%) day 28 responders and/or 4 or more (≥50%) dose-limiting toxicities (ie, adverse drug reactions of grade 3 or higher) were observed (phase I), the trial would be terminated for futility and/or toxicity; otherwise, the trial would be extended to a total of 20 patients (phase II) (sample size estimation, S2).

Adult patients (age ≥18 years) who developed grade II-IV aGVHD following HSCT or following post-transplantation donor lymphocyte infusion [19] were eligible for participation; aGVHD grade was defined according to the criteria established by Harris et al. [20]. Diagnosis of aGVHD was confirmed with a tissue biopsy. SR-aGVHD was defined as aGVHD that progressed after 3 days or did not improve after 7 days on systemic corticosteroid therapy (≥2 mg/kg/day prednisolone or equivalent) [3,4]. Patients who had already received additional therapy for SR-aGVHD were excluded, as were patients with manifestations of moderate or severe chronic GVHD (cGVHD), severe organ dysfunction, uncontrolled infection, serum creatinine level >266 μmol/L (1.87mg/dL), and/or serum albumin level ≤1.5 g/dL.

The treatment schedule for CD3/CD7-IT (S3) consisted of four 4-hour i.v. infusions of 4 mg/m2 administered at 48-hour intervals. GVHD prophylaxis, which consisted primarily of cyclosporine A either alone or in combination with mycophenolate mofetil, was continued during with CD3/CD7-IT therapy. The recommended taper for systemic corticosteroids in patients responding to CD3/CD7-IT was 10% of the starting dose at 3- to 5-day intervals. After study day 28, the rate of steroid tapering was left to local protocols. The use of antimicrobial prophylaxis, preemptive and/or empirical treatment for infection,

and clemastine pretreatment (2 mg i.v.) was left to the discretion of the physician and established local protocols.

Patients were included in the analysis of toxicity and efficacy if they received at least 1 dose of CD3/CD7-IT. The primary endpoints were the overall response rate (ORR; defined as the sum of partial response [PR] and complete response [CR] rates) on day 28 and the occurrence of possible drug-related adverse events (AEs) up to 6 months following treatment with CD3/CD7-IT. The secondary endpoints were the day 28 CR rate, 6-month overall survival (OS), and the incidence of cGVHD. ORR, CR on day 28, and 6-month OS were compared with data recorded for our institutions' historical controls who received either inolimomab-etanercept (n = 21) or infliximab (n = 21) (S4; Table 1) [21]. CR was defined as the resolution of all signs and symptoms associated with aGVHD. PR was defined as an improvement in GVHD stage in all initial GVHD target organs, without complete resolution or emergence of GVHD in any new organ. No response was defined as no change, a mixed response, progressive disease, or the need for salvage therapy before day 28 [22]. The 2014 National Institutes of Health diagnostic criteria were used to assess and score cGVHD [23]. Hematologic and nonhematologic AEs, including cytokine release syndrome (CRS), were graded based on the Common Terminology Criteria for AEs, version 4.0. Capillary leak syndrome was graded as follows using previously defined criteria [24]: grade 1, asymptomatic, not requiring therapy; grade 2, symptomatic but not requiring fluid support; grade 3, respiratory compromise or requiring fluids; grade 4, life-threatening, requiring vasopressor support and/or mechanical ventilation. In the event of a grade 3 AE, subsequent doses with CD3/CD7-IT were to be given only if the patient's toxicity parameters improved or when judged to be in the patient's interest, at the investigator's discretion. Invasive fungal disease (IFD), Epstein-Barr virus (EBV) infection, and cytomegalovirus (CMV) infection were defined in accordance with established guidelines [25–27].

Manufacturing of CD3/CD7-IT. CD3/CD7-IT consists of the murine monoclonal antibodies SPV-T3a (anti-CD3) and WT1 (anti-CD7), each of which is conjugated to recombinant ricin toxin A (RTA). CD3/CD7-IT was manufactured following Good Manufacturing Practices as described previously [15], with the addition of a step to block residual linkers with cysteine and the replacement of deglycosylated plant-derived RTA

with recombinant RTA [17,28]. The immunotoxins were formulated at a concentration of 0.2 mg/mL in an isotonic buffered solution (pH 6.5) and stored frozen at −20°C or below.

In Vitro Laboratory Analyses. Peripheral blood samples were collected before and after treatment to analyze predictive GVHD biomarkers, cytokine levels, immune reconstitution, pharmacokinetics, and the development of human anti-drug antibodies (ADAs). Levels of the biomarkers ST2 (suppression of tumorigenicity 2) and Reg3*α* (regenerating islet-derived protein 3-*α*) were measured at the Icahn School of Medicine at Mount Sinai, New York. A probability score was determined for each patient based on a validated algorithm [29] used to predict the risk for treatment failure and non-relapse mortality among patients with aGVHD. Patients were considered at high risk at p^>.291 after 1 week ± 3 days of treatment with systemic corticosteroids. Serum cytokine levels were measured at Myriad RBM (Austin, TX) using quantitative, multiplexed immunoassays (S5).

Lymphocytes were analyzed by immunophenotyping using flow cytometry. Lymphocytes were gated on $CD45^+$ and low side-scatter cells, and enumeration of helper T cells ($CD5^+$ and $CD4^+$), cytotoxic T cells ($CD5^+$ and $CD8^+$), NK cells ($CD56^+$ and CD5−), and B cells ($CD19^+$) was recorded for each pheno-type per microliter of blood. CD5 was used instead of CD3 to identify and quantify T cells because of potential CD3 modulation by the CD3/CD7-IT treatment. For TCR sequencing, DNA was isolated from whole blood collected in PAXgene tubes (PreAnalytiX, Hombrechtikon, Switzerland). The TCR/3 CDR3 region was then amplified and sequenced using ImmunoSEQ. (Adaptive Biotechnologies, Seattle, WA). Bias-controlled V and J gene primers were used to amplify the rearranged V(D)J segments for high-throughput sequencing (HTS) analysis at approximately 20x coverage [30]. After correcting for sequencing errors using a clustering algorithm, CDR3 segments were annotated using the International ImMunoGeneTics information system, thereby identifying which V, D, and J genes contributed to each rearrangement [31]. The absolute numbers of EBV-associated and CMV-associated T cells were determined by comparing the patients' TCR*β* data with *TCRβ* sequences reported to be specific for EBV and CMV antigens [32]. The serum concentrations of SPV-T3a-RTA and WT1-RTA, as well as the presence of ADAs against either of these immunotoxins, were measured at Celonic AG (Basel, Switzerland) using validated bioluminescence

assays. Pharmacokinetics analyses were performed as described previously (S6 and S7) [17].

Statistical Analysis. Patient characteristics were analyzed using descriptive statistics. The estimated aGVHD response rates along with the 95% Clopper-Pearson exact confidence interval (Cl) are presented. Toxicity was analyzed by tabulating the incidence of AEs and/or infections with a Common Terminology Criteria for AEs grade ≥2. Kaplan-Meier curves were used to analyze OS. The chi-square test was used to compare the ORR and the rates of CR and PR on day 28 after initiation of CD3/CD7-IT therapy, with the corresponding results obtained from institutional historical controls who received either inolimomab-etanercept (n = 21) or infliximab (n = 21) [21]. The 6-month OS rate was compared using the log-rank test.

Within-patient differences in immune-reconstitution were analyzed in the pretreatment, 1-month, 3-month, and 6-month samples using the Wilcoxon matched-pairs signed-rank test. A 2-sided *P* value <.05 was considered statistically significant. Expanded and enriched T cell clones were identified using differential abundance analysis as described by DeWitt et al. [33]. A given clone was determined to be significantly expanded or contracted in 2 samples based on its proportion in each repertoire or time point and was analyzed using the Fisher exact test with Benjamini-Hochberg correction at the 5% level.

Patient and GVHD characteristics

Twenty patients were enrolled in the study between June 2014 and September 2016. Patient, donor, and GVHD characteristics are presented in Table 1. At the time of enrollment, 3 patients (15%) had grade II aGVHD and 17 had grade III or IV aGVHD (85%). Sixteen patients (80%) had involvement of 2 organs, with the GI tract and liver involved in 18 (90%) and 5 (25%) cases, respectively. Baseline albumin levels were low, particularly in the patients with GI GVHD (median, 2.3 g/dL; range, 1.6 to 3.4 g/dL; normal range, 3.5 to 5.0 g/dL). A validated algorithm using serum concentrations of ST2 and Reg3α demonstrated a significant risk for all patients with a mean pˆ>valueof.345; the majority of patients (11 of 20) were classified as high risk for treatment failure and non-relapse mortality [29]. Treatment with CD3/CD7-IT was initiated after a median interval of 8 days (range, 5 to 16 days) after the initial corticosteroid treatment.

Table 1. Patient characteristics and HSCT and GVHD features.

Patient Characteristics and HSCT and GVHD Features

Characteristic	Value
Number of patients	20
Age, yr, median (range)	53 (18-74)
Sex, male/female, n (%)	9 (45)/11 (55)
Diagnosis, n (%)	
Myeloid malignancy	15 (75)
Lymphoid malignancy	5 (25)
Donor type, n (%)	
Matched unrelated donor	13 (65)
Matched related donor	5 (25)
Mismatched unrelated donor	1 (5)
Haploidentical related	1 (5)
Stem cell source	
Peripheral blood stem cells	19 (95)
Bone marrow	1 (5)
Disease Risk Index, n (%)	
Low	0
Intermediate	5 (25)
High	15 (75)
Conditioning regimen, n (%)*	
MAC	6 (30)
RIC	5 (25)
NMA	9 (45)
GVHD prophylaxis, n (%)	
CyA	5 (25)
CyA/MTX	1 (5)
CyA/MMF (post-CyA)	13 (1) (65)
aGVHD, n (%)	
Post-HSCT	19 (95)
Post-donor lymphocyte infusion	1 (5)
aGVHD grade at enrollment, n (%)	
II	3 (15)
III	11 (55)
IV	6 (30)
Organ involvement, n (%)	
Skin	15 (75)
Liver	5 (25)
Intestinal	18 (90)
2 organs involved	16 (80)
Biomarker score at start of CD3/CD7-IT therapy, high risk ($P > .291$), n (%)	11 (55)
Time to aGVHD, d, median (range)	40 (10-308)
Time to treatment with CD3/CD7-IT, d, median (range)†	8 (5-16)

MAC, myeloablative conditioning; NMA, nonmyeloablative conditioning; RIC, reduced-intensity conditioning; CyA, cyclosporin A; MTX, methotrexate; MMF, mycophenolate mofetil.

* NMA conditioning consisted of fludarabine (Flu)-total body irradiation (TBI), RIC regimens were Flu-busulfan (Bu)- and Flu-melphalan (Mel)-based, and MAC regimens were Cy-TBI-, Flu-Mel-TBI-, or Flu-AraC-Amsa/Cy-TBI-based. † Relative to the initial corticosteroid treatment.

GVHD Response and Patient Outcomes

The median duration of follow-up after therapy with CD3/CD7-IT was 292 days (range, 3 to 889 days). Two patients died due to progressive SR-aGVHD before completing the treatment schedule. The remaining 18 patients (90%) received all 4 scheduled doses at 48-hour intervals. On day 28, ORR was 60% (12 of 20 patients), with a 95% Cl of 36% to 81%; 10 patients (50%; 95% Cl, 27% to 73%) achieved CR (Figure 1). In the 12 responding patients, corticosteroids could be tapered according to protocol (S8; Figure 7). ORR was 55% (6 of 11) in patients with a high-risk biomarker profile. At the 6-month time point, 12 patients had survived, corresponding to an OS of 60% (95% Cl, 36% to 78%) (Figure 1); survival was 64% (7 of 11) in patients with a high-risk biomarker profile (S9; Figure 8). Ten of the 12 surviving patients had achieved a PR or CR. Causes of death for the 8 patients who died during the trial were refractory aGVHD in 4 patients, refractory GVHD with infection in 3 patients, and pseudomembranous colitis in 1 patient.

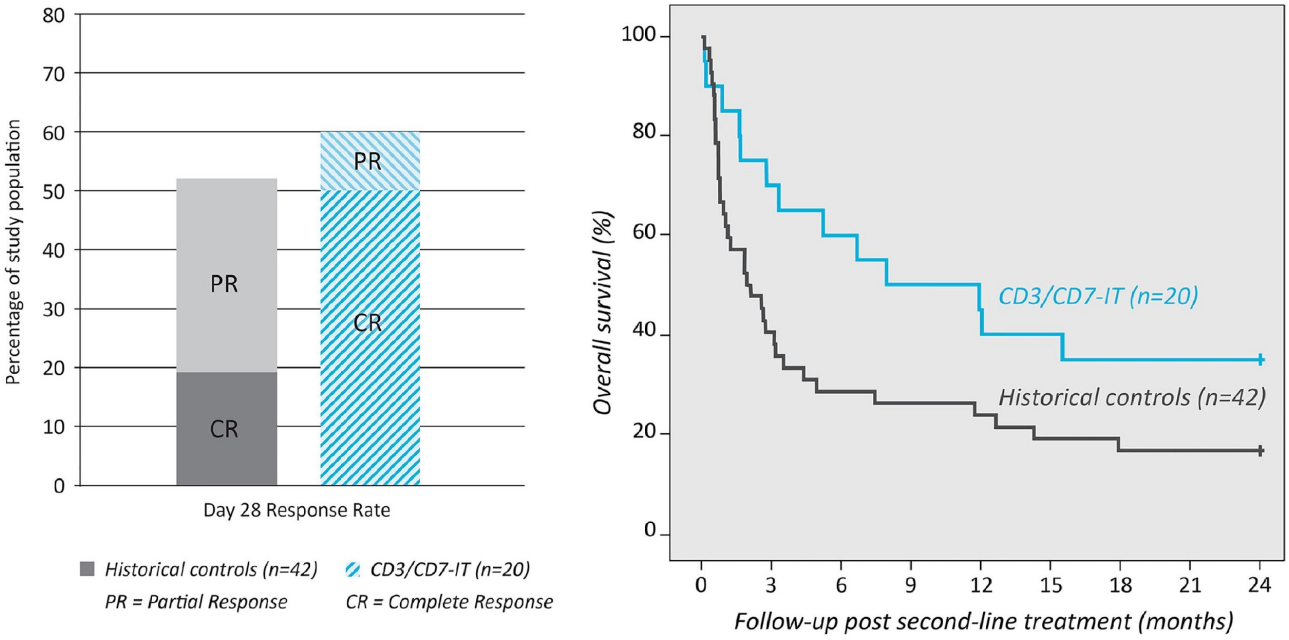


Figure 1. Overview of the response rate at day 28 (top) and OS after treatment with CD3/CD7-IT (bottom) compared with historical controls.

The difference between patients who received CD3/CD7-IT and the historical controls was statistically significant, with improvements in both the CR rate (P = .012) and 6-month OS (P = .021). All survivors reached the 24 month milestone.

The outcomes achieved with CD3/CD7-IT were favorable compared with the outcomes reported for the cohort of 42 patients included immediately adjacent to the start of the trial. Specifically, the CR rate was 50% versus 19% ($P = .012$), and the 6-month OS was 60% versus 29% ($P = .021$). To compensate for differences in aGVHD severity at the start of treatment, the foregoing analysis was repeated after adjustment for overall aGVHD grading. After adjustment for aGVHD grade [20], the CR and OS rates remained significant ($P = .032$ and .034, respectively) (S10). At the 2-year follow-up, OS was still better in the study cohort compared with the historical controls (35% versus 16.7%; $P = .047$ and .09, respectively). Three of the 12 patients (25%) who survived to the 6-month time point developed cGVHD, which was mild in 2 patients and severe in 1 patient. Relapse occurred in 3 patients who underwent transplantation for acute myelogenous leukemia with adverse risk features at a median of 4 months after CD3/CD7-IT therapy.

Safety

The Data and Safety Monitoring Board reviewed the pre-planned interim analysis of the first 8 patients, based on which they concluded that no major safety concerns had arisen and that the observed risk-benefit balance warranted continuation of the study. In general, CD3/CD7-IT was well tolerated and found to be safe, with no suspected unexpected serious adverse reactions or serious AEs related to the study drug reported. Although no clinically significant infusion-related reactions were recorded, 2 patients who had not received pre-treatment experienced chills that resolved quickly after clemastine treatment (grade 2 AE). Most of the patients had elevated levels of markers of macrophage activation/recruitment (MCP-1 and MIP-lβ), and this increase was most prominent after the first infusion; however, only the 2 aforementioned patients who experienced chills also had an increase in IL-6 levels [34,35]. The remaining patients had no increase in IL-6, IL-8, IL-10, or IFN-γ concentrations, nor did they develop clinical signs corresponding to CRS (S5).

Several of the 20 patients developed a limited number of possible treatment-related AEs, including hypoalbuminemia, microangiopathy, and/or thrombocytopenia (Table 2). Hypoalbuminemia was present in all 20 patients at baseline (grade 2 or 3 in 80% of the patients) and may have worsened in 8 patients due to treatment with CD3/CD7-IT. These 8 patients developed mild peripheral edema, which could be easily managed with diuretics in all but 1 patient. One patient required treatment with an albumin infusion and diuretics

for generalized edema and marked weight gain; thus, this patient was classified as having grade 2 capillary leak syndrome. Fifteen patients (75%) had a preexisting low platelet count (grade 3 or 4 in 25% of cases), and thrombocytopenia either occurred or worsened in 14 patients (70%). Although various other causes might have contributed to the development of thrombocytopenia, the time course was at least suggestive of a possible relationship with CD3/CD7-IT in 9 patients. Nevertheless, the thrombocytopenia was transient, did not result in a bleeding event, and rarely required platelet transfusion. Early EBV and CMV infections (within 3 months) were observed in 3 patients each (with 2 patients positive for both EBV and CMV); however, no EBV or CMV disease occurred. Although only 40% of patients received mold-active antifungal prophylaxis, IFD was not observed in any of the patients. Nevertheless, as expected in this setting, the number of infections and AEs was relatively high. Two patients developed a *Clostridium difficile* infection, and 1 of them died due to pseudomembranous colitis. Moreover, although 5 patients developed bacteremia (with infection by enterococci in 2 patients, staphylococci in 2 patients, and *Klebsiella oxytoca* in 1 patient), the incidence rate (25%) was not higher than that reported in historical controls [21].

After treatment with CD3/CD7-IT, ADAs against SPVT3a-RTA and/or WT1-RTA were detected in 10 out of 20 patients (50%). In 4 of these 10 patients, the titers were ≥20,000 at any given point (S7); nonetheless, no cases of serum sickness were reported. The emergence of ADAs was considered of little clinical relevance, because ADAs typically form after 9 to 10 days, whereas CD3/CD7-IT is currently offered as a 1-week treatment option only, and its serum half-life is only 9 hours.

Grade 2*	Grade 3	Grade 4
Anemia (1)	Thrombocytopenia (3)	Thrombocytopenia (5)
Abdominal pain (1)	Neutropenia (1)	
Thrombocytopenia (1)	Elevated bilirubin (2)	
Neutropenia (1)	Myopathy (1)	
Microangiopathy (1)	Microangiopathy (1)	
Chills (2)	Hypoalbuminemia (1)	
Capillary leak syndrome (1)		
Hypoalbuminemia (1)		

Summary of AEs Potentially Related to Treatment.

The numbers in parentheses refer to the number of patients who experienced the indicated AE. Grading of each AE is based on version 4.0 of the Common Terminology Criteria for AEs, with the exception of capillary leak syndrome, which was graded using the system described by Messmann et al. 24.

Pharmacokinetics

Pharmacokinetics analysis revealed a mean serum half-life and mean maximum concentration of CD3/CD7-IT of 8.59 ± 3.04 hours and 1231 ± 671 μg/L, respectively (S6), which is consistent with previously published data [17].

Immune Reconstitution and Antiviral Immunity

Consistent with its intended effect, treatment with CD3/CD7-IT led to a profound depletion of T cells and NK cells, with rapid recovery starting as early as the second week after treatment (Figure 2A and andB).B). Importantly, no significant effect on the absolute B cell count was observed (Figure 2C). No apparent patterns were seen in terms of treatment-induced changes in the relative proportions of naïve, memory, effector, and effector memory T cells before and after treatment start, as well as no decrease in or reversal of the CD4:CD8 ratio. In addition, the absolute count of regulatory T cells (Tregs) and the percentage of Tregs in the $CD4^+$ cell population showed normal variation, with no obvious upward or downward trends observed at 28 days after initiation of treatment or during the remainder of the follow-up period.

HTS was performed on the CDR3 region of the *TCRβ* genes in PBMCs before and, when possible, at 1,3, and 6 months after treatment with CD3/CD7-IT. HTS can determine the total T cell count, the diversity of the T cell repertoire, and the sequences of the TCR CDR3 regions in all T cells in a given sample. The T cell diversity in a sample is characterized by the number of unique T cell clones present in the sample, which is reflected by the number of unique CDR3 sequences identified using HTS. Before the start of treatment with CD3/CD7-IT, the patients had low T cell diversity that further decreased after the first month, most likely due to a reduction in the absolute number of T cells. T cell diversity rebounded steadily by 6 months post-treatment, with a diverse T cell repertoire that included several new polyclonal T cell populations (Figure 2D–H).

We next examined whether CD3/CD7-IT treatment affects antivirus T cell clones. To do so, we analyzed the development of EBV- and/or CMV-specific T cell clones in patients following treatment with CD3/CD7-IT. Antiviral T cell clones were identified by screening for a validated list of 164 and 854 TCRβ sequences encoding receptors that recognize CMV- and EBV-specific antigens, respectively (S11) [32].

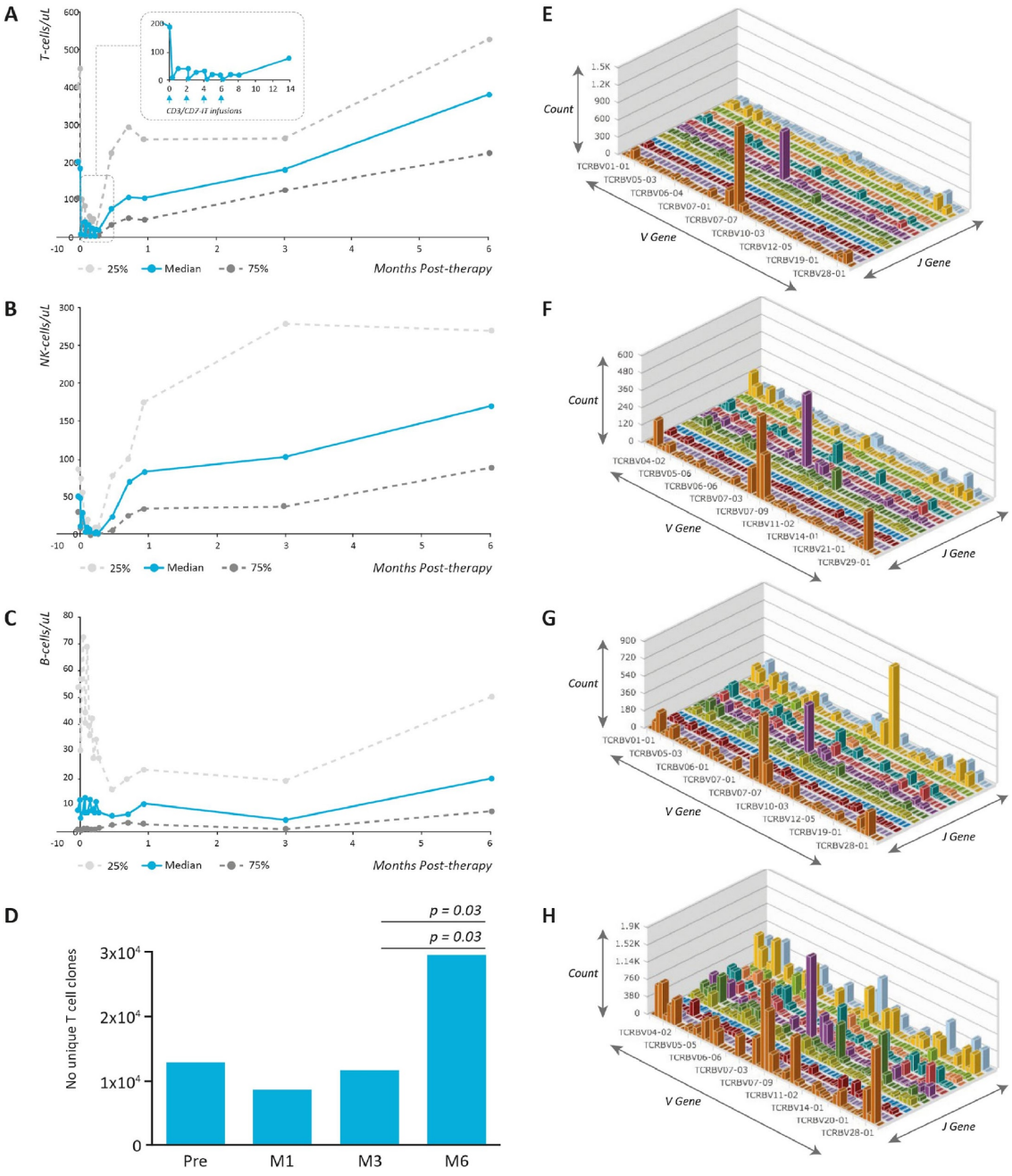


Figure 2. CD3/CD7-IT induces rapid immune reconstitution with a diverse T cell repertoire. **(A-C)** Time course of the median T cell count (A), median NK cell count **(B)**, and median B cell count **(C)** for all patients. In each plot, the blue line represents the median value, and the lower and upper gray dotted lines represent the 25th and 75th percentiles, respectively. **(D)** Summary of the absolute number of unique T cell clones before administration of CD3/CD7-IT (Pre) and at 1, 3, and 6 months after treatment. The number of unique T cell clones was measured using the total number of unique CDR3 sequences. The *P* values are based on the Wilcoxon matched-pairs signed-rank test. The significant increase in unique T cell clones at 6 months after CD3/CD7-IT therapy reflects an increase in the diversity of expanded T cells. **(E-H)** Representative histograms showing the T cell repertoires in a single patient before CD3/CD7-IT therapy **(E)** and at 1 month **(F)**, 3 months **(G)**, and 6months **(H)** after therapy.

Serology was positive for EBV in 95% of patients and 85% of donors and for CMV in 40% of patients and 35% of donors. Infections occurred only in those patients with positive serology. Four patients experienced EBV and/or CMV infection after treatment with CD3/CD7-IT, including 2 patients with either EBV or CMV infection and 2 patients with both EBV and CMV infections) (Figure 3A and C). All these patients demonstrated increased numbers of postinfection EBV- and CMV-associated clones, suggesting that the antiviral T cell response was not negatively affected by treatment with CD3/CD7-IT.

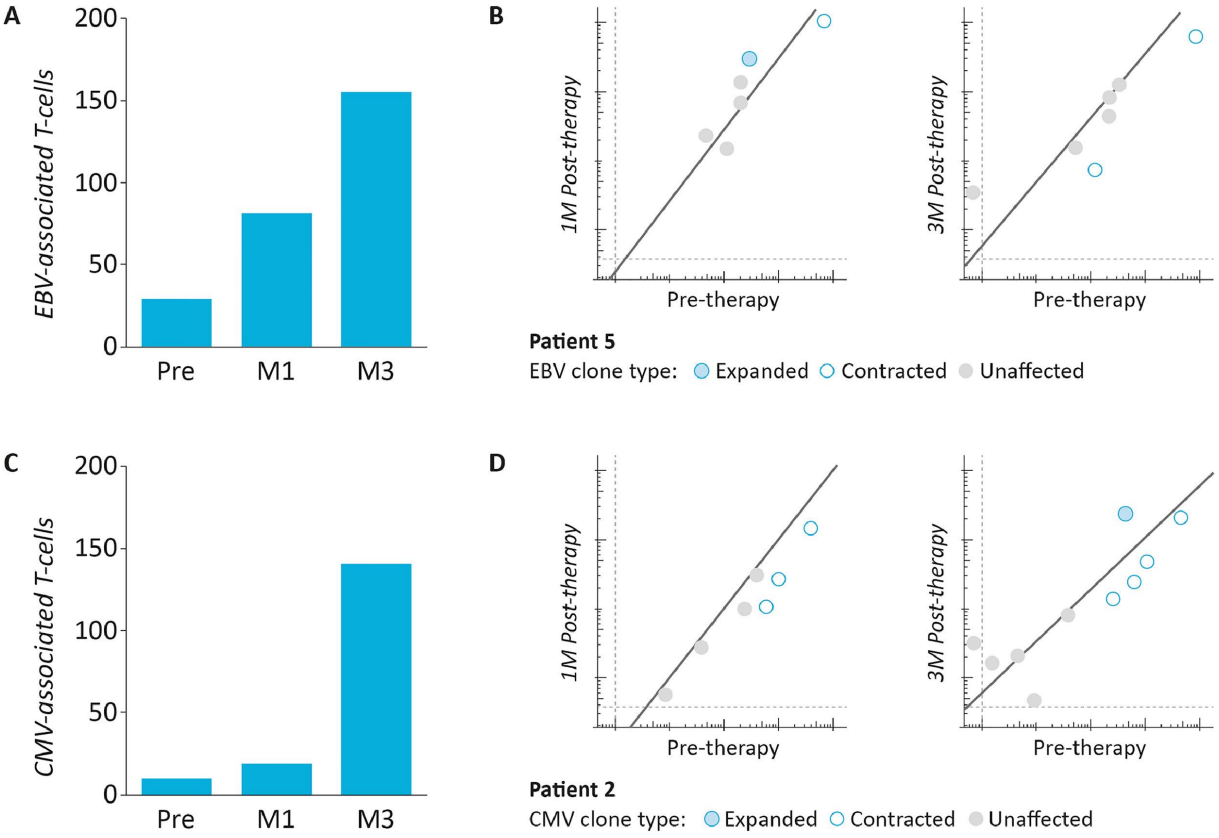


Figure 3. CD3/CD7-IT does not affect the fraction of anti-virus EBV- and CMV-associated T cell clones. (A and C) Summary of the absolute numbers of anti-EBV **(A)** and anti-CMV **(C)** T cells in patients who tested positive for viral infection after treatment. In each patient group, the number of virus-associated T cells was measured before and after treatment. **(B and D)** Plots showing the differential abundance analysis of unique anti-EBV **(B)** and anti-CMV **(D)** T cell clones. Shown are representative graphs of 2 patients who tested positive for the respective viral infection before treatment. Screening samples were compared with samples obtained at 1 month and 3 months after therapy with CD3/CD7-IT. This pairwise comparison confirms that the majority of the respective CMV- and EBV-associated clones neither expanded nor contracted as a result of therapy. In each plot, the solid gray diagonal line indicates equal numbers of clones in both samples (no change). Clones positioned between the dotted gray lines and the respective *x*- or *y*-axis were not present in other samples (eg, present before therapy but not after therapy).

Finally, we performed a differential analysis of unique antiviral T cell clones by performing pairwise comparisons between samples taken directly before treatment with CD3/CD7-IT and samples obtained at 1 month and 3 months after treatment in patients who tested positive for a viral infection before the start of treatment. This analysis revealed that at the start of treatment, the EBV- and CMV-associated T cell clones were distributed equally throughout the entire T cell population in terms of clonal abundance; moreover, these clones did not expand or contract as a result of therapy with CD3/CD7-IT (Figure 3B–D). Similar results were obtained when we analyzed samples from patients who had antiviral T cells at the start of treatment but did not develop a viral infection; our data (not shown) suggest that these patients may have acquired these antivirus clones from a seropositive donor. Taken together, these results indicate that CD3/CD7-IT does not negatively affect the proportions of anti-EBV or anti-CMV T cell clones, suggesting that this treatment does not appear to put these patients at greater risk of acquiring an infection with these opportunistic viruses.

DISCUSSION

Here we report the results of a multicenter phase I/II trial to study the in vivo safety and efficacy of using CD3/CD7-IT therapy in patients with SR-aGVHD. Our results show that CD3/CD7-IT has promising efficacy, with an ORR of 60% on day 28; specifically, 50% of our patients achieved a CR, and the 6-month OS rate was 60%. These results are superior to the outcomes reported for our institutional historical controls (Figure 1) and are notable given the patients' high-risk profile: 85% with severe SR-aGVHD, 90% with GI involvement and 55% with a high-risk biomarker profile. A pooled analysis of second-line therapies showed that only 32% of patients achieve CR, with a corresponding 6-month survival rate of 49% [1]. In addition, our phase II results closely match those reported for other drugs currently under investigation for SR-aGVHD, including brentuximab vedotin and ruxolitinib, which have been shown to achieve CR in approximately 30% of patients [36].

This study has several limitations that should be acknowledged. First, the sample size was relatively small, and we did not include a randomized comparator arm. In addition, the study population was heterogeneous with respect to age, conditioning regimen, donor type, and GVHD prophylaxis regimens used. Nonetheless, the study population is representative

of patients with SR-aGVHD treated at our institutions and consisted primarily of patients with underlying high-risk features.

CD3/CD7-IT therapy appears to be safe. Despite the presence of the anti-CD3 mAb SPV-T3a, CD3/CD7-IT induced a mild infusion reaction in 2 patients, neither of whom had received preinfusion clemastine. In addition, we observed no toxicity related to CRS or rhabdomyolysis as has been reported with other RTA-based immunotoxins [37,38]. We did consider hypoalbuminemia, microangiopathy, and thrombocytopenia as possibly related to CD3/CD7-IT; however, these AEs primarily involved worsening of preexisting conditions, and we considered these events as likely related to the underlying SR-GVHD and/or the concomitant use of a calcineurin inhibitor. Nonetheless, given the potential toxic effects of immunotoxins, it remains possible that CD3/CD7-IT may have contributed to these events, and this possibility merits consideration in future studies.

As expected in the clinical setting of this study, infections were relatively common; however, the incidence of infection did not differ substantially from that in previous reports or in our institutional controls [21,39,40]. The multifaceted immune defects due to the presence—and treatment—of GVHD itself, the disruption in the mucosal barrier due to GI GVHD, and/or dysbiosis can explain the majority of these infections, particularly the C *difficile* infections and enterococcal bacteremia [41]. Although only one-half of our patients received mold-active antifungal prophylaxis, we observed no cases of IFD. More importantly, despite the profound depletion of T cells and NK cells, the incidence of EBV/CMV infections was relatively low (15%) [21,39], and no cases of post-transplantation lymphoproliferative disorder or CMV disease occurred in our patients. This may be explained by the fact that virus-specific T cells were relatively spared by the treatment, and that immune reconstitution occurred within 6 months after the start of treatment. In the second week of treatment, the T cell and NK cell counts began to rise, particularly in patients who achieved remission of SR-aGVHD; at 3 months, these cell counts were similar to those normally seen following HSCT [42]. This increase in cell numbers was also accompanied by a simultaneous and significant increase in the diversity of T cell clones. Thus, therapy with CD3/CD7-IT allows the patient's immune system to recover after remission is achieved, and the immune reconstitution after therapy seems favorable compared with other treatment modalities that rely on in vivo T cell depletion, such as antithymocyte globulin and alemtuzumab [43,44].

Other immunotoxin-based treatments, such as H65-RTA (anti-CD5, ricin A chain) and denileukin diftitox (CD25, diphtheria toxin), have been clinically evaluated for treating aGVHD [13,45]. CD3/CD7-IT may offer advantages compared with these previous therapies. First, the combination targets multiple antigens on the same target cell, a strategy that tends to be more efficacious than the use of single immunotoxins [46–53]. In addition, CD3/CD7-IT has a clear preference for recently activated T cells, as well as the NK cells that may play a role in the efferent phase of aGVHD [17]. Finally, CD3/CD7-IT has a dual mechanism of action, in that the anti-CD3 mAb SPV-T3a provides added immunosuppression by binding to the CD3/TCR complex via a mechanism independent of RTA-induced cell killing (SI; Figure 1) [17].

In conclusion, the results of our phase I/II study involving patients with high-risk SR-aGVHD show that CD3/CD7-IT provides a high rate of clinical remission and rapid immune reconstitution following treatment. Based on these results, a phase III study is currently being designed to examine the potential value of including CD3/CD7-IT in the treatment of SR-aGVHD.

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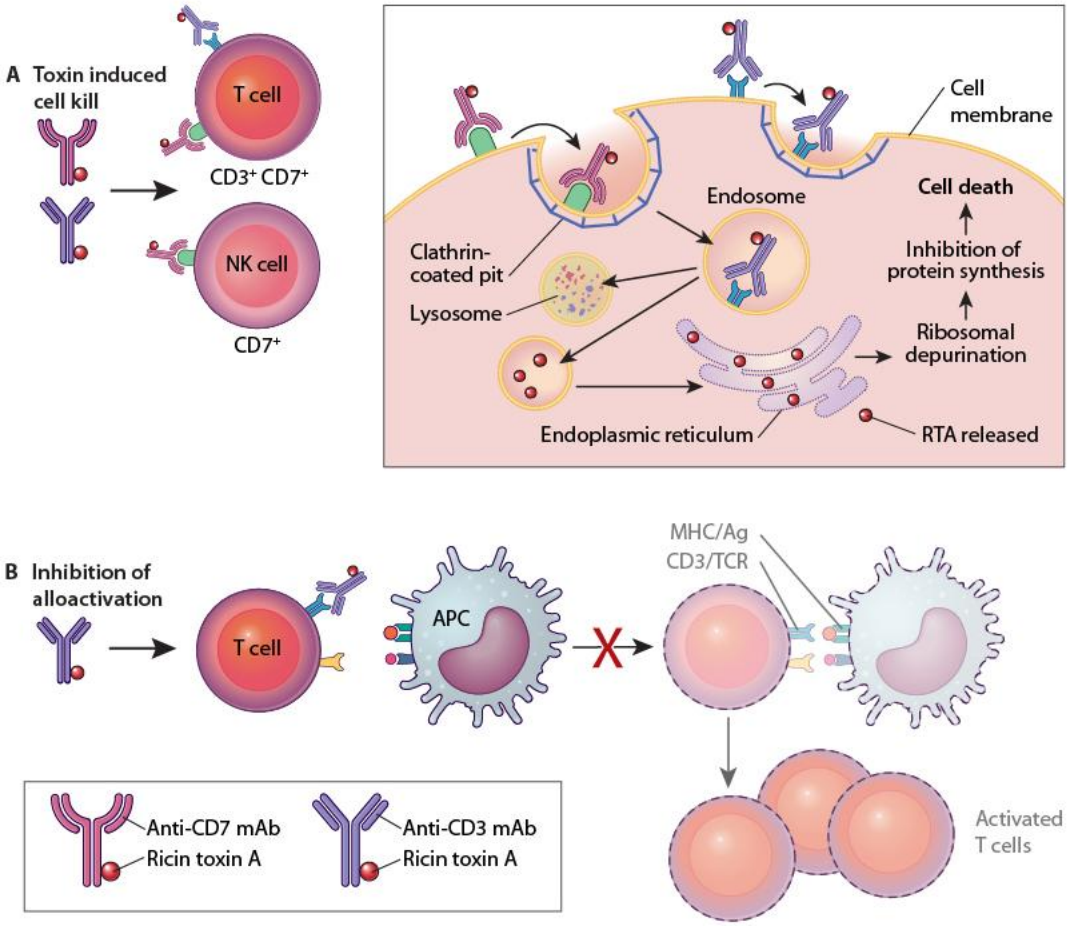
SUPPLEMENTAL MATERIALS

Supplemental Figure 1. Mechanism of action CD3/CD7-IT. CD3/CD7-IT consists of two murine monoclonal antibodies (mAbs). One mAb is directed against CD3, and the other is directed against CD7, and both mAbs are conjugated to a recombinant ricin toxin A (RTA) chain. These immunotoxins induce apoptosis in target cells by inhibiting protein synthesis, resulting in the synergistic depletion of both T cells and NK-cells **(A)**. In addition, the anti-CD3 antibody blocks the T-cell receptor (TCR), preventing T-cell activation and the release of inflammatory cytokines **(B).**



CHAPTER 6

Idelalisib Given Front-line for Treatment of Chronic Lymphocytic Leukemia Causes Frequent Immune-Mediated Hepatotoxicity

Benjamin L. Lampson,[1] Siddha N. Kasar,[1] Tiago R. Matos,[1] Haesook Kim,[2] Elizabeth A. Morgan,[3] Laura Rassenti,[4] Matthew Davids,[1] David C. Fisher,[1] Arnold Freedman,[1] Caron A. Jacobson,[1] Philippe Armand,[1] Jeremy Abramson,[5] Thomas Kipps,[4] Joshua Fein,[1] Stacey Fernandes,[1] John Hanna,[1] Jerome Ritz,[1] Jennifer R. Brown[1]

Thesis' author contribution: T.R.M. performed the research and analyzed data, performed statistical analyses and wrote the manuscript.

Affiliations:

[1] Division of Hematologic Malignancies and Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA

[2] Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA

[3] Department of Pathology, Brigham and Women's Hospital, Boston, MA

[4] Division of Hematology/Oncology, Department of Medicine, University of California, San Diego and Moores UCSD Cancer Center, La Jolla, CA

[5] Center for Lymphoma, Massachusetts General Hospital Cancer Center, Boston, MA

ABSTRACT

Idelalisib is a small molecule inhibitor of PI3Kδ with demonstrated efficacy for the treatment of relapsed/refractory chronic lymphocytic leukemia (CLL). To evaluate idelalisib as front-line therapy, we enrolled 24 subjects in a phase II study consisting of two months of idelalisib monotherapy followed by six months of combination therapy with idelalisib and the anti-CD20 antibody ofatumumab. After a median follow-up of 14.7 months, hepatotoxicity was found to be a frequent and often severe adverse event. Nineteen subjects (79%) experienced either grade ≥1 ALT or AST elevation during the study, with 13 subjects (54%) experiencing grade ≥ 3 transaminitis. The median time to onset of transaminitis was 28 days, occurring before the introduction of ofatumumab. Younger age and mutated immunoglobulin heavy chain (IGHV) status were significant risk factors for the development of hepatotoxicity. Multiple lines of evidence suggest that this hepatotoxicity is immune mediated. A lymphocytic infiltrate was seen on liver biopsies taken from two subjects with transaminitis, and levels of the pro-inflammatory cytokines CCL-3 and CCL-4 were higher in subjects experiencing hepatotoxicity. All cases of transaminitis resolved either by holding the drug, initiating immunosuppressants, or both, and rates of recurrent toxicity were lower in patients taking steroids when idelalisib was reinitiated. A decrease in peripheral blood regulatory T cells was seen in patients experiencing toxicity on therapy, which is consistent with an immune-mediated mechanism. These results suggest that caution should be taken as drugs within this class are developed for CLL, particularly in younger patients who have not received prior disease-specific therapy.

INTRODUCTION

A major recent advancement in therapy for chronic lymphocytic leukemia (CLL) has been the development of small-molecule inhibitors of the p110 δ isoform (p110δ) of phosphatidyl-inositol-3-kinase (PI3K). These inhibitors block signaling from a variety of prosurvival B-cell surface receptors that converge on the PI3K pathway. Idelalisib is a highly selective oral inhibitor of p110δ that, in combination with anti-CD20 monoclonal antibodies, has shown efficacy for the treatment of relapsed/refractory CLL. Even in heavily pretreated patient populations, responses to these drug combinations are both frequent and durable, with overall response rates of 70% to 80% and median progression free survival in the range of 18 to 19 months.[1-3] This success in relapsed, refractory disease has prompted evaluation of idelalisib in the upfront setting, with particular interest for older patients who may have multiple other medical comorbidities. Given this, tolerability is crucial. Major toxicities of p110δ inhibition with idelalisib are enterocolitis, transaminitis, and pneumonitis. In the relapsed/refractory setting, these toxicities have been manageable, with grade ≥3 diarrhea/colitis seen in ∼14% of patients, grade ≥3 transaminitis in 14%, and any-grade pneumonitis in 3%.[4] However, the rates of such toxicities when idelalisib is used as upfront therapy for previously untreated disease have not been well established. A phase 2 study has examined front-line idelalisib in patients age 65 or older, with rates of grade ≥3 diarrhea/colitis up to 42% and grade ≥3 transaminitis up to 23%.[5,6] These data suggest that toxicity rates may be higher in the front-line setting.

A better understanding of the pathogenesis of these adverse events may facilitate treatment or avoidance of them. An autoimmune mechanism for idelalisib-associated enterocolitis has been hypothesized. Mice with genetic inactivation of p110δ develop an autoimmune colitis.[7] Histopathologic data from patients with idelalisib-associated colitis show an intraepithelial lymphocytosis.[8,9] Some patients with late-onset idelalisib-associated diarrhea that does not respond to antidiarrheal or empiric antimicrobial therapy are anecdotally responsive to steroids.[10] It is unknown if idelalisib-related transaminitis occurs via a similar mechanism.

Given the known efficacy of idelalisib in relapsed/refractory CLL, but also given concern about its toxicities, we report here an initial safety analysis of a phase 2 clinical trial of front-line idelalisib used in combination with the anti-CD20 monoclonal antibody

ofatumumab, with the finding that an immune-mediated transaminitis in this setting is frequent and often severe.

METHODS

The study was initiated on June 16, 2014 (Study 13-309; ClinicalTrials.gov identifier: NCT02135133). Of the 24 patients enrolled in the trial, 23 had CLL and 1 patient was later shown to have lymphoplasmacytic lymphoma and removed from study. The target enrollment was 50 subjects between the Dana-Farber Cancer Institute and Massachusetts General Hospital Cancer Center, but all up-front combination studies of idelalisib were discontinued by the sponsor in March 2016. This analysis is based on data up to November 2015. Institutional review boards at each study site approved the protocols. All authors had full access to study data and were involved in data interpretation, manuscript preparation, revision, and final approval.

Eligibility criteria. Eligible patients were older than 18 years and had previously untreated CLL with an indication for treatment according to the 2008 International Workshop on Chronic Lymphocytic Leukemia guidelines.11 Immunoglobulin heavy chain variable (IGHV) mutated disease was present if the IGHV sequence differed from its germline counterpart by greater than 2%. Subjects were required to have measurable disease (lymphocytosis > 5000 cells / μL or palpable / CT measurable lymphadenopathy >1.5cm) and an Eastern Cooperative Oncology Group performance status of 0-2. Exclusion criteria included the following: prior systemic therapy for CLL, serum creatinine ≥ 2.0 times the upper limit of normal (ULN), total bilirubin ≥ 1.5 ULN, alanine aminotransferase ≥ 2.5 ULN, alkaline phosphatase ≥ 2.5 ULN, positive testing for active hepatitis B or C, active chronic infections requiring treatment, and clinically significant cardiovascular disease. All patients provided written informed consent.

Study treatments. A schematic of the trial design is provided in supplemental Figure 1. For the first fifty-six days, subjects received idelalisib 150mg twice daily, followed by six months of idelalisib plus ofatumumab combination therapy, then followed by idelalisib monotherapy continued indefinitely until progression or toxicity. The initial dose of ofatumumab was 300mg; all subsequent doses were 1000mg. Infection prophylaxis was not initially required, but the protocol was subsequently amended to require prophylaxis

against *Pneumocystis jirovecii* pneumonia (PJP) and HSV/VZV after two cases of PJP occurred.

After identification of hepatotoxicity as a frequent adverse event, the trial protocol was modified in September 2014 to enhance early recognition of transaminitis and standardize treatment. Liver function tests were monitored twice weekly from week 3 to 16. The development of grade 1 transaminitis was treated with prednisone 40mg daily. Idelalisib was held for the development of grade 2 transaminitis or worsening of grade 1 transaminitis while on steroids. Grade ≥3 transaminitis was treated with prednisone 1mg/kg in addition to discontinuation of idelalisib. For patients with grade ≥3 transaminitis without immediate response to steroids, mycophenolate mofetil was considered.

Correlative studies. Blood samples were obtained from enrolled subjects and processed by the CLL Research Consortium Tissue Core at the UC San Diego Moores Cancer Center. Ficoll-Hypaque density-gradient centrifugation was used to obtain mononuclear cells. ZAP-70 status and immunoglobulin heavy chain variable (*IGHV*) mutation status were assessed by the Consortium Tissue Core as per established criteria (see Rassenti et al[12,13] for ZAP-70 and Ghia et al,[14] Widhopf et al,[15] Giudicelli et al,[16] and Lefranc et al[17] for *IGHV*). Sequences with <98% homology to the corresponding germline *IGHV* gene were considered mutated.

Peripheral blood mononuclear cells were isolated from 16 subjects at baseline, 15 subjects at day 28 (±14 days, depending on when toxicity developed), and 5 subjects at day 130 (±21 days, depending on when toxicity developed). At these time points, the patients were experiencing toxicity, but the drug had not yet been held or steroids initiated. Mass cytometric (CyTOF) analysis was performed with a panel of monoclonal antibodies targeting 26 surface-membrane and 9 intracellular markers. The Wilcoxon matched-pairs signed rank test was used to compare percentages of T-cell subsets obtained from CyTOF analysis as well as cytokine concentrations; any samples without a matched baseline time point were not included in significance calculations, and samples with values from day 28 and day 130 were used twice.

Cytokine analysis was performed on serum collected from subjects at the indicated time using the Magnetic Luminex Performance Assay (catalog number FCSTM03-13, R&D Biosystems). Each sample was analyzed in duplicate. Concentrations reported are the average of all values. Mann-Whitney *U* test was used for statistical comparison.

Statistical analysis. All patients who received any amount of study treatment were included in the analysis. The median time on therapy was 7.7 months (range, 0.7-16.1 months) and median follow-up time was 14.7 months (range, 1.2-16.8 months). All reported p values are two sided, and no adjustments have been made for multiple comparisons.

RESULTS

Patient Characteristics

At the time of data cutoff, 24 patients had enrolled. Baseline subject characteristics are provided in Table 1. The 24 subjects enrolled had a median age of 67 years (range, 58 to 85 years) and included 6 women and 18 men. Seventeen subjects (71%) had high-risk Rai stage 3-4 disease, and 29% had bulky lymphadenopathy defined by the presence of at least 1 lymph node ≥5 cm. From the 21 patients with bone marrow biopsy specimens at enrollment, lymphocytes comprised a median of 80% (range, 35% to 95%) of the intertrabecular space. Two patients (8%) had del 11q, and an additional four patients (17%) had either del 17p, aTP53 mutation, or both.

Frequency, Severity, and Timing of Hepatotoxicity

Multiple subjects developed severe hepatotoxicity. In a representative index case, the patient suddenly developed a grade 3 alanine aminotransferase (ALT) and asparate aminotransferase (AST) elevation on day 28 of idelalisib monotherapy (Figure 1A). The drug was stopped. Despite the drug being held, the transaminitis worsened, reaching a maximum AST of 1251 U/L and ALT of 2237 U/L on day 35. On day 34, the subject underwent a liver biopsy, and on day 35, steroids were initiated. The liver function tests normalized after 3 weeks of steroid treatment.

Characteristics	Statistics, *n* (%)
N	24
Age -- yr	
Median	67.4
Range	57.6-84.9
Sex – no	
Male	18 (75%)
Female	6 (25%)
Rai Stage	
0	0 (0%)
I-II	7 (29%)
III-IV	17 (71%)
Lymphocytes in bone marrow at enrollment	*n*=21
Median	80%
Range	35-95%
β_2-microglobulin	
Median	4.9
Range	2.4-13.7
IGHV	
Mutated > 2%	12 (50%)
Unmutated	12 (50%)
***NOTCH1* c.7541-7542delCT**	
Mutated	3 (13%)
Unmutated	17 (71%)
Unknown	4 (17%)
***TP53* mutation or 17p deletion**	
No	20 (83%)
Yes	4 (17%)
11q deletion	
No	22 (92%)
Yes	2 (8%)
13q deletion	
No	8 (33%)
Yes	16 (67%)
Trisomy 12	
No	18 (75%)
Yes	6 (25%)

Table 1. Baseline demographic and clinical characteristics of enrolled patients.

Cumulatively, 19 subjects (79%) experienced at least grade 1 ALT or AST elevation during the study, with 13 subjects (54%) experiencing grade ≥3 transaminitis. The median time to initial development of any grade ≥2 transaminitis was 27 days (range, 14-133 days). This was before the initial administration of ofatumumab on day 56, exonerating ofatumumab as the cause. At week 5, the time of maximum incidence, 11 subjects (46%) had an elevated ALT, with 5 subjects (21%) experiencing grade 3 ALT elevations and 3

subjects (13%) experiencing grade 4 ALT elevations (Figure 1B). The fraction of subjects with ALT abnormalities decreased over time, but this reflected active intervention rather than self-resolution of the process. The pattern of liver injury was hepatocellular, with more severe elevations in the transaminases compared with alkaline phosphatase and bilirubin (supplemental Figure 2). Patients could be divided into 4 groups based on the adverse effects they experienced (Figure 1C). In addition to the 12 subjects who developed early grade ≥2 transaminitis around day 28 of idelalisib therapy, a second group of 4 subjects developed a delayed grade ≥1 hepatotoxicity around day 130. A third group of subjects did not experience hepatotoxicity but discontinued the study due to other adverse events (colitis and rash). A fourth group of subjects tolerated the therapy well, with 4 subjects remaining on therapy for >1 year without experiencing transaminitis, pneumonitis, or colitis.

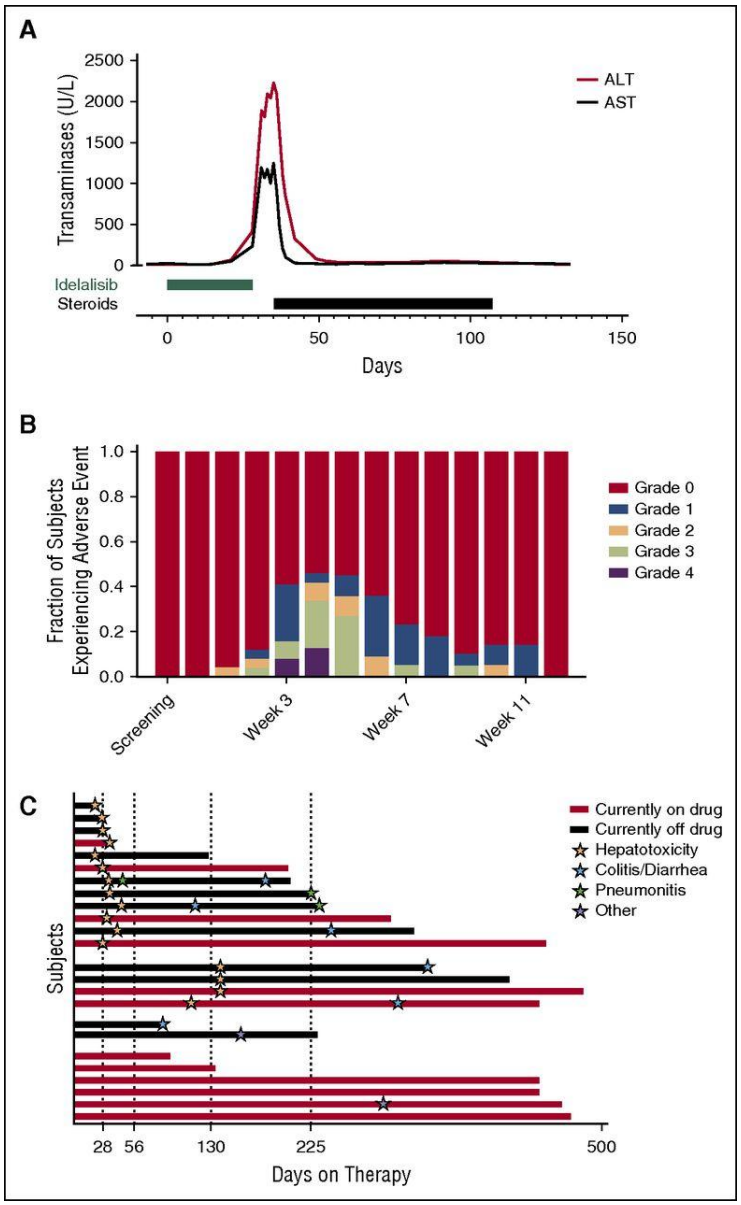


Figure 1: Transaminitis is frequent and severe in subjects receiving idelalisib monotherapy. (A) An index case of idelalisib-related transaminitis. (B) The fraction of subjects experiencing an ALT elevation of the indicated grade at the indicated time. (C) Swim plot of toxicities experienced by all subjects enrolled in the trial.

Frequency of Other Toxicities

The incidence of any grade adverse event, regardless of whether the event was considered related to the study treatment, was 100%. The incidence of any adverse event of grade 3 was 50% and any adverse event of grade 4 was 38% (Table 2). Transaminase elevations were the most frequent toxicity. Diarrhea and colitis were also frequent, with 46% of patients experiencing diarrhea/colitis (17% grade ≥3). The median time to onset of grade ≥2 diarrhea/colitis was 228 days (range, 100-392 days). Pneumonitis was seen in 13% of patients (8% grade ≥3), with a median time to onset of 117 days (range, 40-223 days). Hematologic toxicities included anemia (8%, with 4% grade ≥3), neutropenia (46%, with 29% grade ≥3), and thrombocytopenia (8%, with 0% grade ≥3). Opportunistic infections included 2 cases of *P jirovecii* pneumonia, 1 case of *Aspergillus* pneumonia, 1 brain abscess due to an unknown organism (thought likely to be *P jirovecii*) that resolved on trimethoprim/sulfamethoxazole, 1 case of cytomegalovirus colitis, and 1 case of herpes simplex virus esophagitis. There were no deaths on study or during the follow-up period.

Clinical Risk Factors for Early Hepatotoxicity

A younger age was a significant risk factor for the development of early hepatotoxicity (Figure 2A). The median age of subjects who did not have any hepatotoxicity was 72 years, whereas the median age of subjects who did have early hepatotoxicity was 61 years ($P = .02$). All subjects under age 65 required systemic steroids for immune-mediated toxicities at some point during the trial. A total of 75% of patients with mutated *IGHV* experienced early hepatotoxicity, whereas only 25% of patients with unmutated *IGHV* experienced early hepatotoxicity ($P = .039$). The combination of age and *IGHV* status was highly predictive of early hepatotoxicity, with an area under the receiver-operating characteristic curve of 0.9762 (Figure 2B). Measurements of disease burden, including β_2-microglobulin, LDH, absolute lymphocyte count, Rai stage at baseline, intertrabecular bone marrow involvement at screening, and computed tomography–assessed lymph node size burden were not predictive of early hepatotoxicity (supplemental Figure 3).

Event	Any Grade	Grade 3 or 4
ALT Increase	19 (79%)	13 (54%)
AST Increase	19 (79%)	10 (42%)
Colitis / Diarrhea / Enteritis	11 (46%)	4 (17%)
Blood Bilirubin Increase	10 (42%)	1 (4%)
Rash	8 (33%)	3 (13%)
Alkaline Phosphatase Increase	8 (33%)	0 (0%)
Nausea / Vomiting	8 (33%)	0 (0%)
Abdominal Pain	7 (29%)	0 (0%)
Constipation	7 (29%)	0 (0%)
Myalgia	5 (21%)	1 (4%)
Arthralgia	5 (21%)	0 (0%)
Cough	5 (21%)	0 (0%)
Edema	5 (21%)	0 (0%)
Chills	4 (17%)	0 (0%)
Dysgeusia	4 (17%)	0 (0%)
Night Sweats	4 (17%)	0 (0%)
Insomnia	4 (17%)	0 (0%)
Weight Loss	4 (17%)	0 (0%)
Lung Infection	3 (13%)	3 (13%)
Hyperglycemia	3 (13%)	2 (8%)
Pneumonitis	3 (13%)	2 (8%)
Hypotension	3 (13%)	1 (4%)
Infusion-related Reaction	3 (13%)	1 (4%)
Anorexia	3 (13%)	0 (0%)
Dizziness	3 (13%)	0 (0%)
Dry Mouth	3 (13%)	0 (0%)
Dry Skin	3 (13%)	0 (0%)
Dyspnea	3 (13%)	0 (0%)
GERD	3 (13%)	0 (0%)
Hyponatremia	2 (8%)	2 (8%)
Anemia	2 (8%)	1 (4%)
Hypertension	2 (8%)	1 (4%)
Oral Mucositis	2 (8%)	1 (4%)
Alopecia	2 (8%)	0 (0%)
Back pain	2 (8%)	0 (0%)
Bronchial Infection	2 (8%)	0 (0%)
Depression	2 (8%)	0 (0%)
Nasal Congestion	2 (8%)	0 (0%)
Platelet Count Decreased	2 (8%)	0 (0%)
Polyuria	2 (8%)	0 (0%)
White Blood Cells Decreased	2 (8%)	0 (0%)

Table 2. Adverse events experienced by at least two subjects during the trial.

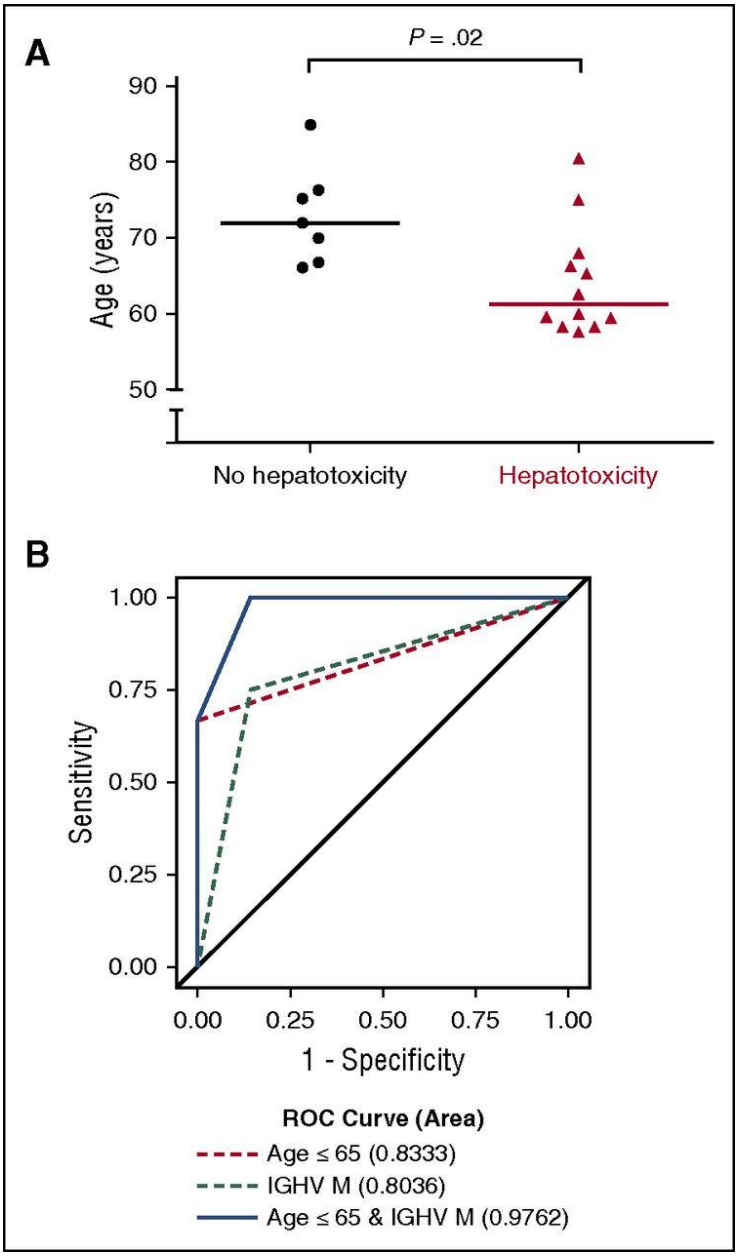


Figure 2: Clinical factors are associated with the development of early hepatotoxicity. (A) Significant difference in age at enrollment between subjects who experienced no toxicity on trial and those who experienced early hepatotoxicity (p=0.02). **(B)** Receiver-operated curves (ROC) for mutated IGHV status, age ≤ 65 years, or a combination of the two for predicting the occurrence of early hepatotoxicity.

Histologic Findings on Liver Biopsy of Affected Subjects

No viral etiology for the transaminitis could be identified, and no other drug culprits were suspected. All subjects tested negative for hepatitis B surface antigen and hepatitis C antibody at enrollment. Hepatitis B surface antibody alone was positive in 5 subjects, consistent with immunization. Hepatitis B surface antibody and core antibody were positive at enrollment in 2 subjects who developed transaminitis, but one of these subjects was on monthly intravenous immunoglobulin infusions (a possible source of false-positive serologies), and in both cases, the viral load remained undetectable. Upon developing transaminitis, 4 subjects underwent repeat testing for hepatitis A, B, and C, as well as EBV and cytomegalovirus, which in all cases was negative.

In order to determine the cause of hepatotoxicity, 2 patients who developed severe transaminitis underwent liver biopsy. These specimens were compared with specimens taken from a normal liver and from the liver of an otherwise healthy patient with CLL. No significant mononuclear infiltrate was seen in healthy liver, whereas clusters of $CD20^+$ periportal lymphocytes and scattered intraparenchymal $CD8^+$, perforin-negative cytotoxic T cells were seen in the patient with CLL, consistent with previous reports.[18] However, biopsy specimens from the CLL patients experiencing idelalisib-related transaminitis showed an increased infiltrate of $CD8^+$ cytotoxic T cells that also stained positive for perforin, indicating an activated state (Figure 3A). Similar biopsy findings were noted in sections of duodenum taken from a subject on trial experiencing idelalisib-related enteritis, with $CD8^+$ cytotoxic lymphocytes infiltrating the lamina propria (Figure 3B).

Use of Steroids to Treat Hepatotoxicity

All cases of transaminitis resolved by holding the drug, initiating immunosuppression (1 subject required mycophenolate mofetil in addition to steroids), or both. Many subjects were initiated on steroids around day 28, and a second group of subjects required steroids around day 130 (supplemental Figure 4). The median time from initiation of steroids to reduction of transaminitis to grade $\leq$1 was 8 days (range, 3-21 days). Patients who developed hepatotoxicity but were able to remain on study drug at the time of data cutoff had a short time from onset of transaminitis to initiation of steroids (median, 0 days; range, 0-4 days) and had long steroid tapers (median, 129 days; range, 31-235 days). In comparison, subjects who developed hepatotoxicity and eventually had to discontinue drug

had a longer time to steroid initiation (median, 7 days; range, 0-17 days) and shorter steroid tapers (median, 70 days; range, 7-198 days). By the end of the trial, 16 subjects (67%) had received steroids for transaminitis and 19 subjects (79%) had received steroids for presumed autoimmune toxicities.

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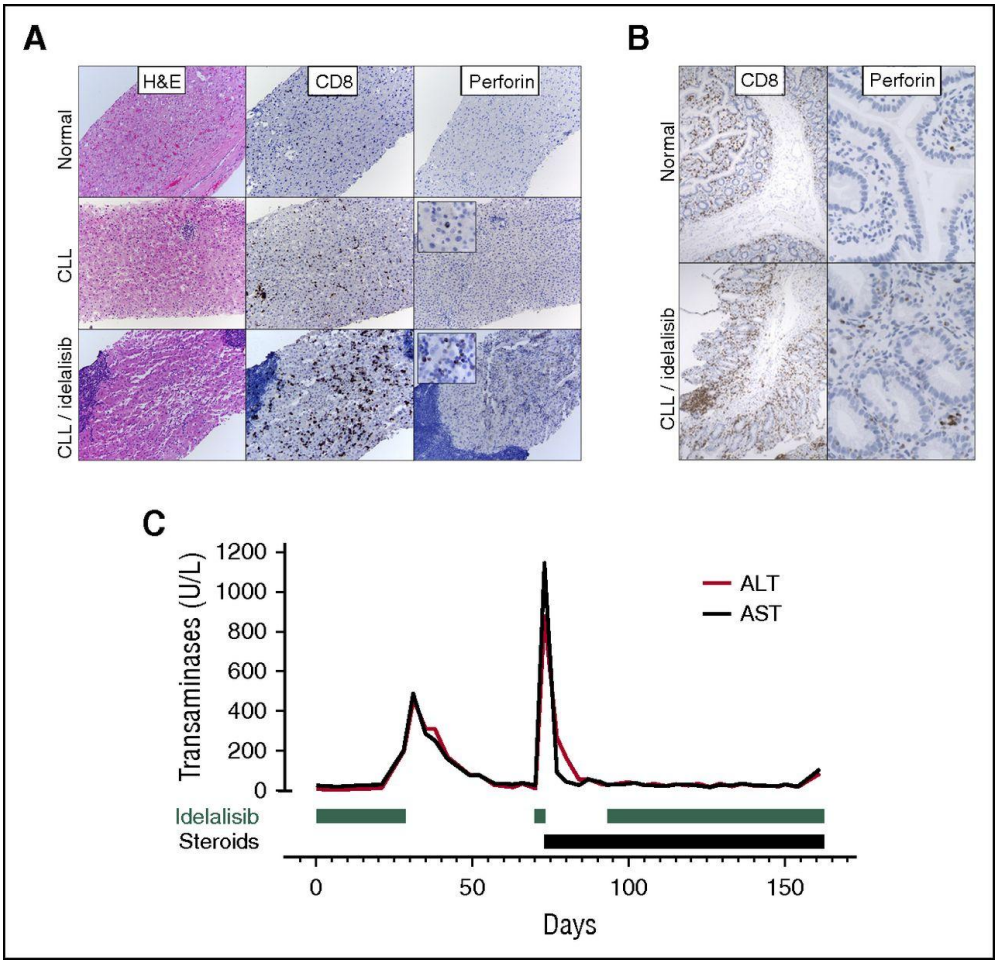

Figure 3: Tissue biopsies and response to steroids suggest an immune-mediated cause for hepatotoxicity. (A) Liver biopsy from a subject with persistent transaminitis after discontinuation of idelalisib and **(B)** duodenal biopsy from a subject with diarrhea both reveal an activated (perforin-positive) $CD8^+$ lymphocytic infiltrate, which is not present in controls. **(C)** Transaminase levels over time for a subject who, after an unsuccessful attempt at idelalisib re-introduction, was successfully re-introduced to the drug while on steroids.

In 12 instances, subjects who developed grade ≥2 transaminitis were rechallenged with idelalisib after it was held for a median of 21 days (range, 10-43 days). Transaminases had normalized for at least 1 week prior to the reintroduction of idelalisib. Six subjects developed recurrent transaminitis. In all cases of recurrent transaminitis, the elevated liver enzymes developed within 1 to 4 days of re-exposure to idelalisib (for example, Figure 3C). A total of 5 subjects were rechallenged while off steroids, and 4 developed recurrent transaminitis (grade 2, 1; grade 3, 2; and grade 4, 1). Seven subjects were rechallenged while on steroids, and 2 developed recurrent transaminitis (grade 3, 1; grade 2, 1). This protective effect of steroids was also noted when all episodes of recurrent transaminitis, pneumonitis, or colitis were examined in aggregate. In total, after an initial episode of transaminitis, pneumonitis, or colitis, resumption of idelalisib led to 22 subsequent, separate additional grade ≥2 toxicities (including only transaminitis, pneumonitis, or colitis). Of these, 17 occurred while subjects were off steroids (grade 2, 9; grade 3, 7; grade 4, 1), and 5 occurred while subjects were on steroids (grade 2, 3; grade 3, 2; and grade 4, 0).

Cytokine Analysis

We measured concentrations of a panel of cytokines in serum obtained from patients at baseline and at day 28, the time of maximum incidence of hepatotoxicity. At day 28, patients experiencing hepatotoxicity, compared with those without hepatotoxicity, had significantly higher levels of the proinflammatory cytokines CCL-3 (median, 1983 pg/mL vs 1429 pg/mL, $P = .01$; Figure 4A) and CCL-4 (median, 2351 pg/mL vs 303 pg/mL, $P = .002$; Figure 4B), consistent with an underlying inflammatory process as the cause of the transaminitis. Subjects with and without hepatotoxicity showed no significant difference in the other tested cytokines (CCL-2, CXCL-5, and vascular endothelial growth factor) at baseline or at day 28 (supplemental Figure 5).

Changes in Regulatory T Cells on Idelalisib

Inactivation of p110δ in mice decreases the number and function of regulatory T cells (Tregs).[19] Through the use of mass cytometry, we investigated the effects of idelalisib on T cells in patients exposed to idelalisib. Samples of peripheral blood mononuclear cells had been collected from patients at baseline, day 28, and day 130 (at the time of late hepatotoxicity). At day 28, the fraction of $CD3^+$ T cells that were $CD4^+$ helper cells trended higher, whereas $CD8^+$ cytotoxic cells trended lower (supplemental Figure 6A-B).

Decreases in the Treg population were frequent. At baseline, the median percentage of total $CD4^+$ T cells that were $CD3^+CD4^+$ $FoxP3^+CD25^{hi}$ Treg cells was 5.8%. This decreased to a median of 4.0% after 28 days on therapy and 2.8% after 130 days on therapy, although these decreases were not statistically significant (Figure 4C). Out of 19 matched pairs, 13 patients (68%) experienced a decrease in Treg percentage while on therapy, with a median relative loss of 42% of the Treg fraction. When patients were stratified by toxicity at the time of sample collection (including those with transaminitis or colitis), those without toxicity had a median Treg percentage of 5.7%, whereas those experiencing toxicity had a significantly lower median Treg percentage of 2.3% (p=.03, Figure 4D). In patients experiencing toxicity on idelalisib, there was a trend toward a lower Treg:$CD4^+$ ratio (supplemental Figure 6C).

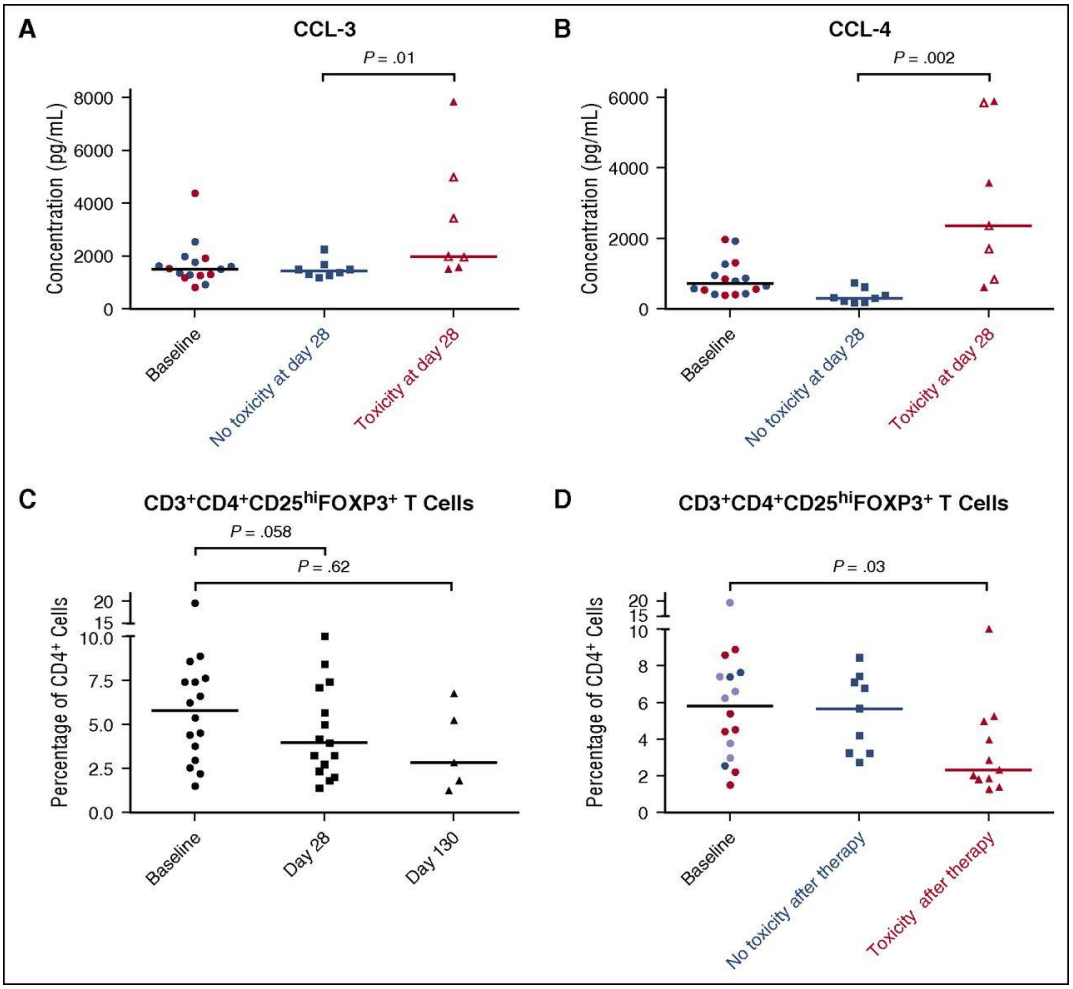

Figure 4. Increased inflammatory cytokine levels and decreased regulatory T-cell levels are associated with the development of toxicities while on idelalisib. (A) Serum CCL-3 and **(B)**

CCL-4 levels in subjects at various time points on idelalisib therapy. Baseline values indicated in red are subjects who experienced toxicity at day □28, and blue represents subjects who did not experience early hepatotoxicity. Open symbols represent levels drawn when subjects have held idelalisib for 3 to 9 days due to toxicity; closed symbols represent samples drawn while subjects remain on idelalisib. **(C)** Percentage of $CD4^+$ T cells that are $FoxP3^+CD25^{hi}$ regulatory T cells in subjects on idelalisib. **(D)** Percentage of $CD4^+$ T cells that are CD31CD41 $FoxP3^+CD25^{hi}$ regulatory T cells in subjects on idelalisib, stratified by toxicity. At baseline, values indicated in red are subjects who experienced toxicity at day □28, blue symbols are subjects who did not experience toxicity, and purple symbols are subjects who experienced delayed toxicity and thus contributed data points to the no toxicity group at day □28 and the toxicity group at day □130.

Discussion

Idelalisib is a p110δ inhibitor that has shown impressive efficacy for the treatment of relapsed, refractory CLL. Here, we found that idelalisib used as upfront therapy for CLL caused an early, severe (grade ≥3) hepatotoxicity in 54% of patients. Subjects who are younger and subjects with IGHV mutated disease were more likely to experience this early hepatotoxicity. Steroids were effective at treating the transaminitis once it developed, and the incidence of recurrent hepatotoxicity was lower if subjects were receiving steroids at the time of reintroduction of the study drug. In keeping with preclinical data, idelalisib caused a reduction in the number of Tregs in the peripheral blood, particularly in patients experiencing toxicities.

Multiple lines of evidence point to an autoimmune mechanism as the cause of the hepatotoxicity seen. Perhaps surprisingly, younger age was identified as a risk factor for early hepatitis, consistent with the view that younger subjects may have a more robust immune system than older subjects. An activated T-cell infiltrate is seen on liver biopsy specimens of patients who experienced idelalisib-related hepatitis, but not in otherwise healthy patients with CLL. Immunosuppression with steroids (and, in a single case, mycophenolate mofetil) was effective at treating the transaminitis and decreasing the incidence of subsequent transaminitis upon re-exposure to the drug. The observation that the transaminitis takes weeks to develop but then rapidly recurs upon re-exposure to idelalisib is suggestive of liver injury secondary to an adaptive immune response.[20] Cytokine analysis demonstrated increased levels of CCL-3 and CCL-4, which

are known to play proinflammatory roles in murine[21] and human[22] immune-mediated hepatitis. CyTOF analysis of the peripheral blood did show a decrease in Tregs, which would correlate with an inflammatory state.

Preclinical evidence also supports the conclusion that on-target inhibition of p110δ by idelalisib can cause autoimmune toxicities and furthermore that the mechanism underlying this could be due to effects on Tregs. Mice with genetic inactivation of p110δ develop an autoimmune colitis characterized by numerous intraepithelial lymphocytes.[7,23] Systemic p110δ inactivation has broad effects on the immune system, decreasing B-cell function and number as well as decreasing the ability of naive T cells to differentiate into both Th1 and Th2 subtypes.[7,24,25] However, on an organismal level, this impaired effector cell function is often counterbalanced by inhibition of Tregs, as p110δ is also critical for the survival and function of Tregs.[19] For example, despite the fact that both $CD8^+$ and $CD4^+$ T cells from p110δ-deficient mice demonstrate reduced production of cytotoxic mediators and interferon-γ, respectively, mice with genetic inactivation of p110δ are resistant to tumorigenesis. Adoptive transfer experiments showed that p110δ inactivation in Tregs is both necessary and sufficient to confer this resistance to tumor growth.[26] In agreement with this, germline genetic mutations that disrupt Treg function in mice and humans, including FOXP3, lead to autoimmune syndromes with hepatitis, enteritis, and pneumonitis.[27,28]

The rates of grade ≥3 transaminitis reported here are higher than rates previously reported for this drug, as summarized in Table 3 . In the phase 1 trial, subjects were the most heavily pretreated, with a median of 5 prior therapies, and the rate of grade ≥3 hepatotoxicity was the lowest, at 2%. [29] When toxicity data were aggregated from 8 clinical trials examining idelalisib used as later-line treatment of indolent B-cell malignancies, the rate of grade ≥3 transaminitis was 14%.[4] In addition to the trial presented here, one other published trial examined idelalisib in the upfront setting, given to patients age 65 or older. In the cohort treated with combination rituximab therapy, 67% of subjects experienced a transaminase elevation of any grade (23% grade ≥3)[6]; in the cohort treated with idelalisib alone, 24% experienced a transaminase elevation (22% grade ≥3).[5] Thus, as the median age and number of prior therapies increased, the frequency of immune-mediated adverse events decreased. This may reflect the long-lasting effects that some CLL therapies, and the disease itself, can have on the immune system, as well as an

accruing immune senescence with age. For example, for at least 2 years after fludarabine therapy, absolute $CD4^+$ and $CD8^+$ T-cell counts remain less than half pretreatment levels.[30] In keeping with these observations, when idelalisib was given to healthy young volunteers, toxicity was frequent, with 5 subjects out of 24 (21%) experiencing grade 3 transaminitis after only 7 to 9 days on 150 mg idelalisib twice daily.[31] The association between *IGHV* mutation status and early hepatotoxicity may reflect a unique interaction between mutated *IGHV* neoplastic cells and T-cell subsets.[32] For example, 2 studies have previously reported that patients with mutated *IGHV* CLL have reduced numbers of Tregs.[33,34] We did find a lower baseline median percentage of Tregs in our patients with *IGHV* mutated disease (data not shown), although the difference was not statistically significant.

	Phase I	**Overall Relapsed**	**Upfront patients ≥ 65yo with rituximab**	**Upfront patients ≥ 65yo idelalisib monotherapy**	**Current Trial**
Number of subjects	54	760	64	41	24
Median Prior Therapies	5 (2-14)	≥1	0	0	0
Median Age	63 (37-82)	66 (21-91)	71 (65-90)	71 (65-84)	67.4 (58-85)
Median Time on Therapy (mos)	15 (0.2-48.7)	-	22.4 (0.8-45.8)	9.3 (1.4-17.4)	7.7 (0.7-16.1)
Grade ≥3 Transaminitis	1.9%	14%	23%	22%	54%
Grade ≥3 Colitis/Diarrhea	5.6%	14%	42%	27%	13%
Any grade pneumonitis	5.6%	3%	3%	5%	13%
Reference	Brown 2014	Coutre 2015	O'Brien 2015	Zelenetz 2015	Lampson 2015

Table 3. Idelalisib-related toxicities compared across multiple clinical trials.

Transaminitis is seen when other drugs within this class are tested in the upfront setting, as would be expected if hepatotoxicity is due to on-target p110δ inhibition. TGR-1202 is a p110δ inhibitor with hepatotoxicity seen in 2% of patients treated for relapsed, refractory disease.[35] However, a recent trial combining TGR-1202 with obinutuzumab and

chlorambucil for CLL treatment had rates of grade ≥3 transaminitis of 28%.[36] Eighty-three percent of the subjects in this 18-patient trial were treatment naive. Our group recently reported preliminary results of the phase 1 portion of a trial of the p110δ/γ inhibitor duvelisib in combination with fludarabine, cyclophosphamide, and rituximab for the upfront treatment of CLL. We observed a 29% rate of grade ≥3 transaminitis, occurring at time points similar to those reported here.[37] In this duvelisib trial, the combination of p110δ inhibition with chemoimmunotherapy or concomitant p110γ inhibition may have decreased the overall rates of autoimmune toxicity.

Limitations of the study include the small number of patients enrolled and the correspondingly small number of samples available for correlative studies. Our analysis here focuses on the characteristics of the early hepatotoxicity seen at day 28 and may not be applicable to other toxicities seen with the drug, including enterocolitis, pneumonitis, and the delayed hepatotoxicity occurring around day 130. Correlative studies regarding the mechanism of drug-mediated hepatotoxicity were analyzed retrospectively, and while these findings are important for future hypothesis generation, they cannot show causation.

While this manuscript was in preparation, Gilead closed seven randomized trials of idelalisib in B-cell malignancies (5 in treatment-naive patients) due to an excess of infectious deaths. In our study, opportunistic infections were noted prior to mandating prophylaxis. Further studies will be required to determine any relationship between these infectious toxicities and the autoimmune toxicities seen here, but both may relate to the immunologic effects of inhibiting p110δ. As additional drugs are developed within this class, subjects should be closely monitored for infection as well as transaminitis (and other autoimmune phenomena), with a low threshold for infectious prophylaxis for the former and immunosuppressants for the latter, based on the likely immune-mediated origin of this toxicity.

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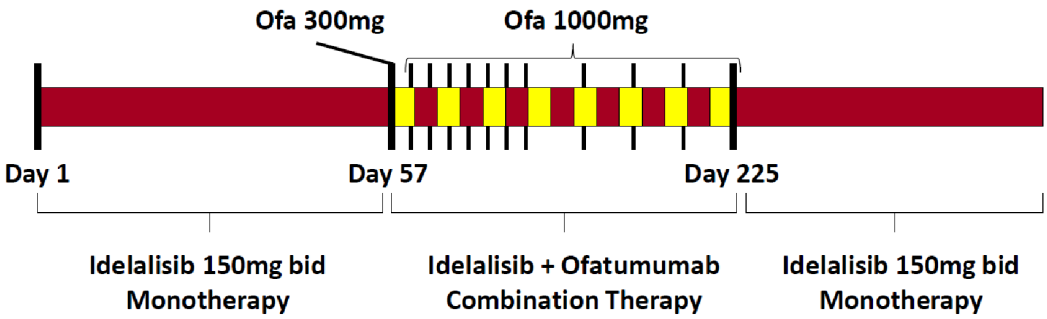
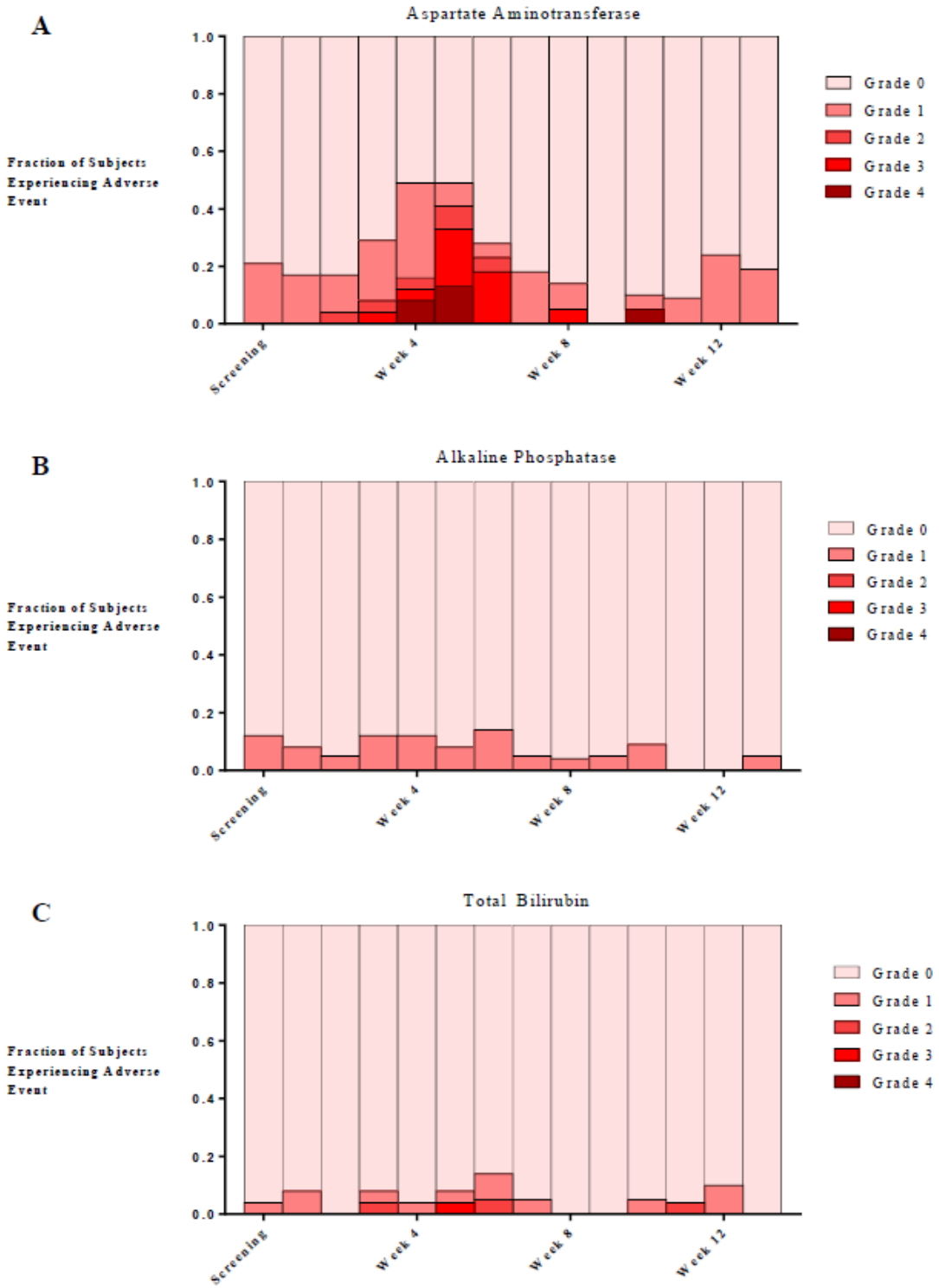
SUPPLEMENTAL MATERIALS

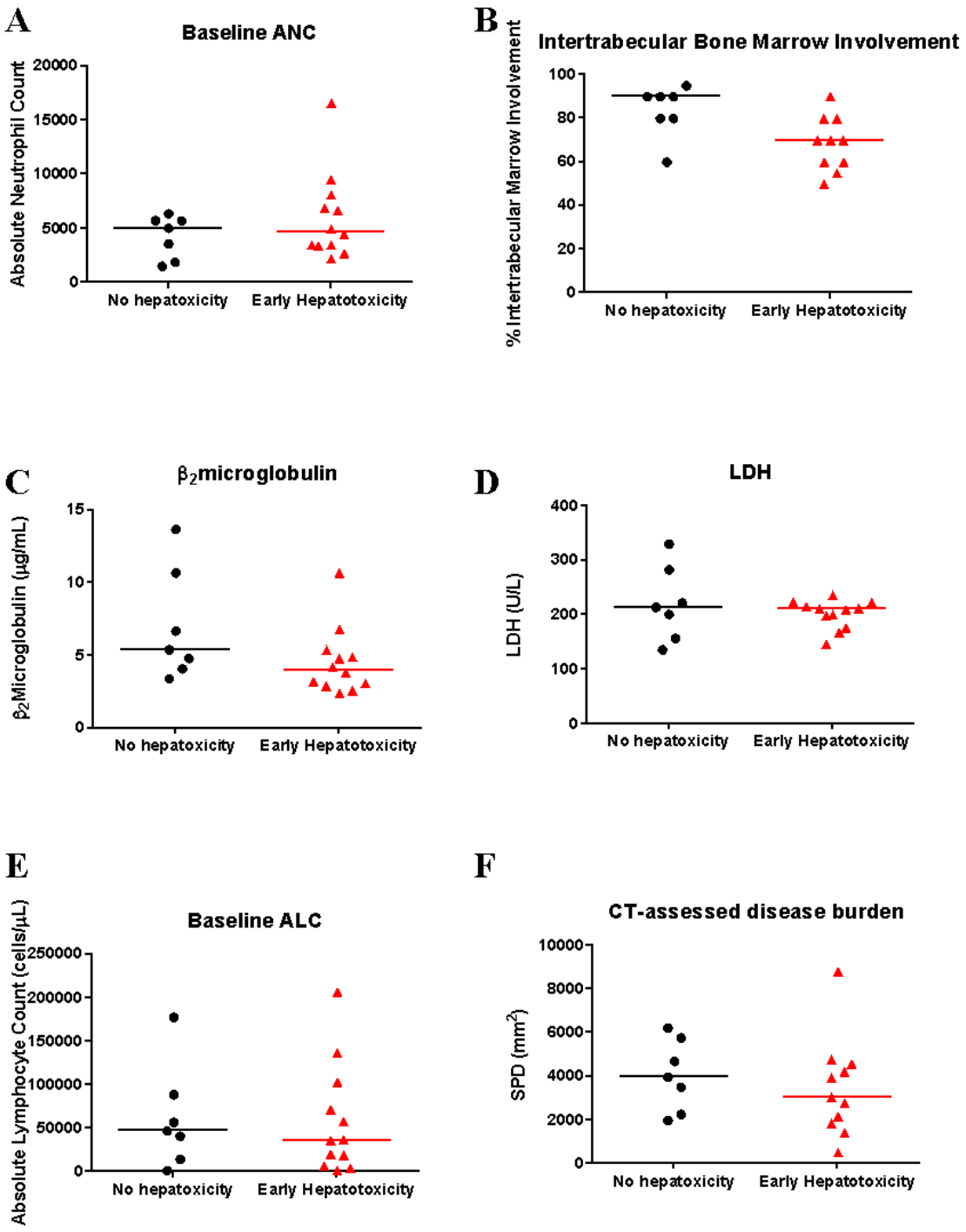
Supplemental Figure 1. Design of idelalisib-ofatumumab combination trial. Idelalisib monotherapy is followed by idelalisib plus ofatumumab combination therapy and then followed by idelalisib monotherapy until disease progression. The first 8 doses of ofatumumab were given on a weekly basis; the last four on a monthly basis.



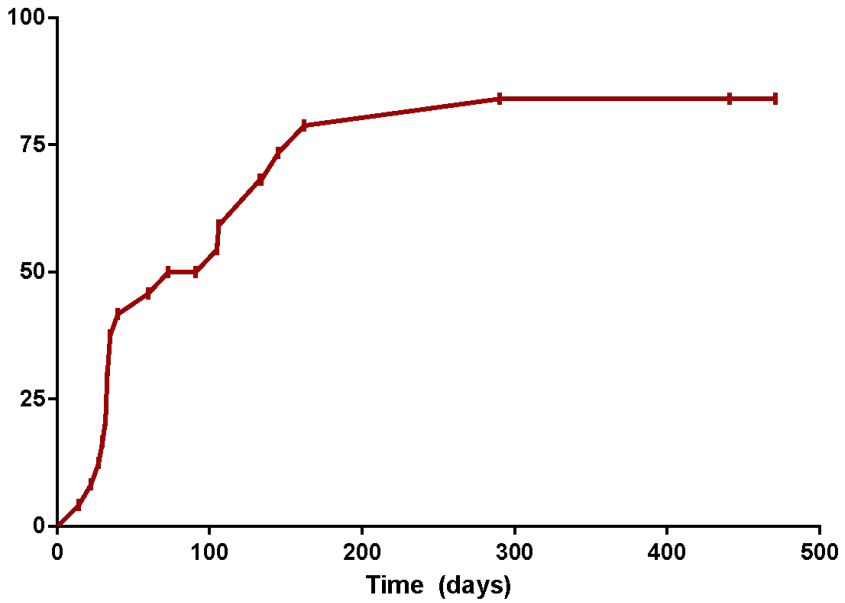
Supplemental Figure S2: Idelalisib-mediated liver injury shows a hepatocellular pattern. The fraction of subjects experiencing **(A)** AST, **(B)** alkaline phosphatase, or **(C)** total bilirubin elevations of the indicated grade at the indicated time.



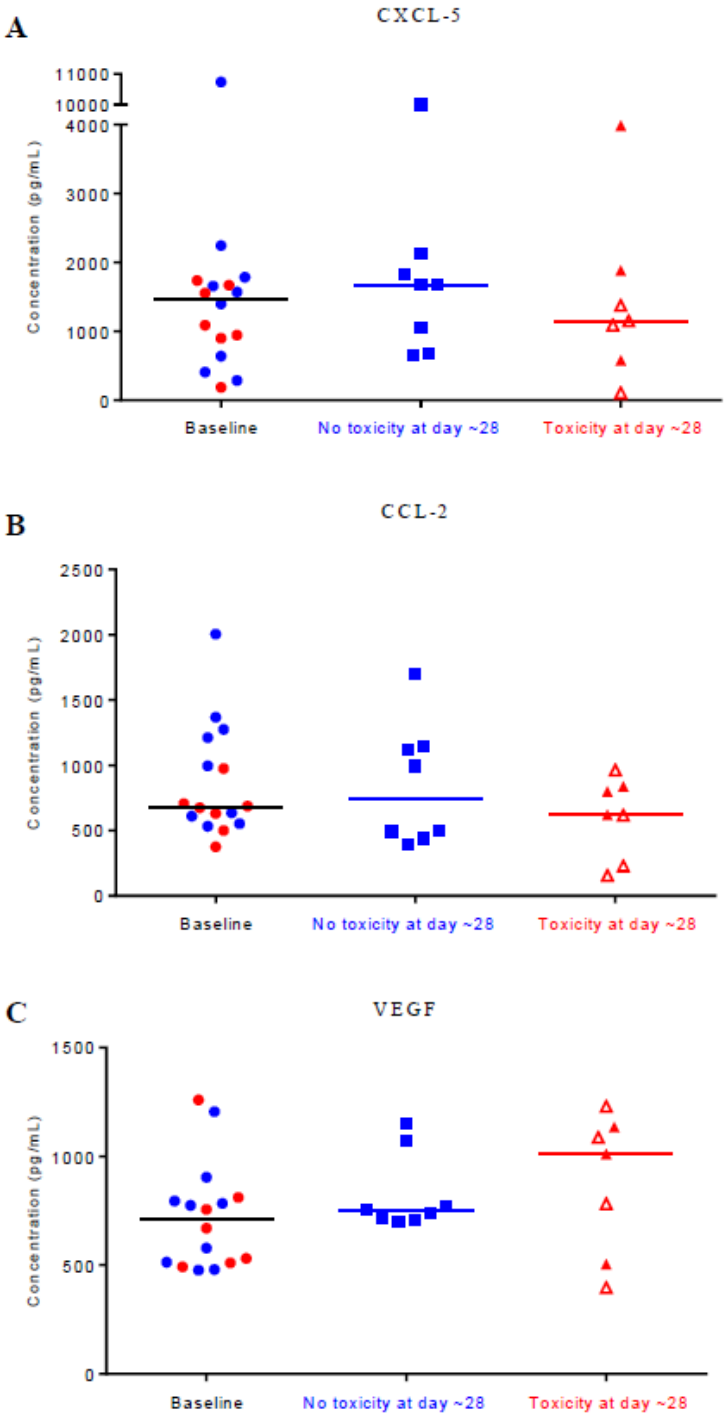
Supplemental Figure S3: Participant characteristics that do not predict early hepatotoxicity. There is no significant difference in **(A)** baseline absolute neutrophil count (ANC), **(B)** intertrabecular bone marrow involvement, **(C)** β2-microglobulin, **(D)** lactate dehydrogenase (LDH), **(E)** baseline absolute lymphocyte count (ALC), or **(F)** CT-assessed disease burden (as measured as the sum of the product of the diameters (SPD) of six representative target lesions) between subjects who experienced early hepatotoxicity and those who did not.



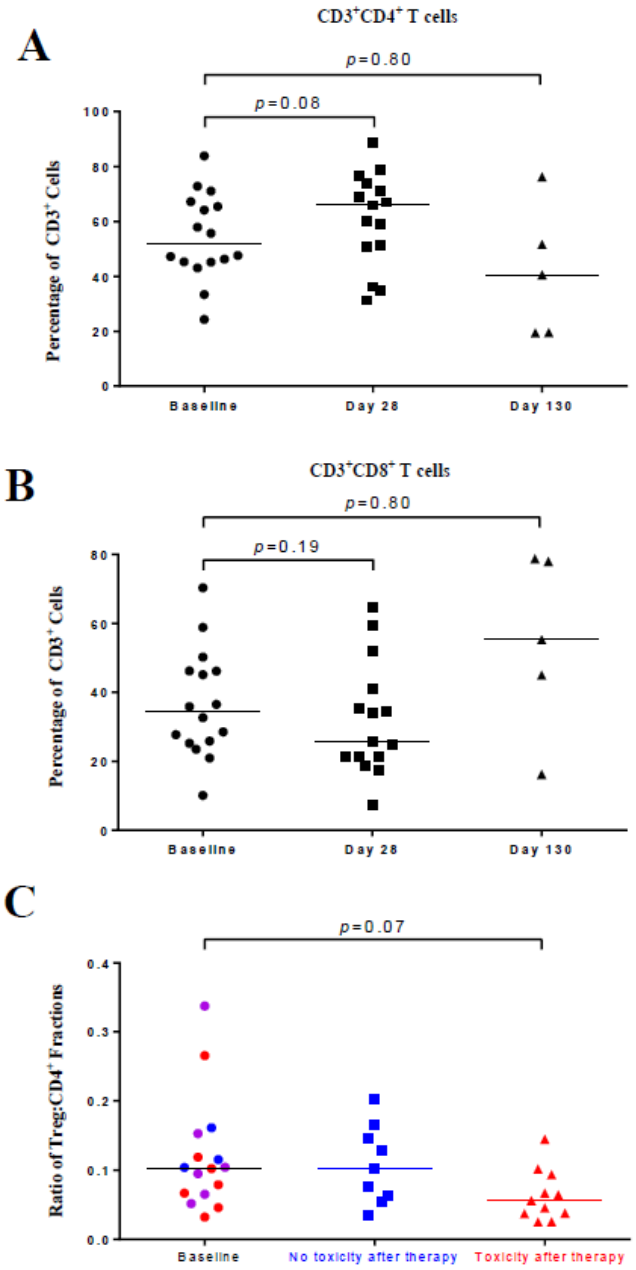
Supplemental Figure S4: Time to initiation of steroids. Kaplan-Meier time-to-event curve representing time to first exposure to systemic steroids for each enrolled subject.



Supplemental Figure S5: Additional serum cytokine levels in patients on idelalisib. Changes in **(A)** CXCL-5, **(B)** CCL-2, and **(C)** VEGF levels are not associated with the development of early hepatotoxicity. Open symbols represent levels in subjects who have had idelalisib held for 3-9 days. Baseline values indicated in red are subjects who experienced toxicity at day ~28, and blue represents subjects who did not experience early hepatotoxicity.



Supplemental Figure S6: Additional T cell subsets in patients on idelalisib. Absolute percentage of $CD3^+$ T cells that are **(A)** $CD4^+$ and **(B)** $CD8^+$ in subjects on idelalisib. **(C)** Ratio between fraction of $CD4^+$ T cells that are Tregs to fraction of $CD3^+$ T cells that are $CD4^+$, stratified by toxicity. At baseline, values indicated in red are subjects who experienced toxicity at day ~28, blue symbols are subjects who did not experience toxicity, and purple symbols are subjects who experienced delayed toxicity and thus contributed data points to the no toxicity group at day ~28 and the toxicity group at day ~130.



CHAPTER 7

7.1 Discussion

CD4 Regulatory T cells (Treg) have been discovered over twenty years ago and been shown to play a critical role in maintaining immune homeostasis.

During an autoimmune process the immune system reacts against the self-healthy cells and tissues of an organism. There are many examples of autoimmune diseases, either happening systemically or locally focused, as for example type 1 diabetes, lupus erythematosus or psoriasis. Interestingly the presence of autoreactive cells in the organism does not always cause an autoimmune response. In fact, the presence of autoreactive cells and autoantibodies circulating in the blood of healthy individuals without causing harm seems to be physiological.[1,2] Similarly, one could extrapolate that we possibly would find host-reactive cells and antibodies after HSCT in many individuals that do not present symptoms or signs of GVHD. Autoreactive cells can remain in circulation without causing any harm, but when encountering cells expressing their cognate antigen will become activated and deleterious. This physiologic phenomenon of healthy people remaining tolerant to autoreactive cells, remains unclear. Understanding the regulatory mechanisms in healthy individuals could provide some clues on tolerance after transplantation.

A main factor contributing to autoimmunity seems to be the tissue antigens inducing auto-aggressive responses, which can be due to a systemic dysfunction or local immune mechanisms through transient factors (such as concomitant infections) or genetic encoding. While generally autoimmunity is not yet fully understood, the scarce inclusion of a wide range of autoreactive repertoire in the thymus and the systemic deficiency of $FoxP3^+$ Treg cells are known factors to result in various forms of autoimmunity.[3]

Various Treg subsets have been identified, however, the heterogeneity of Treg subpopulations during development remained uncharacterized. In **chapter 3**, by using mass cytometry, we reveal the Treg subsets heterogeneity that is not detected in the routine

characterization of Treg by flow cytometry with a limited set of markers. We also show that Treg heterogeneity varies considerably among individuals and in patients after allogeneic hematopoietic stem cell transplantation (alloHSCT). Although this heterogeneity is based on the variable expression of functional markers, further studies are needed to establish the extent to which this phenotypic heterogeneity reflects actual functional differences between different Treg metaclusters and the ability of different Treg metaclusters to regulate different immune cells and immune networks in vivo. The main limitation was the inability to define each individual cluster and compare them between samples. Since Treg is known to be capable of functional plasticity it will also be important to examine the stability of distinct metaclusters in vitro and in vivo. As Treg directed therapies are evaluated in patients with autoimmune diseases as well as graft-versus-host disease (GVHD), it may be important to examine the effects of these interventions on Treg heterogeneity as well as the number of circulating Treg.

Tregs are mostly (including in this thesis) defined by the expression of FoxP3 and CD25 (Interleukin-2 receptor alpha chain). These two markers are often associated with immune regulation, although it is known that Tregs are plastic and capable of downregulating FoxP3 expression. Hence, other markers to define regulatory T cells should be considered. The limitation of characterizing Tregs simply on bases of 1 or two markers, might lead us to an incomplete conclusion. Various human studies have identified Tregs by correlating the frequency of $CD4^+$ $CD25^+$ Tregs in blood with the levels of FoxP3 messenger RNA (mRNA) in peripheral blood mononuclear cells.[4-7] We need to consider that those studies are subjected to current limitations of cytometric analysis to identify and study the relative low number of circulating regulatory T cells in individuals after allogeneic HSCT, who often have severe lymphopenia.

Regulatory T cells remain the most studied regulatory subset. Preclinical animal studies, where the Tregs were eliminated or transferred in high numbers into immunocompromised animals, showed clearly the powerful suppressive function of Tregs. Additionally, there were suppression assays created, allowing to test the suppressive capacity of Tregs in vitro, by co-culturing Tregs with effector CD8 T cells or antigen-presenting cells. It clarified the direct cell-dependent suppression of effector cells.[8] However, the translation of these mechanisms into for example FoxP3 Treg cell adoptive transfers or in vivo Treg stimulations to treat autoimmune diseases, has so far been unsatisfactory. It should make

us consider expanding the preconceived notion that there is only one major set of regulatory cells and that these cells always bear the task of regulation. A more open and broader view should enable us to find other cell subsets that can also adaptively assume regulatory immune functions.[9]

Continuous technological advancements led to novel high-throughput tissue screenings and generation of biological therapies with small molecule compounds or monoclonal antibodies (mAb). One of the biggest successes was the development of anti-TNF-α blockers that have been implemented world wide for various autoimmune diseases.[10] Followed by the generation of many small molecule inhibitors of inflammation (e.g. TYK2, IRAK4, SYK, JAKs, TPL2, RIPs and BTK) and large molecules that inhibit cytokine functions (e.g. IL-1, IL-6, IL-12, IL-17, and IL-23). Despite the exponential growth of successful immunomodulatory biological therapies, we are still facing significant clinical limitations. Firstly, current biological therapies are immunomodulatory, alleviating symptoms but do not reverse the loss of immune tolerance by generation of a cure. Second, these therapies are extremely costly, making it currently financially unsustainable to be used widely on the population and through endless years. As a result, mostly only patients that are resistant to conventional therapies get access to biologicals, which means that they also have had a severe form of the disease for many years. The use of biologicals at disease early-set could potentially limit the disease evolution to some of the severe irreversible systemic symptoms. Thirdly, these agents are not successful in refractory autoimmune diseases and patients can become unresponsive to therapy.[11,12] Lastly, by reducing systemic inflammation, these treatments can have significant adverse safety risks as susceptibility to malignancies or to opportunistic infections.[13-15]

In **chapter 4**, we used mass cytometry combined with machine learning techniques assess the expression of 33 phenotypic and functional markers in all major lymphocyte subsets at single-cell resolution.[16,17] Application of this marker panel following in vitro stimulation with IL-2 for 15 minutes was used to monitor direct activation of different signaling pathways in each well-defined lymphocyte subset and to correlate signaling activity with an expression of functional intracellular proteins as well as surface cytokine receptors. Comparison of a wide range of IL-2 concentrations subsequently identified specific subsets of T cells and NK cells that respond to either physiologic or supraphysiologic concentrations of this important immune cytokine. The profound but highly selective

immunologic effects of prolonged low-dose IL-2 therapy demonstrated in our studies support further clinical evaluation of this approach in various settings where selective enhancement of CD4 Tregs may be needed to restore or maintain immune tolerance.

It is also interesting to consider the Treg setpoint theory, which stipulates that the naturally homeostatic set point for Tregs is already highly suppressive at a steady state, and it is therefore not possible to increase the Treg suppressive activity. However, we still do not yet understand well how to make the cells perform at their best in vivo. Would it be possible to manipulate the efficiency of the physiological range of Treg suppression? Much research is still needed to try to test the possibility of boosting the Treg population that are already at their physiological steady-state Treg setpoint.

A key approach in the usage of immunotherapeutic Treg cells is 1) expanding the in vivo number of Tregs with low-dose IL-2; 2) infuse ex vivo expanded Tregs; or 3) from adoptive transfer from a donor. Small clinical trials have had some effective preliminary positive findings when adoptive Tregs are transferred with the purpose of preventing acute GVHD. Larger studies are crucial to validate such results. On the other hand, limited studies have also tested in vivo Treg expansion by using low-dose IL-2, with no obvious benefits to prevent acute GVHD. There are numerous limitations to make adoptive Treg infusion widely available as a prophylactic or therapeutic option. The work-up of the donor and ex vivo expansion of Tregs are extremely time consuming, and it requires specialized technologies and equipment. The time required for expansion of Tregs can lead to delays, making adoptive therapy of Tregs often timely incompatible with the new onset of GVHD. A possible alternative for the future could be availability of “off-the-shelf” Tregs. Using Tregs from a third healthy donor, it has the potential of introducing a highly diverse repertoire of Tregs, with various T cell receptors, and standard suppressive and proliferative activity. The endogenous Tregs of the patient have likely been exposed to immunosuppressive therapies, having possibly a lower suppressive capacity, and limited TCR diversity. Regardless, it is still to be studied what could be the dosing schedules and optimal dose of adoptive transfer.

Treg focused treatments for chronic GVHD are largely concentrated in low-dose IL-2 to induce in vivo expansion of the Tregs. Most clinical studies conducted so far are single center, with low numbers of patients, and treating patients that are resistant to current

standard therapies, such as high-doses of steroids. Still, those studies show that low-dose IL-2 is well-tolerated, does not increase malignant relapse or infections, and present very promising effective results. One of the main observed challenges for this in vivo expansion approach was patient compliance, since the low-dose of IL-2 has to be often intracutaneously administered. Here too, it is essential to conduct multicenter studies, with larger numbers of patients to validate the preliminary results and test different dosing schedules and optimum duration of the therapy. Newer studies could also focus on testing low-dose IL-2 as a prophylactic strategy for chronic GVHD. It remains to be determined what could be the long-term effects on the immune system from prolonged administration of low-dose IL-2.

The prognosis for patients who develop acute GVHD is poor, particularly in cases of severe steroid-refractory aGVHD (SR-aGVHD). At present, no standard second-line therapy is approved for SR-aGVHD, and none of the available treatment options seems to provide convincingly superior results with on average only 30% complete responders.[18,19] We reported in **chapter 5** the results of a multicenter phase I/II trial to study the in vivo safety and efficacy of using CD3/CD7-IT therapy in patients with SR-aGVHD. This study had several limitations; First, the sample size was relatively small, and we did not include a randomized comparator arm. In addition, the study population was heterogeneous with respect to age, conditioning regimen, donor type, and GVHD prophylaxis regimens used. Nonetheless, the study population is representative of patients with SR-aGVHD treated at our institutions and consisted primarily of patients with underlying high-risk features. Nonetheless, the results show that CD3/CD7-IT provides a high rate of clinical remission and rapid immune reconstitution following treatment. Based on these results, a phase III study is proceeding to examine the potential value of including CD3/CD7-IT in the treatment of aGVHD.

An interesting follow-up study could be to determine parameters that are associated with response to CD3/CD7-IT. Using the flow cytometry data available, we could analyze immune subsets, namely T cells in the response to CD3/CD7-IT. There is evidence that a balanced recovery of CD4 and CD8 T cells is necessary to prevent aGvHD, and that immune reconstitution after HSCT favors expansion of CD4+ T cells.[20,21] Whereas, a decrease of the CD4:CD8 ratio is strongly correlated with the occurrence of aGvHD after HSCT.[22] For these reasons, the association between CD4:CD8 ratio with response in our

cohort, and if CD4:CD8 ratio was affected by CD3/CD7-IT should be investigated. Different maturation subsets of $CD4^+$ and $CD8^+$ cells are also associated with the development of aGvHD. In mice, central memory (CM) and effector memory (EM) $CD4^+$ T cells do not mediate aGvHD, while naïve $CD4^+$ T cells do play an essential role in this process.[23-25] In humans, increased amounts of all CM, EM, and naïve $CD4^+$ and $CD8^+$ subsets have been correlated with the pathogenesis of aGvHD.[26] Despite this current ambiguity, I would be curious about the effect of CD3/CD7-IT on $CD4^+$ and $CD8^+$ maturation subsets in relation to response. Naturally, I am specifically interested in Tregs as they are crucial in aGvHD pathophysiology.[27] As mentioned before a lack of Treg reconstitution is associated with severe aGvHD, whereas enhanced Treg reconstitution is capable of preventing aGvHD.[28-30] High-throughput T cell receptor sequencing allows to estimate the diversity of the complete T-cell repertoire, known as clonal diversity. Low clonal diversity is associated with higher rates of aGvHD as well as leukemic relapse in patients after allo-HSCT.[31] Furthermore, aGvHD is characterized by the expansion of autoreactive T-cell clones, which lowers clonal diversity.[32] Hence, I hypothesize that response may be correlated with clonal T cell diversity.

The comparison of the T-cell compartment reconstitution among different aGvHD grades and conditioning regimens should also be performed, since these aspects are associated with the rate of steroid resistance and incidence of aGVHD.[33-35] Finally, assessing if CD3/CD7-IT was effective among different aGvHD grades (II to IV) and conditioning regimens.

Taken together, those results could help early identifying patients that are likely, or unlikely, to respond to CD3/CD7-IT enabling a patient-tailored aGvHD treatment. Such prospective analysis of the immune reconstitution could identify several factors that are associated with response to therapy with CD3/CD7-IT. Those results would naturally have to be confirmed in larger cohorts.

Idelalisib is a small molecule inhibitor of PI3Kδ with demonstrated efficacy for the treatment of relapsed/refractory chronic lymphocytic leukemia (CLL). PI3K is a downstream signaling of the T cell receptor and CD28, having a crucial part in T cell activation, cytokine secretion, survival, clonal expansion and motility.[36,37] In mice studies, the inactivation of PI3Kδ in regulatory T cells lead to enhance secretion of IL-10, which

promoted the cytotoxic T cell mediated tumor depletion and lowered the suppressive capacity of Tregs.[38,39] Ex-vivo treatment of peripheral blood isolated T cells with idelalisib shows a preferential blockage of Treg cells even in lower doses when comparing with effector CD8 and CD4 T cells.[40] Confirming this results, a PI3Kδ isoform (IC-87114) showed inhibition of Tregs versus effector CD4 T cells.[41] These findings suggest that PI3Kδ regulate the Treg suppressive activity without compromise of anti-function of cytotoxic T lymphocytes.

To evaluate idelalisib as front-line therapy for CLL, we enrolled 24 subjects in a phase II study (**chapter 6**). A serious side effect of idelalisib was autoimmune toxicity. Considering our vast knowledge in studying single-cells in the context of a diverse immune system, we used our translational methodology in this clinical study, hoping to unveil potential causes of lack of immune tolerance. The observation that the transaminitis takes weeks to develop but then rapidly recurs upon re-exposure to idelalisib is suggestive of liver injury secondary to an adaptive immune response. Cytokine analysis demonstrated increased levels of CCL-3 and CCL-4, which are known to play proinflammatory roles in murine and human immune-mediated hepatitis.[38] CyTOF analysis of the peripheral blood did show a decrease in Tregs, which would correlate with an inflammatory state. We presented the first evidence that patients with CLL being treated with idelalisib have reduced circulating Treg numbers. Treg cells are less responsive to T cell receptor stimulation than effector T cell subsets, but still are dependent on continuous stimulation for their suppression capacity and proliferation in vivo.[42,43] Mice studies demonstrated that Treg and effector T cells IL-2 dependent proliferation is independent of PI3Kδ.[38,44] The analysis of the expression of cell proliferation marker Ki-67 in circulating Tregs, effector CD4 and CD8 T cells in chronic lymphocytic leukemia patients receiving idelalisib therapy found that idelalisib therapy of patients reduced the expression of Ki-67 more in Tregs than effector T cells.[40] Hence, the fact that idelalisib preferably inhibits Treg proliferation might be due to the Tregs hyporesponsiveness to TCR when comparing to counterparts effector CD4 and CD8 T cells.

PI3Kδ signaling regulates the cytotoxic function of effector CD8 T cells, but in less extent when effector CD8 T cells are already differentiated into a memory phenotype, then they become less dependent on PI3Kδ signaling.[45,46,47,48] Such intrinsic differences seen in

dependency of PI3Kδ signaling within cells might explain the molecular basis of different sensitivity to therapy with idelalisib when comparing effector CD8 T cells and Treg cells.

Inhibition of PI3Kδ signaling has shown to block the TCR and co-stimulation receptor–induced expression of important Treg stability and activation markers including FOXP3, CD25, CTLA-4, ICOS, PD-1, and CD39.[40] The idelalisib treatment of a combine culture of effector T cells and Tregs during their activation process, clarifies the impaired capacity of Tregs to secrete IL-10 and consequent increase of TNF-α and IL-2 secretion by the effector T cells. In vitro suppression assays validated that idelalisib mostly affects the differentiation and activation process of Treg cells.[40] On contrary, if Tregs were previously activated to the co-culture with Idelalisib, Treg would keep their suppressive function capacity. Together, PI3Kδ signaling seems to be necessary for human Treg differentiation and activation but not necessarily affect suppression function of previously activated Treg cells.

Limitations of our study in chapter 6 include the small number of patients enrolled and the correspondingly small number of samples available for correlative studies. Our analysis here focuses on the characteristics of the early hepatotoxicity seen at day 28 and may not be applicable to other toxicities seen with the drug. Correlative studies regarding the mechanism of drug-mediated hepatotoxicity were analyzed retrospectively, and while these findings are important for future hypothesis generation, they cannot show causation. Further studies will be required to determine any relationship between infectious toxicities and the autoimmune toxicities, but both may relate to the immunologic effects of inhibiting p110δ. As additional drugs are developed within this class, subjects should be closely monitored for infection as well as transaminitis (and other autoimmune phenomena), with a low threshold for infectious prophylaxis for the former and immunosuppressants for the latter, based on the likely immune-mediated origin of this toxicity. On the other hand, the distinct role of PI3Kδ signaling in effector T cells and Tregs could be used potentially be used to preferentially downregulate the suppressive capacity of regulatory T cells in malignancies, while simultaneously sustaining the cytotoxic T cell effects.[38,39,45] Mice studies show in vivo, that PI3Kδ inhibitors but not PI3Kα- or PI3Kβ inhibitors, enhances the cytotoxic T lymphocyte infiltration in tumors while decreasing the proliferation and numbers of Treg cells.[38,39,41,49]

A common limitation of all studies here presented, like most of current hematological research, is the fact that we concentrate on studying blood circulatory Treg cells. However, GVHD like many autoimmune diseases associated with defected or low Treg counts, affect predominantly solid tissues. We require a greater progress in our research strategies in order to reach a greater understanding of the function of tissue Tregs. It could lead to innovative treatment methods that improve Treg homing to the affected tissues.

As an example, until recently, it was assumed that aGVHD was primarily mediated by donor effector T cells. However, in a publication from last year, we show that host tissue-resident T cells may play a previously unappreciated pathogenic role in acute GVHD.[50] While blood of patients with aGVHD contained primarily donor-derived T cells, most T cells in the skin were host derived. Host T cells were present in all skin and colon acute GVHD specimens studied, yet were largely absent in blood. We observed acute skin GVHD in the presence of 100% host T cells. Analysis demonstrated that a subset of host T cells in peripheral tissues were proliferating ($Ki67^{+}$) and producing the proinflammatory cytokines IFN-γ and IL-17 in situ. Comparatively, the majority of antigen-presenting cells (APCs) in tissue in acute GVHD were donor derived, and donor-derived APCs were observed directly adjacent to host T cells. Furthermore, we demonstrated in a humanized mouse model that host skin-resident T cells could be activated by donor monocytes to generate a GVHD-like dermatitis.[50] It is equally interesting to conduct similar studies focusing on the tissue Tregs of the recipients.

Most current medications for GVHD have many systemic side effects, so Treg-based therapies are a potential therapeutic option. Patients awaiting solid organ transplantation, as well as others with autoimmune conditions and immune dysregulation disorders, will benefit from medications that induce long-term immune tolerance.

7.2 Conclusion

This thesis provides novel insights into the heterogeneity, function, and development of Treg cells, through the means of translational research in clinical studies. Regulatory T cells (Tregs) are the silent heroes within our healthy immune system. Treg cells are as pluripotent as they are complex. Tregs are able to suppress a range of effector cell types through several mechanisms, both direct and indirect, ensuring peripheral tolerance and immune homeostasis. The intricate balance between immune response and regulation can be upset by both a reduced number of Tregs as well as a decline in their functionality, potentially leading to autoimmune-related pathology. Transplants, such as hematopoietic stem cell transplantation, are strictly dependent on a balanced immune system reconstitution. When this careful balance is upset, as is the case all too often, the newly engrafted immune system can turn against its host and target the recipients' own tissues. This affliction is named graft-versus-host disease (GVHD), a severe immunopathological disease. Newer therapies for GVHD, aim to restore the immune system equilibrium and tolerance. The translational research methodology here developed can be reapplied in future clinical studies, aiming to unveil defects that contribute to other immune dysfunctions.

7.3 Future Directions

Up to today, there is not yet an approved and validated T cell therapy or tolerogenic vaccination to treat autoimmune diseases. Tolerogenic vaccine development has been problematic given the scarcity of known biomarkers, limited knowledge about most effective mechanisms of action and heterogeneity in human autoimmune disorder pathogenesis. Antigen-specific immunity, unlike general immune repression, prevents the specific autoantigen pathogenic immunological responses that cause autoimmune diseases while leaving the remaining immune system unaffected. Hence, antigen-specific immunity is the most promising next step in the treatment of autoimmune diseases and transplant rejection. Tolerogenic vaccines are getting popular as the the future of therapeutics.[51-55]

Three of the most pressing limitations compromising the success of antigen-specific immune tolerance are 1) the fact that most autoantigens related to a certain autoimmune disease are not yet identified, despite growing scientific knowledge.[56-58] Even harder when trying to identify the personalized autoantigen of a host prior transplantation. 2) we also do not know yet how many autoantigens are relevant in each specific autoimmune disease or if it varies per individual. 3) could we reach immune tolerance when reversing autoimmunity based on a single autoantigen or do we need pluripotent targeted strategies. As a result, treatments that promote not only antigen-specific but also tolerance towards specific organs are expected to be the most effective in clinical studies.

Immune tolerance requires regulatory T cells in order to maintain a healthy immune system. Many therapies with the intuition to manipulate the function or numbers of Tregs keep being studied and developed, hoping to address immune defects seen in many autoimmune diseases and during rejection after transplantation. Ex vivo and in vivo expansion techniques are designed to boost Treg suppressive capacity and cell numbers in order to suppress alloreactive effector T cells while maintaining graft-versus-tumor effect and preventing infections. Treg infusion will reduce GVHD while retaining GVT activity in some cancers, but not in others, according to preclinical research. Treg administration has been shown in preclinical studies to reduce GVHD while retaining GVT function only in some illnesses. Clinical studies of adoptive Treg treatment or in vivo Treg enhancement in patients after alloHSCT haven't yet revealed an elevated risk of malignant

disease relapse. Various methodologies are being used to try to figure out the optimal use of low-dose IL2 for maximal response, including the ideal dosing, regime schedule and duration of treatment. Novel formulations of IL-2 are now being explored to generate in vivo Treg expansion, with the intent of enhancing the efficiency of implementation. Two examples of new IL-2 formulations are AMG-592 (aldesleukin) and IgG-IL-2(N88D)2. IgG-IL-2(N88D)2 is a bivalent fusion protein between IgG and two human IL-2 molecules with aspartic acid (D) replaced by asparagine (N) at position 88. Aldesleukin is a IL-2 mutein developed to persist with higher half-life and greater selectively for Treg cells when comparing with previous recombinant IL-2.[59] In comparison studies using both humanized mice and cynomolgus monkeys IgG-IL-2(N88D)$_2$ showed greater Treg proliferation and induction in comparison to aldesleukin.[60] Aldesleukin was also outperformed by a newer mouse IL-2R (CD25) and fusion protein of mouse IL-2 at controlling autoimmunity in a nonobese diabetic mouse model and promoting Treg expansion.[61]

Adoptive Treg treatment still relies mainly on polyclonal Tregs with large specificities, possibly causing unintended consequence of immune suppression against opportunistic pathogens and malignant cells. Different approaches to adoptive Treg therapy are seeking to identify the proper dosing, duration and scheduling regimen for Treg administration. That being said, there are numerous technical and financial challenges that must be addressed before ex vivo Treg expansion can become a routine form of treatment. Much thinking still needs to be put on developing a method for manufacturing engineered Tregs at a fair cost that will serve to large numbers of patients. The most important move will be to produce sufficient quantities of pure, functional and stable regulatory T cells. Apart from using IL-2 for expansion of Treg cells, other Treg-specific surface receptors, such as components of the tumor necrosis factor receptor (TNFR) family, have been tested in mice models (in vivo) achieving successful Treg expansion.[62]

The holy grail in GVHD therapy, would be generation of antigen-specific regulatory T cells capable of encouraging immune tolerance while sustaining the graft-versus-tumor effect. With prolific development of engineering techniques to generate synthetic receptors, the chimeric antigen receptors (CARs), there is are several studies investigating

the Tregs with CARs capable of enhanced antigen-specific immunosuppression.[63.64] HLA A2-specific CAR-modified Tregs have been shown in preclinical GVHD model to sustain the expression of FoxP3, keeping their suppressive functions, while being even more efficient than polyclonal Tregs protecting from GVHD induced by HLA-$A2^+$ T cells.[65] If the pathways underlying GVHD and GVT are better understood, tissue-specific CAR-modified Tregs for GVHD treatment could lead to lower undesired off-target effects.

In summary, Treg therapies are well-tolerated, safe, which seems to be a promising option to continue exploring for the future. Both Treg adoptive treatments and in vivo Treg expansion are promising immune modulatory strategies with the intuit of preventing and treating GVHD. Ex vivo Treg expansion approaches and higher precision in Treg targeting can furthermore have a huge impact in treating autoimmune disorders and promote immune tolerance when transplanting solid organs.

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LIST OF PUBLICATIONS

Included in this Thesis

Published Peer - Reviewed articles

Maturation and phenotypic heterogeneity of human CD4+ regulatory T cells from birth to adulthood and after allogeneic stem cell transplantation.

Tiago R. Matos[1,2,3,4], Masahiro Hirakawa[1,2], Ana C. Alho[1,2,3], Lars Neleman[4], Luis Graca[3], Jerome Ritz[1,2]

[1]Division of Hematologic Malignancies and Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.

[2]Harvard Medical School, Boston, Massachusetts, USA.

[3]Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal.

[4]Amsterdam University Medical Centers, Department of Dermatology, University of Amsterdam, Amsterdam, Netherlands.

Published in Frontiers in Immunology, accepted November 2020.

Phase I/II Trial of a Combination of Anti-CD3/CD7 Immunotoxins for Steroid-Refractory Acute Graft-versus-Host Disease.

Christoph Groth[1], Lenneke F.J. van Groningen[2,3], Tiago R. Matos[4], Manita E. Bremmers[2], Frank W.M.B. Preijers[5], Harry Dolstra[5], Christian Reicherts[1], Nicolaas P.M. Schaap[2,3], Eric H.G. van Hooren[6], Joanna IntHout[3,7], Rosalinde Masereeuw[8], Mihai G. Netea[9], John E. Levine[10], George Morales[10], James L. Ferrara[10], Nicole M.A. Blijlevens[2,3], Ypke V.J.M. van Oosterhout[6], Matthias Stelljes[1], Walter J.F.M. van der Velden[2,3]

[1]Department of Medicine A/Hematology and Oncology, University Hospital of Muenster, Muenster, Germany.

[2]Department of Hematology, Radboud University Medical Center, Nijmegen, The Netherlands; Radboud Institute of Health Sciences, Radboud University Medical Center, Nijmegen, The Netherlands.

[3]Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

[4]*Department of Hematology, Radboud University Medical Center, Nijmegen, The Netherlands.* [5]*Department of Laboratory Medicine, Laboratory for Hematology, Radboud University Medical Center, Nijmegen, The Netherlands.*

[6]*Xenikos B.V., Nijmegen, The Netherlands.*

[7]*Radboud Institute of Health Sciences, Radboud University Medical Center, Nijmegen, The Netherlands; Section of Biostatistics, Department for Health Evidence, Radboud University Medical Center, Nijmegen, The Netherlands.*

[8]*Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands.*

[9]*Department of Internal Medicine, Radboud University Medical Center, Nijmegen, The Netherlands.* [10]*Tisch Cancer Institute, The Icahn School of Medicine at Mount Sinai Hospital, New York, NY.* [11]*Department of Hematology, Radboud University Medical Center, Nijmegen, The Netherlands; Radboud Institute of Health Sciences, Radboud University Medical Center, Nijmegen, The Netherlands.*

Published in Biology of Blood and Marrow Transplantation, November 2018.

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

Masahiro Hirakawa,[1,2] Tiago R. Matos,[1,2,3] Hongye Liu,[1,2] John Koreth,[1,2] Haesook T. Kim,[4,5] Nicole E. Paul,[1] Kazuyuki Murase,[1,2] Jennifer Whangbo,[1,2,6] Ana C. Alho,[1,2,3] Sarah Nikiforow,[1,2] Corey Cutler,[1,2] Vincent T. Ho,[1,2] Philippe Armand,[1,2] Edwin P. Alyea,[1,2] Joseph H. Antin,[1,2] Bruce R. Blazar,[7] Joao F. Lacerda,[3] Robert J. Soiffer,[1,2] and Jerome Ritz[1,2]

[1]*Division of Hematologic Malignancies and Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.* [2]*Harvard Medical School, Boston, Massachusetts, USA.* [3]*Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal.* [4]*Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.*

[5]*Harvard T. H. Chan School of Public Health, Boston, Massachusetts, USA.*

[6]*Division of Hematology/Oncology, Boston Children's Hospital, Boston, Massachusetts, USA.* [7]*Division of Blood and Marrow Transplantation, University of Minnesota, Minneapolis, Minnesota, USA.*

Published in Journal of Clinical Investigation Insight, November 2016, Volume 1, Number 18

Idelalisib given front-line for treatment of chronic lymphocytic leukemia causes frequent immune-mediated hepatotoxicity.

Benjamin L. Lampson[1], Siddha N. Kasar[1], Tiago R. Matos[1], Elizabeth A. Morgan[2], Laura Rassenti[3,4], Matthew S. Davids[1,4], David C. Fisher[1], Arnold S. Freedman[1], Caron A. Jacobson[1], Philippe Armand[1], Jeremy S. Abramson[5], Jon E. Arnason[6], Thomas J. Kipps[3,4], Joshua Fein[1], Stacey Fernandes[1,4], John Hanna[1], Jerome Ritz[1], Haesook T. Kim[7], and Jennifer R. Brown[1,4]

[1]Division of Hematologic Malignancies and Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA.

[2]Department of Pathology, Brigham and Women's Hospital, Boston, M.

[3]Division of Hematology/Oncology, Department of Medicine, University of California, San Diego, La Jolla, CA.

[4]CLL Research Consortium, Moores UCSD Cancer Center, La Jolla, CA.

[5]Center for Lymphoma, Massachusetts General Hospital Cancer Center, Boston, MA.

[6]Division of Hematology/Oncology, Beth Israel Deaconess Medical Center, Boston, MA.

[7]Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA

Published in Blood Journal, July 2016; 128(2): 195–203.

Other Publications during PhD

Published Peer - Reviewed

Labeling and tracking of immune cells in ex vivo human skin.

Feline E Dijkgraaf[1], Mireille Toebes[1], Mark Hoogenboezem[2], Marjolijn Mertz[3], David W Vredevoogd[1], Tiago R Matos[4], Marcel B M Teunissen[4], Rosalie M Luiten[4], Ton N Schumacher[5]

[1]Division of Molecular Oncology and Immunology, Oncode Institute, The Netherlands Cancer Institute, Amsterdam, the Netherlands.

[2]Research Facility, Sanquin Amsterdam, Amsterdam, the Netherlands.

[3]BioImaging Facility, The Netherlands Cancer Institute, Amsterdam, the Netherlands.

[4]Department of Dermatology and Netherlands Institute for Pigment Disorders, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam, the Netherlands.

[5]Division of Molecular Oncology and Immunology, Oncode Institute, The Netherlands Cancer Institute, Amsterdam, the Netherlands.

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Peripheral host T cells survive hematopoietic stem cell transplantation and promote graft-versus-host disease

Tiago R Matos[1], Sherrie J Divito[1], Anders T Aasebø[2], Pei-Chen Hsieh[1], Matthew Collin[3], Christopher P Elco[4], John T O'Malley[1], Espen S Bækkevold[2], Henrik Reims[5], Tobias Gedde-Dahl[6], Michael Hagerstrom[7], Jude Hilaire[7], John W Lian[1], Edgar L Milford[8], Geraldine S Pinkus[7], Vincent T Ho[9], Robert J Soiffer[9], Haesook T Kim[10], Martin C Mihm[1], Jerome Ritz[9], Indira Guleria[8], Corey S Cutler[9], Rachael A Clark[1], Frode L Jahnsen[2], Thomas S Kupper[1]

[1]*Department of Dermatology, Brigham and Women's Hospital, Boston, Massachusetts, USA.*

[2]*Department of Pathology, University of Oslo and Oslo University Hospital-Rikshospitalet, Oslo, Norway.*

[3]*Newcastle University, Translational and Clinical Research Institute, Newcastle upon Tyne, United Kingdom.*

[4]*Department of Pathology and Laboratory Medicine, Brown University, Providence, Rhode Island, USA.*

[5]*Department of Pathology, Oslo University Hospital-Rikshospitalet, Oslo, Norway.*

[6]*Department of Hematology, Institute of Clinical Medicine, University of Oslo and Oslo University Hospital-Rikshospitalet, Oslo, Norway.*

[7]*Department of Pathology and.*

[8]*Renal Transplant Program, Division of Renal Medicine, Brigham and Women's Hospital, Boston, Massachusetts, USA.*

[9]*Division of Hematological Malignancies and Stem Cell Transplantation and.*

[10]*Department of Data Sciences, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.*

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Is targeting circulating T blood cells a therapeutic option for vitiligo?

Tiago R. Matos[1]

[1]*Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherland.*

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Skin-resident memory T cells as a potential new therapeutic target in vitiligo and melanoma

Marcella Willemsen[1], Rugile Linkutė[1], Rosalie M Luiten[1], Tiago R Matos[1]

[1]Department of Dermatology and Netherlands Institute for Pigment Disorders, Amsterdam University Medical Centers, University of Amsterdam, Cancer Center Amsterdam, Amsterdam Infection & Immunity Institute, Amsterdam, the Netherlands.

Published in Pigment Cell Melanoma Research, September 2019; 32(5):612-622.

Tissue patrol by resident memory CD8 + T cells in human skin

Feline E Dijkgraaf[1], Tiago R Matos[2], Mark Hoogenboezem[3], Mireille Toebes[1], David W Vredevoogd[1], Marjolijn Mertz[4], Bram van den Broek[4], Ji-Ying Song[5], Marcel B M Teunissen[2], Rosalie M Luiten[2], Joost B Beltman[6], Ton N Schumacher[7]

[1]Division of Molecular Oncology and Immunology, Oncode Institute, The Netherlands Cancer Institute, Amsterdam, the Netherlands.

[2]Department of Dermatology and Netherlands Institute for Pigment Disorders, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam, the Netherlands.

[3]Research Facility, Sanquin Amsterdam, Amsterdam, the Netherlands.

[4]BioImaging Facility, The Netherlands Cancer Institute, Amsterdam, the Netherlands.

[5]Animal Pathology, The Netherlands Cancer Institute, Amsterdam, the Netherlands.

[6]Division of Drug Discovery and Safety, Leiden Academic Centre for Drug Research, Leiden University, Leiden, the Netherlands.

[7]Division of Molecular Oncology and Immunology, Oncode Institute, The Netherlands Cancer Institute, Amsterdam, the Netherlands.

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A primary role for human central memory cells in tissue immunosurveillance

Ahmed Gehad[1], Jessica E. Teague[1], Tiago R. Matos[1,2,3], Victor Huang[1], Chao Yang[1], Rei Watanabe[1], John T. O'Malley[1], Cornelia L. Trimble[4], Thomas S. Kupper[1], and Rachael A. Clark[1]

[1]Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA;

[2]Instituto de Medicina Molecular, Faculty of Medicine, University of Lisbon, Lisbon, Portugal;

[3]Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; and

[4]Department of Gynecology and Obstetrics, Johns Hopkins Medical Institutions, Baltimore;

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The Challenge of Effective Communication among Scientists.

Tiago R. Matos[1]

[1]*Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherland.*

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Clinically resolved psoriatic lesions contain psoriasis-specific IL-17-producing αβ T cell clones.

Tiago R. Matos[1,2,3], John T. O'Malley[1], Elizabeth L. Lowry[1], David Hamm[4], Ilan R. Kirsch[4], Harlan S. Robins[4], Thomas S. Kupper[1], James G. Krueger[5], and Rachael A. Clark[1]

[1]*Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA.*

[2]*Instituto de Medicina Molecular, Faculty of Medicine, University of Lisbon, Lisbon, Portugal.*

[3]*Academic Medical Center, Department of Dermatology, University of Amsterdam, Amsterdam, The Netherlands.*

[4]*Adaptive Biotechnologies, Seattle, Washington, USA.*

[5]*Department of Dermatology, Rockefeller University, New York, New York, USA.*

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Recurrence Rates Over 20 Years in the Treatment of Malignant Melanoma: Immediate Versus Delayed Reconstruction.

Pieter G.L. Koolen[1], Tiago R. Matos[2], Ahmed M.S. Ibrahim[1], Jie Sun[3], Bernard T. Lee[1], Robert A. Frankenthaler[4], and Samuel J. Lin[4]

[1]*Division of Plastic Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Mas.*

[2]*Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, Boston, Mass.*

[3]*Harvard School of Dental Medicine, Boston, Mass.*

[4]*Division of Otolaryngology-Head and Neck Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Mass.*

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Research Techniques Made Simple: High-Throughput Sequencing of the T-Cell Receptor

Tiago R. Matos[1], Menno A. de Rie[1], and Marcel B. M. Teunissen[1]

[1]*Department of Dermatology, Academic Medical Center, University of Amsterdam*

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Research Techniques Made Simple: Mass Cytometry Analysis Tools for Decrypting the Complexity of Biological Systems

Tiago R. Matos[1,2,3], Hongye Liu[1,2], Jerome Ritz[1,2]

[1]*Division of Hematologic Malignancies, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.*

[2]*Harvard Medical School, Boston, Massachusetts, USA.*

[3]*Academic Medical Center, Department of Dermatology, University of Amsterdam, Amsterdam, The Netherlands.*

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Research Techniques Made Simple: Experimental Methodology for Single-Cell Mass Cytometry.

Tiago R. Matos[1,2,3], Hongye Liu[1,2], Jerome Ritz[1,2]

[1]*Division of Hematologic Malignancies, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.*

[2]*Harvard Medical School, Boston, Massachusetts, USA.*

[3]*Academic Medical Center, Department of Dermatology, University of Amsterdam, Amsterdam, The Netherlands.*

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Discovery of skin lymphocytes was a game changer in experimental dermatology.

Tiago R. Matos[1,2,3], Menno A. de Rie[3]

[1]*Division of Hematologic Malignancies, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.*

[2]*Harvard Medical School, Boston, Massachusetts, USA.*

[3]*Academic Medical Center, Department of Dermatology, University of Amsterdam, Amsterdam, The Netherlands.*

Published in Experimental Dermatology. August 2017; 26(8):683-684.

Ultraviolet B radiation therapy for psoriasis: Pursuing the optimal regime.

Tiago R. Matos[1,2], Tsui C. Ling[3], Vaneeta Sheth[1]

[1]Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, Boston,

[2]Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

[3] Phototherapy and Photobiology Unit, Dermatology Centre, Salford Royal Hospital, Manchester, UK.

Published in Clinics in Dermatology, September-October 2016. 34(5):563-70.

The dark side of the light: mechanisms of photocarcinogenesis.

Margarida Moura Valejo Coelho[1], Tiago R. Matos[2,3], Margarida Apetato[1]

[1]Department of Dermatology and Venereology, Centro Hospitalar de Lisboa Central, Lisbon, Portugal

[2]Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, Boston.

[3]Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

Published in Clinics in Dermatology, September-October 2016. 34(5):563-70.

The symbiosis of phototherapy and photoimmunology.

Tiago R. Matos[1,2], Vaneeta Sheth[1]

[1]Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, Boston.

[2]Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

Published in Clinics in Dermatology, September-October 2016. 34(5):563-70.

Novel applications of Phototherapy.

Tiago R. Matos[1,2], Vaneeta Sheth[1]

[1]Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, Boston,

[2]Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Published in Clinics in Dermatology, September-October 2016. 34(5):563-70.

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]

[1]Division of Hematologic Malignancies, Dana-Farber Cancer Institute, Boston, MA, USA.

[2]Harvard Medical School, Boston, MA, USA.

[3]Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal.

[4]Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA, USA.

[5]Cancer Vaccine Center, Dana-Farber Cancer Institute, Boston, MA, USA.

[6]Harvard School of Public Health, Boston, MA, USA.

Published in Blood Journal, February 2016, Volume 127, Number 5, 646-57.

Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells.

Rei Watanabe[1], Ahmed Gehad[1], Chao Yang[1], Laura Campbell[1], Jessica E. Teague[1], Christoph Schlapbach[2], Christopher Elco[3], Victor Huang[1], Tiago R. Matos[1,4], Thomas S. Kupper[1,5], and Rachael A. Clark[1,5]

[1]Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.

[2]Department of Dermatology, Inselspital, University of Bern, Bern, Switzerland.

[3]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.

[4]Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal.

[5]Dana-Farber/Brigham and Women's Cancer Center, Boston, Massachusetts.

Published in Science Translational Medicine, September 2015, 7(279): 279ra39.

Communications

Oral presentation

Idelalisib Given Front-line for the Treatment of Chronic Lymphocytic Leukemia (CLL) Results in Frequent and Severe Immune-Mediated Toxicities

Benjamin L. Lampson[1], Tiago R. Matos[1], Haesook T. Kim[2], Siddha N. Kasar[1], Elizabeth A. Morgan[3], Masahiro Hirakawa[1], Joshua Fein[1], Stacey Fernandes[1], Jerome Ritz[1], and Jennifer R. Brown[1]

[1]Division of Hematologic Malignancies and Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA; [2]Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA; [3]Department of Pathology, Brigham and Women's Hospital, Boston, MA; [3]Division of Hematology/Oncology, Department of Medicine, University of California, San Diego, La Jolla, CA;

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

IL-2 Induces Selective Activation of Helios-Positive Regulatory T Cells and CD56bright NK Cells *in Vitro* and in Patients with Chronic Gvhd Receiving Low-Dose IL-2 Therapy

Masahiro Hirakawa, Tiago R Matos, John Koreth, Edouard Forcade, Jennifer Whangbo, Kazuyuki Murase, Hongye Liu, Sarah Nikiforow, Vincent T Ho, Corey S Cutler, MPH1, Philippe Armand, Edwin P. Alyea, Joseph H. Antin, Robert J. Soiffer, and Jerome Ritz

[1]Division of Hematologic Malignancies and Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA

[2]Division of Hematologic Malignancies, Dana-Farber Cancer Institute, Boston, MA

[3]Department of Medical Oncology and Hematology, Sapporo Medical University School of Medicine, Sapporo, Japan

[4]Dana-Farber Cancer Institute, Boston, MA

[5]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA

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

A phase I/II study on the anti-CD3/CD7 immunotoxin combination (T-Guard) for the treatment of steroid-refractory acute GVHD

Christoph Groth[1*], Lenneke F.J. van Groningen[2,3*#], Manita E. Bremmers[2], Frank W.M.B. Preijers[2,4], Harry Dolstra[2,4], Tiago R. Matos[5], Eric G. van Hooren[6], Ypke V.J.M. van Oosterhout[6], John E. Levine[7], James L. Ferrara[7], Nicole M.A. Blijlevens[2,3], Matthias Stelljes[1*], and Walter J.F.M. van der Velden[2,3*]

**Contributed equally. [1]Department of Medicine A/Hematology and Oncology, University Hospital of Muenster, Muenster, Germany; [2]Department of Hematology, Radboud University Medical Center, Nijmegen, the Netherlands; [3]Radboud Institute of Health Sciences, Radboud University Medical Center, Nijmegen, the Netherlands; [4]Department of Laboratory Medicine, Laboratory of Hematology, Radboud University Medical Center, Nijmegen, the Netherlands; [5]Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.; [6]Xenikos B.V., Nijmegen, the Netherlands; [7]Blood & Marrow Transplant Program, University of Michigan, Ann Arbor, MI, USA; The Tisch Cancer Institute, The Icahn School of Medicine at Mount Sinai Hospital, New York, NY, USA.*

59th ASH Annual Meeting & Exposition, Orlando, USA. Blood 2017; December 7, 2017.

Development and phenotypes of Regulatory T cells; from birth to adulthood and after Allogeneic Hematopoietic Stem Cell transplant.

Tiago R Matos[1,2], Hongye Liu[1], Masahiro Hirakawa[1], Ana Cristina Alho[1,2,3] and Jerome Ritz[1]

[1]Division of Hematologic Malignancies, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA. [2]Faculdade de Medicina da Universidade de Lisboa, Instituto de Medicina Molecular, Lisbon, Portugal [3]Intituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal

Hematologic & Immunologic Therapies Seminar, DFCI, USA; March 2015.

Poster presentations

Mass Cytometry Identifies T-Cell Populations Associated with Severe Hepatotoxicity in CLL Patients on Upfront Idelalisib

Alexander R. Vartanov[1], Tiago R. Matos[1,4], Emily M. McWilliams[1,2], Deepti Gadi[1,2], Deepak A. Rao[3], Siddha Kasar[1,2], Benjamin L. Lampson[1], Stacey M. Fernandes[1], Jerome Ritz[1], Jennifer R. Brown[1,2,5]

[1] *Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA;* [2] *Cancer Program, Broad Institute of MIT and Harvard, Cambridge, MA;* [3] *Division of Rheumatology, Immunology, and Allergy, Brigham and Women's Hospital and Harvard Medical School, Boston, MA;* [4] *Faculdade de Medicina da Universidade de Lisboa, Instituto de Medicina Molecular, Lisbon, Portugal;* [5] *Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*

60th ASH Annual Meeting & Exposition, San Diego, USA. Blood 2018; December 3, 2018.

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

Tiago R Matos[1,2], Hongye Liu[1], Masahiro Hirakawa[1], Ana Cristina Alho[1,2,3] and Jerome Ritz[1]

[1]*Division of Hematologic Malignancies, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA.* [2]*Faculdade de Medicina da Universidade de Lisboa, Instituto de Medicina Molecular, Lisbon, Portugal* [3]*Intituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal*

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

Altered Expression of Functional Proteins in CD4 Regulatory T cells During Therapy with Idelalisib

Tiago R. Matos[1], Benjamin L. Lampson[1], Masahiro Hirakawa[1], Siddha N. Kasar[1], Laura Rassenti[2], Thomas J. Kipps[2], Stacey Fernandes[1], Jennifer R. Brown[1] and Jerome Ritz[1]

[1]Division of Hematologic Malignancies and Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA; [2]Division of Hematology/Oncology, Department of Medicine, University of California, San Diego, La Jolla, CA;

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

IL-2, IL-7, IL-15 and IL-6 Induce Differential Activation of Naive and Memory T Cell Subsets

Masahiro Hirakawa, Tiago R Matos, Edouard Forcade, Kathy S. Wang, Eduardo L Espada, Jennifer Whangbo, Carol G Reynolds, Marie J Chammas, Kazuyuki Murase, and Jerome Ritz

[1]Division of Hematologic Malignancies and Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA

[2]Division of Hematologic Malignancies, Dana-Farber Cancer Institute, Boston, MA

[3]Department of Medical Oncology and Hematology, Sapporo Medical University School of Medicine, Sapporo, Japan

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

Awards & Honors

- ESDR Travel Award - Lilly Scholarship. September 2019
- 1st Prize Entrepreneurship in Health and Life Sciences. ACE, IXA, AMC & VUmc. June 2019
- Amsterdam Infection & Immunity Institute Travel Award. May 2018
- Nominated for International Resident Retreat at the IID. SID, ESDR, JSID. April 2018
- International Investigative Dermatology (IID) Travel Award. April 2018
- Nominated for Academy of Future Leaders in Dermatology by ESDR. November 2017
- Best oral presentation. The Amsterdam Infection & Immunity Retreat, Netherlands. October 2017
- Featured at the Meet the Investigator Series of Journal of Investigative Dermatology. July 2017
- Best oral Presentation. 18th Annual NVED Scientific Meeting, Netherlands. February 2017
- ESDR/SID Collegiality Award, USA. May 2016
- European Society for Dermatological Research Poster Prize winner, Netherlands. September 2015
- European Society for Dermatological Research Travel Award, Netherlands. September 2015
- Dana-Faber Cancer Institute Travel Award, USA. June 2015
- Society of Investigative Dermatology Travel Award, USA. May 2015
- Nominated for the Society of Investigative Dermatology Ph.D. retreat for future investigators, USA. May 2015
- Brigham and Woman's Hospital Travel Award, USA. April 2015
- Nominated for the Roche Continents Program, Austria. June 2014