Universidade de Lisboa

Faculdade de Medicina

The role of IL-7 in the Homeostasis of Human Naive and Memory $CD4^+$ T cell subsets

Rita Isabel Silva de Azevedo

Doutoramento em Ciências Biomédicas
Especialidade em Imunologia

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Tese orientada pela Doutora Maria V. D. Soares

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A impressão desta dissertação foi aprovada pela Comissão Coordenadora do Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 18 de Janeiro de 2011.

Dissertação apresentada à Faculdade de Medicina da Universidade de Lisboa, para obtenção do grau de Doutor em Ciências Biomédicas.

A presente dissertação foi realizada na Unidade de Imunologia Clínica do Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa e Division of Infection & Immunity, University College London.

O trabalho aqui apresentado foi realizado com âmbito nos projectos PPCDT/BIA-BCM/61079/2004 e PTDC/SAU-MII/67662/2006, co-financiados pelo fundo comunitário FEDER através dos programas POCI 2010 e PTDC. Bolsa de Doutoramento da Fundação para a Ciência e a Tecnologia (referência: SFRH/ BD/ 29120/ 2006).

Para os meus pais

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ACKNOWLEDGEMENTS

I would like to start by acknowledging my supervisor Doutora Maria Soares for all her support and helpful discussions not only during the writing of this thesis but also throughout the six years that we have worked together. Despite all the peaks and valleys, I want to thank Maria for always believing in me.

I wish to thank Professor Arne Akbar for allowing me to develop part of my PhD project in his lab in the Division of Infection & Immunity, University College London, and for being incredibly supportive and enthusiastic about my work. I will be forever grateful to Arne for making me feel truly welcome in his group and for bestowing on me such a wonderful opportunity to work (and play) in London.

I also have to acknowledge Professora Doutora Ana Espada de Sousa and Professor Doutor Rui Victorino for giving me the opportunity to perform my PhD project in Unidade de Imunologia Clínica, Instituto de Medicina Molecular.

I want to thank Professor Doutor João Lacerda from Serviço de Hematologia, Hospital de Santa Maria, for allowing me to participate in a very interesting project that gave me a new perspective on research and for always showing support and trust in my work.

I must say a big thank you to my colleagues and true friends from Unidade de Imunologia Clínica who took me under their wing, teaching me all the lab techniques that were to be the foundations of my future work and, more importantly, personal lessons that will stay with me throughout my life: Adriana Albuquerque showed me how to juggle hard work and skilfulness, with endless generosity and kindness; Rita Cavaleiro taught me to stand up for myself and for what I think is right, and I'll always be thankful for that; Russell Foxall selflessly shared his time and endless immunological wisdom… and he also starred in the most hilarious moments I have ever witnessed in a lab; Doutora Alcinda Melo always managed to put a smile on my face with her positive outlook on life and all its mishaps. I especially want to thank Rita Tendeiro, not only for being my trusted extra pair of hands during TREC quantification and the final experiments I performed in Lisbon, but most importantly for being a very close and dear friend, i.e. *a minh'ámiga* ☺. I also must thank Ana Luísa Caetano from Unidade de Citometria de Fluxo for performing all the FACS sortings during my time at Instituto de Medicina

Molecular. Ana Luísa went far beyond the call of duty, working til the wee hours of the morning if necessary, to help me out and I'll always cherish her friendship.

Thank you all for making it worth my while to get into work every day, even when the going got really tough indeed.

I wish to acknowledge my surrogate supervisors at the UCL and *cari amici*, Valentina Libri and Diletta di Mitri, for their support, companionship and all the lovely dinners they hosted. They both taught me a lot in the lab and were instrumental in making London feel like home. I must acknowledge Toni for making life in the lab run smoothly through her great organizational skills and generous helpfulness. But most importantly, I want to thank Toni for basically being Toni, a kind-hearted friend who features in my fondest and funnest memories of London. I also wish to thank Elaine, Nicky, Daisy and Simran for their friendly faces and kind words that really helped warm the cockles of my heart in cold London.

I wish to acknowledge Dário Ligeiro from Immunogenetics Laboratory, Centro de Histocompatibilidade do Sul, for performing the spectratyping analysis, and Rui Soares from Unidade de Imunologia Clínica for technical support during TREC quantification.

I want to acknowledge Doutora Helena Ferreira from Hospital Universitário de Santa Maria, Lisboa, for providing the umbilical cord blood samples. I also acknowledge Rémy Cheynier from Institute Pasteur, Paris for providing the pCD3-TREC plasmid, and Professor David Kipling from University of Wales College of Medicine for supplying BIRB796 aliquots.

I acknowledge Fundação para a Ciência e a Tecnologia for the financial support through the PhD Scholarship (SFRH/ BD/ 29120/ 2006), co-financed by POCI 2010 and Fundo Social Europeu.

Last but definitely not least, I would like to thank my friends and family for all their love and endless patience to put up with the crazy hours, weariness and quite frankly grumpiness that came with the territory of surviving this PhD. Most especially of all, I want to thank my parents for their unwavering love and support throughout my life, always motivating me to do my best.

Obrigada por tudo, Mãe e Pai, esta tese é para vocês porque sei que o meu sucesso passado, presente e futuro se deve ao vosso amor e apoio incondicionais.

ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
γ_C	Common γ Chain
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen-Presenting Cell
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3-related Protein
BSA	Bovine Serum Albumin
c-ART	Combination Anti-retroviral Therapy
CCR	CC Chemokine Receptor
CD	Cluster of Differentiation
CDR3	Complementarity-Determining Region 3
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CMV	Cytomegalovirus
Con A	Concanavalin A
DDR	DNA Damage Response
DLI	Donor Lymphocyte Infusion
DMSO	Dimethyl Sulfoxide
DSB	Double Strand Break
EBV	Epstein-Barr Virus
Eomes	Eomesodermin
ERK	Extracellular Signal Regulated Kinase
FACS	Fluorescence-Activated Cell Sorting
FOXO3a	Forkhead Box Protein O3a

GH	Growth Hormone
GVHD	Graft-Versus-Host Disease
GVL	Graft-Versus-Leukemia
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSCT	Haematopoietic Stem Cell Transplantation
hTERT	Human Telomerase Reverse Transcriptase
IFN	Interferon
IGF-1	Insulin-like Growth Factor-1
IL	Interleukin
IL-7Rα	IL-7 receptor α chain
IR	Ionizing Radiation
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
KIR	Killer Immunoglobulin-like Receptor
KLRG1	Killer cell Lectin-like Receptor sub-family G member 1
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MEK	Mitogen-Activated Protein Kinase
MFI	Median Fluorescence Intensity
MKK	Mitogen-Activated Protein Kinase Kinase
NK	Natural Killer Cell
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1

PI	Propidium Iodide
PI3K	Phosphoinositide 3-Kinase
PKB	Protein Kinase B
PKCθ	Protein Kinase C θ
rh	Recombinant Human
RTE	Recent Thymic Emigrant
SDF	Senescence-associated DNA-damage Foci
Ser	Serine
SIV	Simian Immunodeficiency Virus
sj	Signal-Joint
STAT	Signal Transducers and Activators of Transcription
TCR	T Cell Receptor
TERC	Telomerase RNA Component
TGF	Transforming Growth Factor
Thr	Threonine
TNF	Tumor Necrosis Factor
TR	Telomerase RNA
TREC	T cell Receptor Excision Circle
Tyr	Tyrosine
VZV	*Varicella zoster* virus

RESUMO

O principal objectivo deste trabalho é o estudo da homeostasia de linfócitos T $CD4^+$ *naive* e de memória em humanos, com ênfase particular no papel desempenhado pela IL-7 neste processo. Para tal, investigámos os efeitos desta citocina na homeostasia de subpopulações de linfócitos T $CD4^+$ *naive* identificados pela expressão de CD31. Demonstramos pela primeira vez que a IL-7 induz a proliferação preferencial da subpopulação $CD31^+$ de linfócitos T $CD4^+$ *naive* do sangue periférico de adultos. Além disso, a IL-7 promove a manutenção ou mesmo o aumento dos níveis de CD31 em células T $CD4^+$ *naive* $CD31^+$, apesar de não induzir a re-expressão deste marcador na subpopulação $CD31^-$. Os nossos resultados indicam que tanto a proliferação como a manutenção de CD31 induzidas pela IL-7 são dependentes da via de sinalização PI3K.

Neste estudo, também investigámos quais os potenciais mecanismos responsáveis pelo restabelecimento da homeostasia após transplante haploidêntico de células estaminais, particularmente pela manutenção da subpopulação T $CD4^+$ *naive* $CD31^+$. Os nossos dados sugerem que a reconstituição imunológica a longo prazo foi atingida com sucesso num grupo de receptores de transplante haploidêntico, provavelmente através de uma combinação de mecanismos dependentes e independentes do timo, levando ao estabelecimento de subpopulações equilibradas de linfócitos T $CD4^+$ e $CD8^+$, bem como a um repertório de células T diverso.

Por fim, o estudo da homeostasia dos linfócitos T $CD4^+$ de memória teve como base a investigação do potencial impacto da acumulação de linfócitos T $CD4^+$ $CD45RA^+CD27^-$ que se observa durante a infecção por CMV. Analisámos a capacidade replicativa e funcional destas células altamente diferenciadas, assim como o putativo envolvimento da IL-7 na re-expressão de CD45RA em linfócitos T $CD4^+$ de memória. Os nossos resultados demonstram que os linfócitos T $CD4^+$ $CD45RA^+CD27^-$ não constituem uma subpopulação exausta, mantendo potencial replicativo e funcional. No entanto, estas células apresentam características de senescência independentes do comprimento dos telómeros, mediadas parcialmente pela via de sinalização p38 MAPK.

Globalmente, os nossos dados reiteram a contribuição da IL-7 para a homeostasia de linfócitos T $CD4^+$ *naive* e de memória, sugerindo um potencial envolvimento na

manutenção da população T $CD4^+$ *naive* $CD31^+$ em adultos e na indução da expressão de CD45RA em linfócitos T $CD4^+$ de memória.

Palavras-chave: Homeostasia, Interleucina-7, Linfócitos T $CD4^+$, Reconstituição imunológica, Senescência.

SUMMARY

The main focus of this work is to study the homeostasis of human naive and memory $CD4^+$ T cell subsets, particularly assessing the role of IL-7 in this process. For this purpose, we assessed the potentially distinct effects of IL-7 in the homeostasis of naive $CD4^+$ T cell subsets defined by CD31 expression. We describe for the first time the preferential proliferation of the $CD31^+$ subset within adult naive $CD4^+$ T cells in response to IL-7 stimulation. Furthermore, we showed that IL-7-induced proliferation sustained or even increased the level of CD31 expression in $CD31^+$ naive $CD4^+$ T cells, although it did not induce CD31 re-expression in the $CD31^-$ subset. We also demonstrated that both IL-7-induced proliferation and CD31 maintenance were dependent on the PI3K pathway.

Furthermore, we investigated the mechanisms involved in the restoration of T cell homeostasis following haploidentical haematopoietic stem cell transplantation (HSCT), particularly in the maintenance of the $CD31^+$ naive $CD4^+$ T cell pool. Our data suggest that long term immune reconstitution was successfully achieved in a cohort of haploidentical HSCT recipients, likely through a combination of thymus-dependent and -independent mechanisms which gave rise to balanced $CD4^+$ and $CD8^+$ T cell subsets and to a diverse T cell repertoire.

Finally, we focused on memory $CD4^+$ T cell homeostasis in order to clarify the impact of the increasing representation of $CD45RA^+CD27^-$ $CD4^+$ T cells observed during CMV infection. We sought to determine the replicative and functional potential of these highly differentiated cells, as well as the putative involvement of IL-7 in CD45RA re-expression in memory $CD4^+$ T cells. Our results show that $CD45RA^+CD27^-$ $CD4^+$ T cells do not constitute an exhausted subset, retaining replicative and functional potential. However, these cells display senescence-associated traits independent of telomere length, which are at least partly mediated by the p38 MAPK pathway.

Overall, our data reiterates the contribution of IL-7 signalling to naive and memory $CD4^+$ T cell homeostasis, suggesting a role for IL-7 in the maintenance of the $CD31^+$ naive T cell pool throughout adulthood as well as in the induction of CD45RA on memory $CD4^+$ T cells.

Keywords: Homeostasis, Interleukin-7, $CD4^+$ T Lymphocytes, Immune reconstitution, Senescence.

INTRODUCTION

1. Interleukin-7: a key cytokine in T cell homeostasis

1.1. The role of γ_C cytokines in T cell homeostasis

Homeostasis can be defined as the tendency of a system to maintain internal stability through coordinated responses that compensate for environmental changes, allowing the return to a steady-state following perturbation [1]. Although the aim of homeostasis is to achieve equilibrium, its nature is not static but rather dynamic, ensuring stability by continually adjusting to changing conditions [1]. The homeostasis of the immune system operates through a tightly regulated network of sensing and feedback mechanisms that counteract disturbances in order to restore steady-state settings [2]. T cell homeostasis ensures the maintenance of the size and diversity of the T cell pool [3,4]. A typical example is the preservation of relatively constant peripheral T cell numbers in the face of constant antigenic challenge, which is achieved by counterbalancing the proliferation of antigen-specific cells with the contraction of the expanded population during an immune response [5-7]. Likewise, drastic reductions of peripheral T cell numbers, as observed following chemotherapy [8], bone-marrow transplantation [9] and human immunodeficiency virus (HIV) infection [10], exaggerate the response to mechanisms responsible for naive T cell homeostasis under steady-state conditions, i.e. cytokines, in order to restore the size of the T cell pool through lymphopenia-induced proliferation [11]. However certain challenges to T cell homeostasis ultimately prove too disruptive to allow the return to a steady-state. For example, transformed T cells are able to circumvent cell cycle checkpoints and consequently undergo uncontrolled proliferation which cannot be counteracted by homeostatic feedback mechanisms [2]. Several therapeutic approaches have been developed to help restore immune competence following T cell depletion caused by a variety of immune disorders as well as by tumour therapy regimens and following allogeneic stem cell transplantation [12]. Interleukin-7 (IL-7) is a cytokine from the common γ chain (γ_C) family with potential application as a therapeutic approach in a multitude of clinical settings associated with T cell deficiency, such as ageing, HIV infection and following

radio- or chemotherapy utilized in the treatment of tumours or as part of a conditioning regimen for hematopoietic stem cell transplantation (HSCT) [13-17].

T cell homeostasis relies mainly on signals triggered by self-MHC/peptide complexes and members of the γ_C family of cytokines [1]. The γ_C family encompasses cytokines, such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, whose receptor complexes share the γ_C chain (CD132), in addition to various cytokine-specific chain(s) [18]. IL-7 and IL-15 are the key cytokines involved in the maintenance of T cell homeostasis [1]. The homeostasis of naive T cells is dependent on T cell receptor (TCR) interaction with self-MHC/peptide complexes plus IL-7 signalling [19-23]. *In vivo* studies in mice have demonstrated that both $CD4^+$ and $CD8^+$ naive T cells require IL-7 for survival and homeostatic proliferation [19,20,24-26], while *in vitro* studies have shown that IL-7 alone is able to promote the survival and proliferation of human $CD4^+$ naive T cells from umbilical cord blood [27-30]. As for memory T cells, $CD4^+$ and $CD8^+$ T cells are similarly independent of TCR tickling [31,32] but they appear to have distinct γ_C cytokine requirements: both IL-7 and IL-15 are reportedly involved in memory CD8 homeostasis [33-36], whereas IL-7 is considered critical for the generation and maintenance of memory $CD4^+$ T cells [37-42]. Thus, several lines of evidence point to IL-7 as a key cytokine in the maintenance and restoration of naive and memory T cell homeostasis [28-30].

1.2. IL-7 Receptor signalling in T cells

IL-7 was initially described as a growth factor for murine B cell precursors in a bone marrow culture system [43]. It has since been described as a non-redundant cytokine in the development of T cells in mice and humans [44-47], as well as being essential for the survival and proliferation of naive and memory T cells in the periphery [35,48-50]. Several studies have also demonstrated that IL-7 is a homeostatic cytokine able to promote memory $CD4^+$ and $CD8^+$ T cell generation [37,38,51,52]. The presence of IL-7 during culture of tumour-specific $CD8^+$ T cell clones has been shown to promote long-term survival whilst progressively quenching cytotoxic responses, suggesting that IL-7 may play a role in memory induction by supporting the transition from an activated to a resting state [53].

IL-7 is mainly produced by non-lymphoid cells within lymphoid tissues, such as stromal cells in the bone marrow and lymph nodes, and epithelial cells in the thymus and gut [7,43,54-56]. IL-7 production appears to occur in a constitutive fashion without influence

from extrinsic stimuli [25,45]. On the other hand, the production of other γ_C cytokines such as IL-2 and IL-15 increases greatly with the activation of T cells, macrophages and dendritic cells during an immune response [57,58]. The response to IL-7 appears to be tightly regulated by the expression of the α chain of the IL-7 receptor (IL-7Rα, CD127). The expression of IL-7Rα on lymphocytes fluctuates according to the stage of development and/or activation [58]. IL-7 itself [59,60], other γ_C cytokines [59,61] and TCR activation [19,60,62] induce the down-modulation of IL-7Rα expression. Conversely, IL-7Rα is up-regulated in the absence of its cognate cytokine [59,60]. Hence, unlike the receptor chains specific for IL-2 and IL-15, which are up-regulated following activation [63-66], IL-7Rα expression appears to be transiently dampened upon triggering of signalling pathways that promote cell survival. This feedback regulatory mechanism has been suggested to maximise the number of cells that can make use of the limited amount of IL-7 available [59]. However, the *in vitro* survival and proliferation of human naive CD4$^+$ T cells in response to limiting amounts of IL-7 appear to be independent of IL-7Rα expression levels [60], which argues against the *in vivo* "altruistic" model proposed by Park *et al.* [59].

IL-7 stimulation induces several pro-survival pathways, particularly through the modulation of the expression of Bcl-2 family members [67-69], in addition to promoting cell proliferation, growth and metabolic activity [62,70-73]. IL-7 signalling is triggered by ligation of IL-7 to IL-7Rα, inducing the hetero-dimerisation of IL-7Rα with the γ_C chain [74] and consequent activation of the receptor-associated Janus kinases (JAK) -1 and -3 [75]. JAK1 and JAK3, which are respectively associated with the γ_C chain and IL-7R-α, phosphorylate each other and then IL-7Rα, creating docking sites for the signal transducers and activators of transcription (STAT) factors, such as STAT1, -3 and -5 [76-78]. STAT5, the most relevant STAT in IL-7-induced signalling, comprises two isoforms: STAT5a and STAT5b [79]. Both STAT5 isoforms are then phosphorylated by JAK1/3, inducing their homo- or hetero-dimerisation and translocation to the nucleus where they activate the expression of genes involved in cell survival and proliferation [80-84]. The STAT5 signalling pathway promotes cell survival through the modulation of Bcl-2 family members, up-regulating the expression of anti-apoptotic proteins Bcl-2 and Bcl-xL and down-regulating the pro-apoptotic proteins Bax and Bad [85-87]. STAT5 signalling also leads to the inhibition of protein kinase C θ (PKCθ) and subsequently to the down-modulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$, inducing cell cycle entry [88].

Another major pathway induced by IL-7 is the phosphoinositide 3-kinase (PI3K) signalling pathway which plays a key role in regulating cell survival, growth, metabolism and proliferation [89]. The major substrate of PI3K is Akt, a serine/threonine kinase, also known as protein kinase B (PKB) [90]. Activation of PI3K by growth factors or cytokines induces the recruitment of Akt to the plasma membrane where it is phosphorylated on two residues, Thr308 and Ser473, becoming fully activated [91]. The substrates of Akt include several molecules that directly or indirectly impact on cell survival and proliferation, such as pro- and anti-apoptotic Bcl-2 family members, caspases and forkhead transcription factors [92]. Through its targets in the Bcl-2 family, Akt protects mitochondrial membrane integrity and thus prevents the release of factors such as cytochrome c which can trigger apoptosis in response to stress [93]. The phosphorylation of Bax by Akt induces conformational changes that hinder the translocation of Bax to the mitochondrial membrane, blocking pore formation and consequent cytochrome c release [94]. Akt directly phosphorylates the pro-apoptotic protein Bad [95], forcing the dissociation of Bad from Bcl-2 complexes, releasing the latter free to perform its anti-apoptotic functions [96,97]. Akt promotes $CD4^+$ cell survival in part by phosphorylating, and consequently inactivating, the forkhead transcription factor FOXO3a, which leads to the down-regulation of the pro-apoptotic protein Bim [98]. The PI3K pathway is also required for the IL-7-induced increase of GLUT1 expression, a key glucose transporter in T cells, thus promoting glucose uptake and metabolic activity [71,99]. In addition, IL-7 also up-regulates the transferrin receptor CD71 [80,99], the major mediator of iron uptake associated with increased metabolic activity [100-102]. Hence, IL-7 is a pleiotropic cytokine that promotes T cell survival, proliferation, growth and metabolism.

1.3. Therapeutic applications of IL-7

IL-7 has been suggested as a potential therapeutic agent in a variety of settings, particularly in the improvement of immune reconstitution following T cell depletion [25]. In pre-clinical studies performed both in mice [103-107] and in non-human primates [108], IL-7 administration has been shown to accelerate the rate of immune reconstitution following bone-marrow transplantation [103-108]. Furthermore, IL-7 has also been shown to boost T cell homeostasis in simian immunodeficiency virus (SIV)-infected non-human primates [109,110]. Several reports have attributed the beneficial effects of IL-7 therapy on T cell

reconstitution to the enhancement of peripheral T cell expansion [108,109,111], whereas other studies suggest that IL-7 can also increase thymic output [104,105,110,112,113]. The beneficial effects of IL-7 on thymic output of naive T cells might require prolonged IL-7 treatment [113,114]. Furthermore, the impact of IL-7 in thymic function might be more relevant for younger hosts, given that IL-7 administration does not seem to significantly increase thymic output in aged mice [104]. Regardless of the putative enhancement of thymic output, IL-7 administration has been shown to enhance T cell recovery through the preferential expansion of newly generated naive T cells, termed recent thymic emigrants (RTEs), following allogeneic bone marrow transplantation in mice [115,116]. Hence IL-7 therapy appears to induce cycling of RTEs, consequently allowing the maintenance of a diverse TCR repertoire in patients recovering from T cell depletion which might prove particularly relevant following allogeneic stem-cell transplantation [25].

IL-7 administration has been shown to enhance proliferation driven by high- and low-affinity antigens in T cell depleted mice [104]. Hence, it might be particularly useful as a vaccine adjuvant targeting poorly immunogenic antigens, such as those associated with tumours, given its ability to enhance responses to low-affinity antigens [25,104,117,118]. Pre-clinical studies in mice have confirmed that IL-7 can serve as a potent vaccine adjuvant, preferentially enhancing responses to sub-dominant antigens and thus broadening the scope of immune responses [117]. A model of skin graft rejection mediated by a male antigen in athymic T cell-depleted female mice has shown that IL-7 administration ensures restoration of immune competence following transfer of only 1% of the T cell repertoire, whereas 10% of the repertoire is required in the absence of IL-7 [118]. Thus, IL-7 therapy might potentially improve immune reconstitution through peripheral expansion, ensuring the restoration of a diverse TCR repertoire even in the absence of thymic function. The conversion of non-immunogenic antigens into mitogenic stimuli in the presence of increased IL-7 levels is potentially beneficial for the generation of a diverse TCR repertoire following T cell depletion, although it might also favour the development of autoimmunity [119]. Autoimmune diseases have been linked with settings characterised by T cell depletion and the consequent lymphopenia-induced proliferation [120-123], as well as with elevated IL-7 levels [124,125]. Furthermore, chronic elevation of IL-7 levels in mice has been associated with the development of lympho-proliferative disorders [126,127]. *In vitro* studies have also demonstrated that IL-7 promotes the viability, metabolic activity and proliferation of leukemic T cells [71,80,128,129], underlying the importance of taking into

account the potential tumourogenic effects of IL-7 when designing IL-7-based clinical trials as well as the potential therapeutic application of blocking IL-7 signalling in particular clinical settings [130].

Even in T cell-replete hosts, supra-physiological levels of IL-7 have been shown to induce naive $CD4^+$ and $CD8^+$ T cell proliferation, as observed in mice [51,109,131] and in macaques [114], suggesting that the endogenous levels of IL-7 constitute a limiting resource [12]. IL-7-expanded naive $CD4^+$ and $CD8^+$ T cells have been shown to acquire a memory-like phenotype both in immune-competent macaques [114] and following stem cell transplantation in mice [103,115]. Similarly, lymphopenia-induced proliferation in the absence of IL-7 treatment has also been reported to induce naive T cells to acquire a memory-like phenotype [132-138]. However, these memory-like cells have been shown to regain phenotypic and functional characteristics of naive T cells upon reconstitution of the T cell pool [132,133,138] or upon discontinuation of IL-7 treatment in immune-competent hosts [114]. Therefore the beneficial effects of IL-7 administration in the reconstitution of the naive T cell pool following stem cell transplantation might be initially masked by this phenomenon [103,115]. The impact of IL-7 on proliferation is not restricted to the naive subset as it also induces cycling of different memory $CD4^+$ and $CD8^+$ subsets, thus contributing to the maintenance of the whole T cell pool [114]. Moreover, IL-7 enhances the ability of memory $CD4^+$ and $CD8^+$ T cells to produce cytokines, reinforcing its potential use for IL-7 as a vaccine adjuvant [114].

IL-7 serum levels have been shown to be elevated in children following allogeneic bone-marrow transplantation, showing a direct correlation with the degree of T cell depletion [139]. Furthermore, an inverse correlation between IL-7 serum levels and peripheral $CD4^+$ T cell numbers, particularly naive $CD4^+$ T cells, has been reported in HIV-infected individuals [140,141]. Conversely, the restoration of $CD4^+$ T cell numbers following anti-retroviral therapy is associated with a decline in IL-7 levels [140,141]. Other settings involving $CD4^+$ T cell depletion, such as chemotherapy [140] and idiopathic $CD4^+$ lymphopenia [142], have been associated with elevated levels of circulating IL-7, which return to baseline upon recovery of $CD4^+$ T cell numbers. Immune reconstitution appears to be impaired in patients who display lower levels of circulating IL-7 than would be expected for the degree of lymphopenia observed, suggesting that elevated levels of endogenous IL-7 might aid the recovery of T cell homeostasis following T cell depletion [143,144].

The investigation of the potential causes underlying the increase in IL-7 levels during lymphopenia generated conflicting reports arguing either in favour of increased production, or of decreased consumption, of IL-7 following T cell depletion [119]. The increased production hypothesis was substantiated by the presence of significantly increased levels of cell-associated IL-7 within lymphocyte-depleted peripheral lymph nodes from acquired immunodeficiency syndrome (AIDS) patients [141], together with the observation of a greater delay between the recovery of $CD4^+$ T cell numbers and the restoration of steady-state IL-7 levels in patients recovering from CD4 depletion than would be expected if IL-7 consumption was the underlying mechanism [140]. A putative increase of IL-7 production in response to $CD4^+$ T cell depletion would require one or more sensing mechanisms able to monitor $CD4^+$ T cell levels and regulate IL-7 secretion accordingly [119], however no such mechanisms have been so far identified. On the other hand, studies in mice have reported a decline rather than a rise in IL-7 production in response to lymphopenia [25]. In addition, IL-7 production in the lymph nodes has been shown not to be significantly higher in HIV-infected patients than in non-infected individuals [145]. Hence it has been proposed that IL-7 is produced at a fixed constitutive rate and that its levels rise during lymphopenia as a result of reduced consumption due to a decrease in the number of T cells competing for IL-7 [13,58,130].

A major question regarding the potential efficacy of IL-7 therapy is whether IL-7 administration would be of any benefit in settings already associated with elevated IL-7 levels. The IL-7 serum concentration is in the pg/ml range, even in lymphopenic individuals [13], whereas IL-7 levels have been shown to rise to supra-physiological concentrations ($\geq$ 1000 pg/ml) following IL-7 administration in SIV-infected macaques [109]. Furthermore, quantification of IL-7 in the serum might not provide an accurate assessment of the concentration to which T cells are exposed in IL-7-rich microenvironments, as is the case for lymph nodes which contain IL-7-secreting stromal cells [56], and for extracellular matrix-associated IL-7 deposits which increase the tissue availability of IL-7 [146,147]. Hence the elevated IL-7 serum levels observed following T cell depletion are not likely to preclude potential beneficial effects of IL-7 therapy on T cell reconstitution [13,109].

Several clinical trials in humans have sought to evaluate the safety and efficacy of recombinant human (rh) IL-7 IL-7 therapy alone or as an adjuvant for immune-based therapies for cancer or chronic infection [14-17,148]. A clinical phase I trial assessed the

efficacy of a vaccine consisting of autologous tumour cells ectopically expressing IL-7 in a group of patients with disseminated malignant melanoma [148]. Indicators of anti-tumour immunity, such as the number of tumour-reactive cells, assessed both in terms of proliferative and cytolytic responses, could be detected post-vaccination [148]. However, only minimal anti-tumour efficacy was observed [148]. In a pre-clinical study in mice, IL-7 adjuvant treatment following immunization with a lentiviral vector encoding tumour-associated antigens enhanced the survival and proliferation of tumour antigen-specific, as well as naive, $CD8^+$ T cells, thus improving long-term anti-tumour $CD8^+$ T cell responses [149].

In a phase I/IIa clinical trial in HIV-infected patients with persistently low $CD4^+$ counts despite virologic suppression under combination antiretroviral therapy (c-ART), rhIL-7 administration induced the expansion of naive as well as memory $CD4^+$ and $CD8^+$ T cells, which remained functional and produced cytokines in response to HIV antigen [150]. Another trial in HIV-infected patients receiving antiretroviral therapy, rhIL-7 induced $CD4^+$ and $CD8^+$ T cells to enter cycle, increasing their circulating numbers [14]. Thus, the quantitative and functional changes induced by rhIL-7 therapy observed in these studies indicate that rhIL-7 may have potential therapeutic relevance in HIV infection and other settings of lymphopenia.

Phase I clinical trials performed in cancer patients have reported that rhIL-7 administration induces T cell survival and cycling *in vivo*, increasing $CD4^+$ and $CD8^+$ T cell numbers [15-17]. Naive $CD4^+$ and $CD8^+$ T cells were preferentially expanded [15-17]. Specifically, the absolute numbers of CD31-expressing naive $CD4^+$ T cells, a population enriched in RTEs, were increased following IL-7 administration, leading to the generation of a diverse TCR repertoire even in older individuals [16,17]. IL-7's effects upon naive T cell numbers and repertoire diversity appeared to be due to increased proliferation of RTEs rather than augmented thymic output, since they are age-independent and no thymic enlargement was observed [16]. Nevertheless these results do not preclude a potential effect of IL-7 therapy on thymic output. A clinical study in adults assessing the involvement of thymic function in immune reconstitution after autologous transplantation has shown that a thymic contribution is only observed after several months [151], suggesting that any putative effect on thymopoiesis might require a longer time span of IL-7 administration. As observed in pre-clinical studies, IL-7-expanded T cells appear to have enhanced responses to sub-dominant antigens [16]. In contrast, the proportion of senescent $CD8^+$ and

regulatory T cells decreased following IL-7 therapy [15,16]. IL-7Rα expression was down-regulated during continued IL-7 administration, which might constitute a negative feed-back loop that hinders uncontrolled T cell expansion in the presence of excess IL-7 and thus prevents the development of lympho-proliferative disorders in response to this cytokine [16,130].

These studies suggest a potential application for IL-7 therapy in the enhancement and broadening of immune responses in clinical scenarios associated with low naive T cell numbers and a skewed TCR repertoire, such as ageing, HIV infection and following transplantation [16].

1.4. Hematopoietic Stem Cell Transplantation: a major disturbance to T cell homeostasis

The factors and mechanisms underlying the maintenance of T cell homeostasis have been largely unravelled by investigating how T cell populations are modulated upon severe disruption of T cell homeostasis, namely following stem cell transplantation.

Allogeneic HSCT constitutes a suitable and often successful therapeutic approach for patients with leukemia, particularly for patients with high risk factors of relapse [152]. Reconstitution of the T cell pool after HSCT can occur through *de novo* thymic-dependent generation of T cells, or through thymic-independent peripheral expansion of donor T cells infused with the stem cell graft [103]. The recovery of CD4$^+$ T cell numbers following HSCT can be protracted due to impairment of the reconstitution process, for instance damage to IL-7-producing stromal cells induced by the conditioning regimen can hinder thymic function [153], whilst susceptibility to apoptosis might limit T cell peripheral expansion following transplantation [154]. Pre-clinical studies in animal models have suggested that IL-7 therapy may improve immune reconstitution after stem cell transplantation by improving both *de novo* generation and peripheral expansion of CD4$^+$ T cells [104-106,112,116]. Interestingly, a study in T cell-depleted mice following HSCT has shown that donor mesenchymal stem cells transduced with the IL-7 gene improved immune reconstitution through both enhanced thymopoiesis and peripheral T cell expansion, whilst concomitantly preventing GVHD [155].

The ideal donor for HSCT is a genotipically human leukocyte antigen (HLA)-matched related sibling, however approximately 70% of patients lack an HLA-identical sibling

[156,157]. For those patients, potential alternative donors comprise HLA-matched unrelated donors found through the international registries, although this search constitutes a lengthy and laborious process that can take several months, with the chances of finding a suitable unrelated donor ranging from approximately 10% for ethnic minorities to between 60 and 70% for Caucasian patients [158]. Furthermore, the risk of mortality and long-term morbidity following HLA-identical unrelated transplantation are still high [159,160]. On the other hand, the use of genotipically haploidentical related donors, i.e. related donors who only share one haplotype with the patient [152], provides an opportunity for patients to benefit from HSCT when a HLA-matched donor is not available [156]. The advantages of haploidentical related donors are their prompt availability for most patients, allowing an expedited access to more donor cells if donor-derived cellular therapy or even a second transplant are needed, and a potentially enhanced graft-versus-leukemia (GVL) effect [156,157]. Moreover, when several potential haploidentical donors are available, the most suitable donor can be chosen according to relevant criteria, such as age, cytomegalovirus (CMV) status and natural killer (NK) cell alloreactivity [161-163].

The key challenges facing haploidentical HSCT are to effectively overcome the HLA barrier, preventing graft rejection as well as graft-versus-host disease (GVHD), whilst maximizing GVL and improving immune reconstitution [156,157]. Some of these issues have been partly surmounted through depletion of T cells from the graft to evade GVHD, infusion of a high-dose of donor stem cells and/or use of increasingly intensive conditioning regimens to prevent graft failure and malignancy relapse, and resorting to donor lymphocyte infusion (DLI) after transplantation to boost GVL and immune reconstitution [156,157]. The adoption of less toxic conditioning regimens for haploidentical HSCT in conjunction with T cell-depleted grafts and delayed DLI, to prevent GVHD and retain GVL respectively, might circumvent the transplant-related mortality associated with high-dose conditioning regimens [157].

A possible strategy to maximize GVL is to choose a donor who confers NK alloreactivity due to killer immunoglobulin-like receptor (KIR) ligand incompatibility in the graft-versus-host direction. KIR ligand incompatibility has been correlated with enhanced ability of donor NK cells to kill recipient tumour cells and thus with improved GVL, although there are conflicting reports on this matter [157,164]. Nevertheless, NK alloreactivity in the graft-versus-host direction has not been reported to aggravate GVHD and hence its potential beneficial effects for the outcome of haploidentical HSCT might

outweigh the possibility of it being ineffectual [157]. Another approach to improve immune competence following haploidentical HSCT is to infuse tumour- or pathogen-specific donor T cells in order to avert malignancy relapse and opportunistic infections, respectively [156]. In particular, infusion of CMV-specific T cells might prevent CMV reactivation which constitutes a recurrent problem in immune-suppressed transplant recipients [156,157,165].

Therefore, therapeutic approaches employing adoptive cellular immunotherapy with different cell types, such as regulatory T cells, NK cells, mesenchymal stem cells and relevant antigen-specific T cells, might improve the outcome of haploidentical HSCT [156,157]. Nevertheless, immune reconstitution in this setting is still delayed due to the requirement for T cell depleted grafts and intensive conditioning regimens, leading to major imbalances in T cell homeostasis.

2. Immune response: Naive to Memory

2.1. T cell subsets: Markers & Nomenclature

Naive T cells can be defined as mature T cells that have not yet encountered their cognate antigen in the periphery. CD4$^+$ and CD8$^+$ naive T cells continually re-circulate between peripheral blood and secondary lymphoid organs. Once they encounter cells presenting their cognate antigen-MHC complex, naive T cells undergo proliferation and differentiation which induce phenotypic changes conferring suitable migratory and functional properties. Upon antigen clearance, the expanded T cell population undergoes a contraction phase during which most cells perish through apoptosis, although a proportion of the expanded population is preserved to ensure long-term protection against subsequent antigenic challenge. Antigen-experienced cells can be broadly termed memory T cells although they constitute a highly heterogeneous population, differing in cell surface phenotype, functional ability and history of antigen encounter [166-168]. Memory T cells provide more rapid and effective immunity against previously encountered antigens, as they can be activated by lower concentrations of antigen and accumulate, as well as perform effector functions quicker than their naive counterparts upon antigen re-exposure [169-171]. Furthermore, the distinct migratory capacity of memory T cells allows them to enter non-lymphoid tissues, potentially detecting and responding to infection earlier [172,173].

Isoforms of the transmembrane phosphatase CD45, resulting from alternative RNA splicing, were initially considered the crucial markers of naive and memory T cells, with naive cells expressing the CD45RA isoform and memory cells CD45RO [174-176]. These markers are no longer used in isolation to identify naive and memory cells since antigen-primed $CD8^+$ [177,178] and $CD4^+$ [179-181] T cells have been shown to re-express CD45RA. In order to dissect the heterogeneous T cell pool, CD45 isoforms are used in conjunction with other markers associated with lymphocyte differentiation, such as co-stimulatory molecules (CD27, CD28) [177] or chemokine receptors (CCR7) [182], to identify naive and memory T cell subsets within the $CD4^+$ and $CD8^+$ T cell pools.

There has been much debate about the pathway of T cell differentiation, particularly concerning the nomenclature and respective phenotype of each stage of differentiation [183]. Hence terms like “effector” and “memory” may be misleading in as much as the markers used to define them are not universal. A more accurate way to refer to the different T cell subsets is to name the markers that identify them. In this thesis, the $CD4^+$ T cell subsets studied were defined according to the expression of CD45RA together with the expression of the recent thymic emigrant maker CD31 or of the co-stimulatory molecule CD27, and each subset will be referred to by the corresponding phenotype. The terms “naive” and “memory” will be used to respectively describe cells that have yet to encounter their cognate antigen and antigen-experienced cells.

2.2 Naive $CD4^+$ T cell subsets defined by CD31 expression

The output of naive T cells from the thymus begins to decrease in early human adulthood and continues to decline with ageing, a phenomenon termed thymic involution [184]. Although this process limits the replenishment of the peripheral naive T cell pool by RTEs, the size of the naive pool is kept relatively constant throughout adult life [185-188]. Hence peripheral T cell proliferation must contribute at least partly to the maintenance of the naive T cell pool, implying that naive T cells are able to proliferate post-thymically whilst retaining their phenotypic and functional properties [189]. The assessment of the relative contribution of thymic output versus peripheral expansion to naive T cell homeostasis requires markers able to distinguish RTEs from naive T cells which have undergone post-thymic proliferation. The quantification of T cell receptor excision circles (TRECs) has been used to assess the relative proliferative history of T cell populations

[186]. TRECs are stable DNA episomes resultant from the re-arrangement of TCR gene segments during T cell differentiation in the thymus [186,190-194]. Given that TRECs are not replicated upon cell division, they are diluted out by T cell expansion in the periphery [191,195,196]. Thus TREC content has been proposed to constitute an indicator of thymic output, allowing the identification of RTEs [194]. Umbilical cord naive CD4$^+$ T cells can be used as a model for RTEs given their high TREC content [28]. IL-7 has been shown to rescue RTEs from spontaneous apoptosis *in vitro* through the up-regulation of Bcl-2 and Bcl-xL [27,28,30,99,197]. In addition, RTEs have been shown to proliferate in response to IL-7 in an antigen-independent manner more efficiently than naive T cells from adult peripheral blood [28-30]. The ability of IL-7 to promote the homeostatic proliferation of RTEs has been proposed to allow the maintenance of the peripheral naive CD4$^+$ T cell pool whilst preserving a diverse TCR repertoire [28]. IL-7 boosts the proliferative response of RTEs to TCR stimulation whilst preserving their naive phenotype, thus inducing maturation but not differentiation of RTEs [27,197] .

Unlike the memory T cell population, which has been described to comprise of a variety of subsets differing in differentiation stage, migratory ability and functional properties, naive T cells apparently constitute a fairly homogeneous population identifiable by a characteristic surface phenotype, expressing CD45RA, CD62L, CD27, CD28 and CCR7, whilst lacking or displaying low levels of CD45RO, CD95 and CD11a [198,199]. However, the naive CD4$^+$ T cell population has been shown to comprise two subsets with distinct proliferative histories distinguishable by the expression of the platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) [200]. CD31 is a trans-membrane glycoprotein from the immunoglobulin super-family which is expressed by a variety of cell types, including endothelial cells, platelets, monocytes, neutrophils and T cells [201-203].

The expression of CD31 on umbilical cord blood as well as on adult CD31$^+$ naive CD4$^+$ T cells has been shown to be down-regulated upon activation with anti-CD3 and IL-2 [204]. Although this overt TCR activation also leads to the differentiation into a CD45RO$^+$CD62L$^-$ memory phenotype [204], it has been proposed that the CD31$^-$ naive CD4$^+$ T cell subset could result from TCR triggering with low-affinity antigens, which would induce the loss of CD31 without affecting their overall naive phenotype [200,205]. Moreover, CD31$^-$ naive CD4$^+$ T cells have been shown to express higher levels of BFL1/A1, a marker specifically induced by TCR but not cytokine stimulation, than their

$CD31^+$ counterparts [205]. These data raise the possibility that the non-immunogenic signals triggered by self-MHC/peptide complexes which contribute to the survival and homeostasis of naive $CD4^+$ T cells may also play a role in the generation and/or maintenance of the $CD31^-$ naive $CD4^+$ T cell subset [11,22,23,206-209].

CD31 has been shown to be required for the transendothelial migration of neutrophils and monocytes [210]. Hence it might potentially play a role in the transendothelial migration of $CD31^+$ naive T cells into secondary lymphoid organs [200], a proposed site for homeostatic proliferation of naive T cells [211]. Furthermore, CD31 engagement has been shown to inhibit TCR-mediated signal transduction via immunoreceptor tyrosine-based inhibitory motifs (ITIMs) present in its cytoplasmic domain [202], raising the possibility that CD31 might hamper peripheral proliferation of $CD31^+$ naive $CD4^+$ T cells upon TCR triggering with self-MHC/peptide complexes [189].

The absolute numbers, as well as the frequency, of $CD31^+$ naive $CD4^+$ T cells in human peripheral blood decrease with ageing, in parallel with the decline in TREC content [200,205,212,213]. In contrast, the absolute numbers of $CD31^-$ naive $CD4^+$ T cells remain relatively constant throughout adult life despite thymic involution [200,205,212,213]. Nevertheless, the proportion of $CD31^-$ cells within the naive $CD4^+$ T cell population increases with age, allowing the maintenance of naive T cell numbers in the elderly through peripheral expansion [187,188,200,205,212,214]. On the other hand, the proliferation of $CD31^-$ naïve $CD4^+$ T cell subset has been shown to cause a contraction of the naive TCR repertoire, which might contribute to the impaired immune responses to novel antigens observed in the elderly [205,215,216]. However, a more recent study has demonstrated that clonal TCR diversity within the naive $CD4^+$ T cell pool is preserved during ageing despite peripheral expansion [213].

Human $CD31^+$ naive $CD4^+$ T cells have significantly higher levels of TRECs than their $CD31^-$ counterparts, implying that the latter subset has undergone a higher degree of post-thymic proliferation [200,212,213,217]. Nonetheless, the TREC content within $CD31^+$ naive $CD4^+$ T cells has been reported to decrease slightly with age [213] and following IL-7 administration in humans [16]. Furthermore, the absolute numbers of $CD31^+$ naive $CD4^+$ T cells in the elderly are higher than the values estimated by the assessment of thymic output through the quantification of TREC levels [186]. Hence the $CD31^+$ naive $CD4^+$ T cell subset appears to also undergo post-thymic proliferation which is likely induced by a TCR-independent mechanism driven by homeostatic cues, such as γ_C cytokines [189]. Thus,

although the high TREC content and the age-associated decrease in the absolute numbers as well as in the frequency of CD31$^+$ naive CD4$^+$ T cells, together with the observation that a substantial proportion of naive CD4$^+$ T cells from cord blood express CD31 [189,218], suggest that the CD31$^+$ naive CD4$^+$ T cell subset is highly enriched in RTEs [200,205,212,213], CD31 expression alone is probably insufficient to identify RTEs [186,189,213]. The combined assessment of CD31 expression and TREC content within naive CD4$^+$ T cells may constitute a more accurate strategy to identify RTEs and evaluate thymic function [189].

2.3. CD45RA re-expressing memory T cells

In light of reports on viruses able to establish persistent latent infection, the expression of CD45RO appears to better define cells that have been recently primed by cognate antigen, while CD45RA re-expression would identify cells that have not encountered antigen for some time [178,180,181,219-223].

Herpes viruses, such as Epstein-Barr virus (EBV) [224] and cytomegalovirus (CMV) [225], are classical examples of viruses capable of establishing persistent latent infection in humans. Other persistent viruses have developed different strategies to allow coexistence with their hosts which is reflected in the distribution of virus-specific T cells among the memory subsets. During the acute phase of infection, the virus-specific cells have a similar phenotype regardless of the persistent virus studied [181,226,227]. However, during chronic infection each virus-specific pool becomes enriched in distinct memory subsets depending on the respective viral load [181,226,227]. For example, in HIV infected patients with a high viral load, the HIV-specific cells have a phenotype associated with the acute phase of viral infection [227]; on the other hand, controlled HIV infection in long-term non-progressors gives rise to T cell responses associated with repetitive antigen exposure and low viral load [181]. The latter profile is similar to that of CMV- and EBV-specific T cell responses, as these viruses represent persistent, well-controlled infections with only moderate antigen burden [181].

During persistent viral infections, the emergence of a CD45RA re-expressing subset appears to only occur upon resolution of the acute phase of infection and comprises of cells specific for lytic but not latent antigens [178,180,181,219-223]. A report showing that HIV infected individuals lacked HIV-specific CD45RA re-expressing cells, whilst CMV-specific cells from the same patients did re-express CD45RA concluded that there was an

HIV-induced blocking of T cell differentiation with deleterious effects upon the HIV-specific response [228]. These results were re-interpreted by Carrasco *et al* as a consequence of the distinct viral loads associated with CMV and HIV infections, with only the former providing the long term absence of antigen conducive to CD45RA re-expression [220]. Therefore the distribution of the T cell subsets during persistent viral infections appears not to be static but rather to dynamically fluctuate in response to changes in antigen load [227]. In addition, the expression of CD45 isoforms appears to be reversible, with cells reverting to a CD45RA phenotype in the absence of antigen. CD45RA re-expressing cells have been suggested to ensure the persistence of immunological memory against antigens that are no longer present, such as lytic antigens during the latent stage of a persistent infection [220,229]. In agreement with this view, the CD45RA re-expressing subset has been proposed to constitute a quiescent reservoir of memory T cells which can be re-activated to perform effector functions [221]. It is not clear if the same applies for elderly individuals, where these cells show evidence of terminal differentiation [230].

The majority of the reports on the CD45RA re-expressing memory subset focus on $CD8^+$ T cells. The characterisation of this subset in $CD4^+$ T cells is hampered by the very low frequencies observed, with some studies even reporting an absence of CD45RA re-expressing cells within $CD4^+$ T lymphocytes [177,220,231]. CD45RA positivity has been used in conjunction with the lack of CCR7 [182,220,232], CD27 [177,233,234] and/or CD28 [235] to identify the CD45RA re-expressing memory subset. In $CD8^+$ T cells, this subset has been suggested to have marked cytotoxic potential, displaying cytolytic activity together with high levels of FasL, perforin and granzyme B [177,221,222,234,236-239]. $CD8^+$ CD45RA re-expressing cells have also been shown to produce the pro-inflammatory cytokines IFN-γ and TNF-α, but little or no IL-2 and IL-4 [177,222,237]. This subset is characterised by expression of CD57 [177,240], a marker of highly differentiated and cytotoxic cells [241]. Furthermore, the elevated levels of CD57 displayed by $CD8^+$ CD45RA re-expressing cells have been associated with increased susceptibility to apoptosis and replicative senescence [177,242]. The $CD8^+$ $CD57^+$ T cell population is accumulated during chronic immune activation [243-245], such as CMV infection [240,246,247], and is thought to comprise senescence-prone cells that are constantly generated and subsequently driven to cell death by persistent antigenic stimulation [242,248]. Several studies describe the $CD8^+$ CD45RA re-expressing subset as a resting population, exhibiting a slow rate of *ex vivo* turnover [220,221,237]. However, there are conflicting reports concerning the susceptibility to apoptosis

and replicative potential of this subset. $CD8^+$ CD45RA re-expressing cells have been described as an apoptosis-resistant population expressing high levels of Bcl-2 expression [221], whilst another study found them to be apoptosis-prone following activation, a feature associated with low Bcl-2 levels [232]. As for their replicative potential, some studies report that $CD8^+$ CD45RA re-expressing cells are able to proliferate upon activation [220-222,238], whereas others state the opposite [232,237,239]. An argument supporting the maintenance of replicative potential in these cells is the observation that $CD8^+$ EBV-specific $CD45RA^+$ cells have relatively long telomeres in comparison to their $CD45RO^+$ counterparts [221].

$CD8^+$ CD45RA re-expressing cells display high levels of the adhesion molecule LFA-1 [237], whilst expressing concomitantly low levels of the chemokine receptor CCR7 and of L-selectin (CD62L) [182]. Furthermore, this subset has been reported to be significantly under-represented in lymph nodes, whilst accounting for virtually all $CD8^+CD45RA^+$ T cells in peripheral tissues of the same individuals [237]. Their phenotype and tissue distribution led to the speculation that $CD8^+$ CD45RA re-expressing T cells might migrate into extra-lymphoid tissues rather than re-circulate to secondary lymphoid organs [234,237].

$CD4^+$ T cells are pivotal for the generation and maintenance of immunological memory [249-251]. Nonetheless, most studies concerning CD45RA re-expression have been performed on $CD8^+$ T cells, whereas the occurrence of this phenomenon on $CD4^+$ T cells has been largely overlooked due to the relatively small proportion of $CD4^+$ CD45RA re-expressing cells. The $CD4^+$ CD45RA re-expressing subset has been described as terminally differentiated, with short telomeres, lack of proliferative ability and high levels of CD57 expression [181]. Their cytokine production profile is apparently similar to that of the $CD8^+$ CD45RA re-expressing subset, i.e. IFN-γ, but no IL-2 or IL-4 production [252]. Despite their low frequency, virus-specific CD45RA re-expressing $CD4^+$ T cells have been detected through their production of IFN-γ and TNF-α [253].

The mechanisms that induce CD45RA re-expression in T cells, including the signalling pathways and respective molecular targets that are engaged, are yet to be fully understood. CD45RA re-expression has been shown to occur on $CD8^+$ $CD45RA^-CCR7^+$ cells in the presence of IL-7 and IL-15 upon cytokine-driven homeostatic proliferation in the absence of antigen [232]. The $CD8^+$ CD45RA re-expressing subset was proposed to be continuously replenished from proliferating $CD45RA^-CCR7^+$ precursors, seeing that the naturally occurring $CD45RA^+CCR7^-$ subset was prone to cell death and had the lowest

turnover of all the memory subsets [232]. In another study, IL-15 was shown to induce CD45RA re-expression on $CD8^+$ T cells [239]. However CD45RA re-expression could not be induced in the concomitant presence of TCR stimulation [232,239], suggesting that this process may be driven by homeostatic mechanisms in non-inflamed tissues.

The induction of CD45RA re-expression on T cells is likely to entail changes in the transcriptional program. However, the transcription factors potentially driving CD45RA re-expression are yet to be identified. Transcription factors known to be involved in the homeostasis of highly differentiated T cells such as the T-box family members T-bet and eomesodermin (Eomes) [254-256] are likely candidates. T-bet is essential for Th1 lineage commitment and IFN-γ production in $CD4^+$ T cells [255,257]. Eomes drives effector function and IFN-γ production in $CD8^+$ T cells [254]. It is not known if Eomes and/or T-bet are relevant for the differentiation of memory $CD4^+$ T cells. The transcriptional repressor Blimp-1, a well known key regulator of terminal differentiation of B cells [258], was also found to be essential for controlling the late stages of $CD4^+$ and $CD8^+$ T cell differentiation in mice [259,260]. Blimp-1 expression is controlled by γ_C cytokines which are also involved in regulating T cell homeostasis: IL-2 is thought to induce Blimp-1 expression, after which Blimp-1 itself represses IL-2 transcription in a negative feedback loop [261,262], whilst IL-15 does not seem to maintain Blimp-1 expression [261]. Interestingly, $CD45RA^+CD27^-$ cells have been shown to express the highest levels of T-bet, Eomes and Blimp-1 amongst $CD8^+$ T cells during latent human CMV infection [263]. The differential expression of these transcription factors in $CD8^+$ T cell subsets was more dramatic when T-bet levels were compared, with $CD45RA^+CD27^-$ cells displaying significantly higher expression, at both the mRNA and the protein level, than $CD45RA^-CD27^+$ cells [263].

Even though the exact mechanism underlying the expression of CD45RA on memory T cells has not thus far been described, this process has been proposed to depend on homeostatic cues, such as γ_C cytokines, in the absence of antigen stimulation [220,232,239].

3. Immune and Cellular senescence

Immune-senescence encompasses multiple phenotypic and functional abnormalities observed in the elderly associated with impaired protection against infections, increased susceptibility to cancer and autoimmune diseases, and poor vaccine efficacy [264-268]. Although the size of the T cell pool remains relatively stable during ageing, the functional

aptitude and the distribution of the different T cell subsets may suffer striking changes [268]. In particular, the increased frequency of senescent T cells observed during ageing may significantly contribute to the diminished immune function observed in the elderly [269,270]. Cellular senescence is defined as the irreversible loss of replicative capacity [271], thus the accumulation of senescent T cells may compromise the ability to mount an effective immune response and consequently contribute to immune-senescence [272]. Chronic immune activation, namely during CMV infection, has also been shown to induce the accumulation of senescent T cells [243-245,273-277]. One of the factors that can lead to cellular senescence is the progressive decrease in telomere length that occurs with each cell division, a process termed telomere erosion [278,279]. Telomeres are nucleoprotein structures that cap the terminal portions of linear chromosomes, preventing the loss of coding sequences and maintaining chromosomal integrity [280,281]. Telomere erosion can be compensated for by the induction of telomerase activity, an enzyme which is able to add back telomere sequences and thus increase the replicative lifespan by compensating for proliferation-induced telomere shortening [282-285]. Unlike other somatic cells, lymphocytes are able to activate telomerase during development and following activation [286]. However, T cells lose the ability to induce this enzyme after repeated stimulation [287,288], eventually leading to critically short telomeres which in turn trigger either apoptosis or senescence [288-290]. Therefore, persistent T cell activation, together with other stress factors present during chronic immune activation, compromise the ability of telomerase to compensate for the loss of telomere sequences that occurs upon cell division and thus might accelerate the onset of senescence [288].

3.1. Telomeres, Telomerase and Senescence

The dynamic interplay between telomere erosion and the compensatory effect of telomerase activity is critical for the maintenance of immune function [281]. An appropriate balance between cell survival and proliferation on the one hand, and cell death on the other, has to be reached in order to dispose of expanded populations that are no longer needed whilst simultaneously maintaining long-term memory against previously encountered antigens [281]. The need for constant renewal of the existing memory T cell pool constitutes a challenge for the preservation of immune-competence in the elderly. The life-long proliferative stress on memory T cells is reflected in the shorter telomeres observed in T cells from old individuals as compared to those from young individuals

[230,291]. Moreover, both $CD4^+$ and $CD8^+$ memory T cells have shorter telomeres than their naive counterparts, further pointing to cell proliferation as the major driving force behind telomere erosion [286,292-296].

Telomeres comprise repetitive hexameric DNA sequences associated with a variety of telomere-binding proteins and are located at the terminal portions of chromosomes [280,297]. These capping structures maintain the integrity of linear chromosomes by averting chromosomal fusion and rearrangement, as well as preventing the loss of coding sequences during DNA replication [297]. DNA polymerase requires a RNA primer to initiate replication in the 5'-3' direction [298] and it synthesises the leading strand continuously until the end of the linear DNA template [299,300]. In contrast, the synthesis of the lagging strand runs in the opposite direction based on a series of DNA fragments termed Okazaki fragments, each requiring a RNA primer [299,300]. When synthesis is complete, the primers are degraded and the resulting gaps between consecutive Okazaki fragments are filled to form an uninterrupted progeny strand [299,300]. However, because the Okasaki fragments do not start from the very end of the DNA template and the gap left by the most distal RNA primer cannot be filled, the lagging strand synthesis on linear DNA templates is incomplete [299,300]. Hence each round of DNA replication results in the loss of terminal sequences, a phenomenon known as the end-replication problem [299,300]. The presence of telomeres at the ends of linear chromosomes prevents the loss of coding sequences following DNA replication [281]. Instead, telomere length decreases with each round of cell division [278,279]. Hence, the replicative potential of T cells is limited by the telomere erosion brought about by cell proliferation [293], which eventually leads either to apoptosis or replicative senescence [288-290]. Induction of telomerase may initially compensate for telomere shortening but repeated stimulation hinders further activation of this enzyme and ultimately leads to telomere erosion [282,283,285,291]. The telomerase holoenzyme is comprised by a catalytic protein (telomerase reverse transcriptase, TERT) and a RNA template (telomerase RNA component, TERC, or telomerase RNA, TR) [291]. The expression of TERC is ubiquitous, whereas the expression of human (h)TERT is tightly regulated [301,302]. Over-expression of hTERT in $CD4^+$ T cells allows for constitutive telomerase activity which has been shown to slow down the rate of telomere shortening, although it does not prevent telomere erosion and consequent onset of cellular senescence [303].

In resting CD8$^+$ [284] and CD4$^+$ T cells [304] virtually no telomerase activity is detectable, although high levels can be induced by mitogenic stimuli such as TCR stimulation and γ_C cytokines [30,286,305,306]. Although telomerase activity is potently induced in T cells during a primary response to antigen [282,283,285], the ability to activate telomerase is progressively lost upon repeated stimulation which leads to telomere erosion and eventually cell senescence [286,288,291,294]. The mechanisms by which telomerase activity is switched off are not yet fully understood. Type I interferon (IFN-α, IFN-β) has been shown to inhibit telomerase activity in CD4$^+$ T cells, potentially accelerating telomere erosion and thus reducing the replicative lifespan of these cells during secondary immune responses *in vivo* [307]. Furthermore, the progressive differentiation of CD8$^+$ T cells into the highly differentiated CD27$^-$CD28$^-$ phenotype has been shown to be associated with defective telomerase activity upon TCR stimulation [308]. Telomerase deficiency in CD27$^-$CD28$^-$ CD8$^+$ T cells was not reversed by the restoration of CD28 signalling and was accompanied by defective phosphorylation of Akt at serine 473 [308]. Phosphorylation of hTERT by Akt has been shown to induce its translocation to the nucleus [304] and to enhance telomerase activity [309]. These data, together with the observation that Akt inhibition abrogated the induction of telomerase activity [308], indicated Akt(Ser473) phosphorylation as a key trigger of telomerase activity. However a recent study has shown that blocking the senescence-associated inhibitory receptor KLRG1 improved Akt(Ser473) phosphorylation but did not restore telomerase activity in CD27$^-$CD28$^-$ CD8$^+$ T cells [310]. These data suggest that Akt(Ser473) phosphorylation may be necessary but not sufficient to induce telomerase activity, which is apparently regulated through a multi-factorial process possibly involving transcription and post-translational changes of hTERT, translocation between different cellular compartments and access to relevant DNA targets [296,302,311].

Telomerase has been reported to promote cell survival and stress resistance independently of its telomere elongation activity [312-316]. Upon oxidative stress, telomerase can shuttle from the nucleus to the mitochondria which improves overall mitochondrial function [317]. Moreover, the levels of induced telomerase activity and hTERT expression have been shown to inversely correlate with cell death in CD4$^+$ T cells cultured with IL-7, thus implying a role for telomerase in the IL-7-induced survival of human CD4$^+$ T cells [318].

3.2. Immune-senescence in the elderly

Ageing has an adverse impact on various physiological processes, not least of which is on the development and function of the peripheral T cell pool [319]. Thymic involution plays a major role in the decline of immune function during ageing [320]. A decrease in the proportion of thymopoietic epithelial space and a thymosuppressive cytokine environment have been suggested to contribute to the waning thymic function observed with ageing [321,322]. The decreased thymic output of naive T cells is associated with the expansion of the memory population in order to maintain the overall size of the T cell pool, leading to a shift in the ratio of naive to memory T cells in the periphery [320,323,324]. In addition, the residual naive T cells have increased longevity to compensate for the declining thymic replenishment, which leads to age-related defects in T cell function [325]. Naive $CD4^+$ T cells from aged mice have been shown to have defective cell survival, proliferation and IL-2 production following antigen stimulation [326-329]. In contrast, when young naive $CD4^+$ T cells are transferred into aged mice, they show an inferior ability to expand and produce cytokines than the ones transferred into young hosts [330]. Furthermore, naive $CD4^+$ T cells generated from aged stem cells in young mice have been shown to be highly functional [331], suggesting that environmental factors present in both the thymus and the periphery of aged hosts contribute to the impaired naive $CD4^+$ T cell function observed during ageing. Hence the impaired $CD4^+$ T cell responsiveness to novel antigenic challenges observed with ageing is due to a decline both in the number of naive cells and in the functional capability of the naive $CD4^+$ T cells that do persist [326].

Memory $CD4^+$ T cell responses are also compromised in aged mice, featuring defective signalling and proliferation following activation [332]. In humans, the accumulation of $CD28^-$ cells within both the $CD4^+$ and $CD8^+$ T cell populations is a consistent change observed during ageing [270,333-335] that is associated with diminished immune response to pathogens and vaccine efficacy in the elderly [336-338]. T cells lacking CD28 expression have been shown to have a skewed TCR repertoire and defective proliferation in response to antigenic stimulation, whilst displaying enhanced cytotoxic activity [339,340].

The loss of CD28 expression with age is thought to result from recurring activation and cell cycling episodes [268], which is supported by the shorter telomeres observed in $CD28^-$ T cells as compared to their $CD28^+$ counterparts [295,341,342]. In addition to T cell

activation, type I interferon [292,343] and TNF-α [270,344] have also been shown to contribute to the accumulation of $CD28^-$ T cells *in vitro*, with the latter directly inhibiting CD28 gene transcription [344,345]. The accumulation of $CD4^+$ $CD28^-$ T cells at the expense of their naive and memory $CD28^+$ counterparts has been shown to be induced during inflammation and to in turn exacerbate the pro-inflammatory environment, contributing to tissue injury and compromising responses to novel antigens [346,347]. High frequencies of $CD4^+$ $CD28^-$ T cells and elevated TNF-α levels are concomitantly observed during ageing [348-350], chronic inflammation [351,352] and persistent viral infections, particularly CMV infection [353]. TNF-α inhibition has been shown to delay CD28 loss and the onset of senescence on $CD8^+$ T cells *in vitro*, increasing the proliferative ability and telomerase activity on these cells [354]. Anti-TNF-α therapy in rheumatoid arthritis patients has been shown to restore CD28 expression within the $CD4^+$ T cell population [355]. These data suggest that the pro-inflammatory environment observed during ageing and CMV infection may contribute to the accumulation of senescence-prone highly differentiated T cells.

IL-7 has been shown to boost T cell reconstitution through the increase of both thymopoiesis and peripheral T cell proliferation following stem cell transplantation in mice [104,105,112], suggesting IL-7 therapy as a potential approach to improve immune function in the elderly by raising naive T cell numbers.

3.3. CMV infection accelerates immune-senescence

The prevalence of CMV infection increases with age [356]. Although this infection is largely asymptomatic in immune-competent individuals [357], it can cause life-threatening diseases in immune-suppressive settings, such as HIV infection and following bone marrow transplantation [358-361].

The control of CMV infection requires substantial immune resources, with CMV-specific cells constituting a substantial proportion of both the $CD4^+$ and $CD8^+$ T cell pools [362-364]. $CD4^+$ T cells have been shown to directly contribute to the control of CMV infection [365,366], with CMV-specific $CD4^+$ T cells displaying a terminally differentiated phenotype and cytotoxic activity, in addition to IFN-γ and TNF-α production [367].

Ageing and CMV infection induce similar alterations to the subset distribution of the T cell pool as both are associated with a decrease in naive T cells and a concomitant accumulation of cells with effector functions [226,368-371]. CMV infection accelerates the

age-related changes in the TCR repertoire by triggering the expansion of CMV-specific cells at the expense of diversity [235,372]. In the elderly, there is an increase in the proportion of CMV-specific $CD4^+$ cells whilst the frequency of $CD4^+$ T cells with other specificities, such as *Varicella zoster* virus (VZV)-specific cells, diminishes [292]. This enrichment in CMV-specific cells at the expense of other specificities may be behind the reactivation of latent pathogens like VZV often seen in the elderly [268,373,374].

CMV infection is not only characterised by the presence of highly differentiated CMV-specific $CD4^+$ T cells, but also by a bystander effect on the rate of differentiation of $CD4^+$ T cells with other specificities [292]. The pronounced levels of non-specific T cell differentiation observed during CMV infection might be explained by the CMV-induced secretion of IFN-α [375] and TNF-α [353], two cytokines reported to accelerate the loss of co-stimulatory molecules [343-345] and to inhibit telomerase activity [307,354]. Interestingly, $CD4^+$ CMV-specific cells have been reported to have low levels of telomerase activity and to reach growth arrest earlier than cells with other specificities, indicating that these cells are susceptible to replicative senescence [292].

The consequences of the accumulation of highly differentiated CD45RA re-expressing T cells observed during ageing [294,376] and CMV infection [222,235,292] are not clear. These cells might be functionally relevant and grant protection against recurring pathogens; on the other hand they might be smothering the available memory space and directly contributing to immune-senescence. It is therefore of major interest to characterise these cells in detail, determining their functional potential and uncovering the mechanisms behind their generation.

3.4. Cellular Senescence

Cellular senescence is a state of irreversible growth arrest that can be induced in normal somatic cells by critically short telomeres [377,378] or by several other stress factors, such as non-telomeric DNA damage [379-381], over-expression of oncogenes [382], chemotherapeutic agents [383] and oxidative stress [384]. The former type of senescence is called replicative senescence and its onset can be delayed by inducing telomerase activity [385]; the latter is called stress-induced premature senescence and it cannot be bypassed by the ectopic expression of telomerase [386,387], suggesting a telomere-independent mechanism. Cellular senescence was originally described in human fibroblast cultures

when it was observed that these cells could only undergo a limited number of population doublings before they would irreversibly withdraw from the cell cycle [388], a phenomenon that has become known as the Hayflick Limit. The majority of the reports on cell senescence have been performed in fibroblasts, but it has become clear that this phenomenon also occurs in T lymphocytes [389]. The inducible nature of telomerase activity, delaying the rate of telomere loss, and the potential exposure to stresses that can accelerate the onset of cellular senescence make it very difficult to predict the number of population doublings achievable before a T cell becomes senescence *in vivo*, suggesting that the Hayflick Limit does not define the replicative lifespan of T cells *in vivo* [389]. The surface markers for senescent T cells are not well-established, although the expression of CD57 and KLRG1, and conversely the lack of CD27, CD28 and CCR7 expression appear to be relevant [177,182,228,238,242,390,391]. Cellular senescence has been argued to comprise genetic and phenotypic changes that result in altered function of T cells, not necessarily only loss but also gain of function, as is the case for the increased production of the pro-inflammatory cytokines TNF-α and IL-6 [392].

Chronic T cell activation driven by persistent viruses that establish latent infection or by tumour-associated antigens may drive antigen-specific cells to senescence [392]. Cellular senescence has been proposed to constitute a tumour suppression mechanism that prevents the transformation of damaged cells [393,394]; on the other hand, it might lead to the accumulation of senescent cells and thus contribute to age-related loss of tissue function [272,395].

Cellular senescence can be triggered by DNA damage [396]. The DNA damage response (DDR) allows cells to sense damaged DNA and to respond by arresting cell cycle progression [396], providing time to repair the damage and prevent cellular transformation. When repair is not possible, the persisting damage either triggers apoptosis or causes the cell cycle arrest to become irreversible, thus inducing senescence [397]. The DNA damage response can be triggered by DNA double strand breaks (DSBs) or by telomere uncapping, i.e. destabilisation of telomeric loops due to telomere shortening [271,377]. This response induces the recruitment and activation of the ATM/ATR kinases which in turn phosphorylate the H2AX histone, a variant of the histone H2A family, adjacent to the site of damage [271,395]. Phosphorylated H2AX (γ-H2AX) promotes the assembly of DNA repair factors and the phosphorylation of Chk1/2 which leads to the activation of the tumour suppressors p53/p21 [398-400]. The function of H2AX is thought to be primarily related to

DNA damage repair, although H2AX phosphorylation by the mitogen activated protein kinase (MAPK) p38 has been shown to be required for serum starvation- [401] and chemotherapeutic drug-induced apoptosis [402].

MAPKs comprise three major signalling pathways: extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNKs) and p38 MAPKs [403]. The activation of the MAPK pathways is triggered by a variety of extracellular stimuli. The Erk pathway, also known as the mitogen-activated protein kinase/ERK (MEK/ERK) pathway, is thought to be mainly activated by growth-promoting mitogenic factors, whereas the JNK and p38 pathways appear to be activated by environmental stress, including oxidative stress, growth factor withdrawal and pro-inflammatory cytokines such as TNF-α [404]. Although a previous study reported an IL-7-induced activation of p38 [405], the p38 pathway is thought to be activated by the withdrawal of trophic factors such as IL-7 and IL-2 [406-408]. The induction of increasing levels of activated p38 can be achieved through different mechanisms, as was shown in CMV infection which has been reported to lead to the accumulation of activated p38 by both inhibiting the dephosphorylation of p38 and by promoting its phosphorylation in order to induce the host cell changes necessary for viral DNA replication [409].

The activation of MAPK signalling pathways triggers a cascade of kinases, which can either be shared or specific for each MAP kinase. The full activation of each MAPK requires dual phosphorylation on Thr and Tyr residues within the activation loop by the respective MAPK kinases (MAPKKs) [410]. The involvement of each MAPKK in the *in vivo* activation of p38 varies according to the triggering stimulus [410]. The two specific up-stream activators of p38 are the MAPKKs MKK3 and MKK6 [411]. Studies in mouse fibroblasts have shown that MKK3 and MKK6 play redundant but essential roles on the activation of p38 MAPK induced by TNF-α [410]. The abrogation of p38 activation leads to defective cell cycle arrest and promotes tumorigenesis [410]. Together with the observation that p38 is able to activate the tumour suppressor protein p53 [412,413], these data suggest that the p38 pathway may contribute to tumour-suppression. On the other hand, TCR stimulation has been shown to activate p38 by mono-phosphorylation of Tyr in the activation loop via an alternative pathway, independent of the classical MAPK cascade, resulting in altered substrate specificity [414].

The p38 family is composed by four p38 isoforms: p38α, p38β, p38γ and p38δ [415]. The different p38 isoforms are encoded by distinct genes although they have a high degree of

homology [415]. Nevertheless, they vary in substrate specificity and tissue distribution suggesting that the different p38 isoforms might be independently regulated *in vivo* [411]. The dissimilarities between the p38 family members have been further highlighted by the selectivity of p38 inhibitors for certain p38 isoforms. For example, the commonly used SB203580 compound specifically inhibits p38α and p38β [416,417], but not p38γ and p38δ [418,419]. On the other hand, the BIRB796 compound has been shown to inhibit all the p38 isoforms both *in vitro* and *in vivo* [420,421], rendering it a valuable tool for the effective switching off of the p38 signalling pathway.

The substrates of MAPKs encompass a variety of molecular effectors that regulate a wide range of cellular processes, including cell cycle, differentiation and apoptosis. Besides its role in tumour suppression [422,423], the p38 MAPK pathway has been described to have a potential role in inflammatory responses [424]. One of the mechanisms through which the p38 pathway appears to respond to inflammation is a positive feedback loop with TNF-α, wherein this pro-inflammatory cytokine has been shown to trigger p38 activation [404,410], which in turn induces the production of TNF-α [425].

The p38 pathway has been proposed to play a key role in mediating both telomere-dependent and –independent senescence [426]. The mechanisms by which p38 signalling induces cell senescence are yet to be fully characterised, but p38 is known to induce cell cycle arrest by up-regulating $p16^{INK4a}$ expression, which leads to pRb hypo-phosphorylation [427,428], and by phosphorylating p53, which induces $p21^{Cip1}$ expression [422,429]. DSBs generated by γ-radiation have been shown to activate p38 MAPK *in vitro* and consequently induce cell cycle arrest [430]. The p38 pathway has also been shown to be activated *in vivo* by DSBs resulting from V(D)J recombination in mouse thymocytes, inducing a p53-dependent G2/M cell cycle checkpoint to allow DNA repair and maintain genomic stability [431]. In order to allow cell cycle progression and further differentiation of thymocytes, the p38 pathway has to be inactivated [431].

The p38 pathway has also been described as a mediator of cell death via the triggering of intracellular alkalinisation following growth factor withdrawal [406] and by inducing the translocation of the pro-apoptotic protein Bax to the mitochondria during chemotherapeutic drug-induced cell cycle arrest [432]. In addition, p38 has been shown to phosphorylate Bcl-2, decreasing its anti-apoptotic potential and triggering apoptosis following serum deprivation of mouse embryonic fibroblast cultures [433]. The phosphorylation of Bcl-2 by p38 induces apoptosis by promoting cytochrome c release

from mitochondria and caspase activation [434], possibly by abrogating the ability of Bcl-2 to hetero-dimerise with Bax and thus allowing the translocation of the latter to the mitochondria [433,435,436]. Besides suffering post-translational modifications mediated by p38, Bcl-2 has also been shown to be a transcriptional target of p38α on mouse embryonic stem cells [437].

Cellular senescence thus constitutes a complex process which may work as a tumour suppressive mechanism, whilst possibly hindering immune surveillance during ageing [396].

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CHAPTER 1

Role of IL-7 in the homeostasis of human CD31$^+$ naive CD4$^+$ T cells

Introduction

Homeostasis of the T cell pool constitutes a dynamic process encompassing a variety of mechanisms working concertedly in order to constantly adapt to fluctuating conditions. In particular, naive CD4$^+$ T cell homeostasis has to compensate for the decreasing contribution of the thymus to the replenishment of the peripheral naive T cell pool during ageing through post-thymic proliferation of naive T cells [1,2]. In order to maintain the size and diversity of the naive CD4$^+$ T cell pool, this peripheral expansion should be driven by homeostatic mechanisms that induce proliferation, whilst preserving the phenotypic and functional characteristics of naive CD4$^+$ T cells, as well as ensuring an unbiased stimulation in order to maintain a diverse TCR repertoire [2]. The homeostatic γ_C cytokine IL-7 has been shown to promote the survival of recent thymic emigrants derived from umbilical cord blood [3-7], in addition to inducing antigen-independent homeostatic proliferation of cord blood RTEs more potently than of naive CD4$^+$ T cells from adult peripheral blood [5,6,8]. These data suggest that IL-7-mediated homeostatic proliferation of RTEs contributes to the maintenance of the peripheral naive CD4$^+$ T cell pool [5].

The expression of CD31 within naive CD4$^+$ T cells has been proposed to identify a population enriched in RTEs[9-12], whereas naive CD4$^+$ T cells lacking CD31 have been suggested to have undegone homeostatic proliferation driven by low-affinity TCR triggering [9,12]. In the first part of this chapter, we sought to investigate the effects of IL-7 stimulation on naive CD4$^+$ T cell subsets defined by the expression of CD31 from umbilical cord and adult peripheral blood samples. Specifically, we were interested in clarifying the outcome in terms of survival, proliferation and levels of CD31 expression following *in vitro* culture of purified CD31$^+$ and CD31$^-$ naive CD4$^+$ T cell subsets in the

presence of IL-7, in addition to elucidating the signalling pathways mediating these effects.

In this same line of research, concerning T cell homeostasis, we had the opportunity to perform an evaluation of long term immune reconstitution in a clinical setting associated with major disturbances in T cell homeostasis. In the second part of this chapter, our aim was to assess the distribution of naive and memory T cell subsets in a group of five patients who underwent haploidentical HSCT from a related donor, at an average of five years post-transplant, simultaneously comparing their profile to the T cell distribution observed in the respective donors and age-matched controls. We were particularly interested in investigating the mechanisms that drove T cell reconstitution in these patients by determining the relative contribution of thymic output and peripheral expansion of mature T cells. For that purpose, we assessed the expression of CD31 within naive $CD4^+$ T cells, the TREC content and the telomere length in recipients of haploidentical HSCT, as well as in donors and age-matched controls.

Methods

1. Blood samples

1.1. Adult peripheral blood and umbilical cord blood samples

Adult peripheral blood samples from healthy volunteers and umbilical cord blood samples collected immediately after delivery of full-term infants were obtained with approval from the Ethics Board of the Faculty of Medicine of Lisbon. Umbilical cord blood samples were provided by Dr. Helena Ferreira from Hospital Universitário de Santa Maria, Lisboa, with informed consent obtained in accordance with the Declaration of Helsinki.

1.2. Haploidentical HSCT recipients

This study was approved by the Ethics Committee of the Faculdade de Medicina da Universidade de Lisboa. Heparinized peripheral blood and serum samples from five haploidentical related hematopoietic stem cell transplantation recipients were obtained four to six years post-transplant through collaboration with Dr. João Lacerda from Serviço de Hematologia, Hospital de Santa Maria. In parallel, samples were collected

from the respective donors, who were always one of the parents, and age-matched healthy controls.

2. Purification of lymphocyte subsets

Mononuclear cells from adult peripheral blood and from cord blood were isolated by Ficoll-Hypaque density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). $CD4^+$ T cells were negatively selected using the EasySep Human $CD4^+$ T Cell Enrichment Kit (StemCell Technologies, Vancouver, BC) according to the manufacturers' instructions. $CD4^+$ T cells were subsequently sorted into $CD31^+$ and $CD31^-$ naive subsets using a FACSAria flow cytometer (BD Biosciences, San Jose, CA) after staining with CD45RA, CD45RO, CD4, and CD31 for 30 minutes at 4ºC in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma-Aldrich).

3. *In vitro* cell culture

Cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated human AB serum (Sigma-Aldrich, St Louis, MO), 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine (Invitrogen), in the presence or absence of recombinant human (rh) IL-7 (10 ng/mL; R&D Systems, Minneapolis, MN) or rhIL-2 (10 U/mL; obtained through the National Institutes of Health (NIH)/ AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH [IL-2] from Hoffman-La Roche). PI3K and MEK/ERK activity were respectively blocked by incubation of cells for 1 hour at 37°C before IL-7 stimulation with either 10 μM LY294002 or 10 μM PD98059 (both from Calbiochem, Merck Biosciences, Nottingham, United Kingdom) or the equivalent volume of the vehicle control dimethyl sulfoxide (DMSO; Sigma-Aldrich) alone. LY294002, PD98059 and DMSO were re-added to the culture at day 4.

4. Flow cytometric analysis

4.1. Surface staining

Cells resuspended in PBS containing 1% BSA (Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich) were stained for 20 minutes at room temperature with the following anti–human monoclonal antibodies: CD4 phycoerythrin–cyanin 7 (PE-Cy7;

clone L3T4), CD8 peridinin chlorophyll protein (PerCP; clone RPA-T8), CD45RA fluorescein isothiocyanate (FITC) or allophycocyanin (APC; clone HL100), CD45RO PE (clone UCHL1), CD62L APC-Cy7 (clone DREG 56), CD27 FITC (clone O323) and CD31 PE or APC (clone WM59) from eBioscience (San Diego, CA); CD38 PE (clone HB7), CD25 APC (clone 2A3) and CD3 PerCP (clone SK7) from BD Biosciences; and CD127 PE (IL-7Rα; clone 40131; R&D Systems).

4.2. Intracellular staining

Intracellular staining for Bcl-2 FITC (clone 124; Dako, Glostrup, Denmark), Ki67 FITC (clone B56; BD Biosciences) and Foxp3 PE (clone PCH101; eBioscience) was performed using fixation and permeabilization reagents from eBioscience. Samples were acquired on a BD FACSCanto flow cytometer (BD Biosciences) after fixation with 1% formaldehyde (Sigma-Aldrich). Data were analyzed using FlowJo software version 8.1.1 (TreeStar, Ashland, OR).

4.3. Apoptosis assessment

Apoptosis was assessed using 7-aminoactinomycin D (7-AAD) viability Staining Solution (eBioscience) or Annexin V/Propidium Iodide (PI) detection kit (BD Biosciences). For the former assay, cells resuspended in PBS were incubated with 7-AAD for 5 minutes at 4ºC. As for the latter, cells resuspended in 1x Binding Buffer were incubated with Annexin V antibody and PI for 15 minutes at room temperature. Samples were immediately acquired on a BD FACSCanto flow cytometer (BD Biosciences). Data were analyzed using FlowJo software version 8.1.1 (TreeStar, Ashland, OR).

4.4. Proliferation assessment by CFSE dilution assay

Cells were labeled with 0.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes-Invitrogen, Carlsbad, CA) at 37°C for 15 minutes in the dark, quenched with ice-cold culture medium at 4°C for 5 minutes, and washed 3 times before culture. Samples were acquired on a BD FACSCanto flow cytometer (BD Biosciences) after fixation with 1% formaldehyde (Sigma-Aldrich). Data were analyzed using FlowJo software version 8.1.1 (TreeStar, Ashland, OR).

4.5. Assessment of STAT-5 phosphorylation

Cells were surface stained and stimulated with 50 ng/mL of rhIL-7 for 15 minutes, fixed with 2% formaldehyde at 37°C for 10 minutes, and placed on ice. Cells were then

permeabilized with ice-cold 90% methanol (Sigma-Aldrich) at 4°C for 30 minutes and incubated with anti–phospho-STAT-5 (pY694) antibody coupled to Alexa Fluor 488 (BD Biosciences) at room temperature for 1 hour. Samples were immediately acquired on a BD FACSCanto flow cytometer (BD Biosciences). Data were analyzed using FlowJo software version 8.1.1 (TreeStar, Ashland, OR).

4.6. Telomere length measurement by Flow-FISH

Telomere length was measured using a modified version of the fluorescent *in situ* hybridization coupled to flow cytometry (Flow-FISH) protocol that was previously described [13,14]. In brief, PBMCs were surface stained using CD4 FITC (clone RPA-T4; BD Pharmingen), CD8 biotin (clone OKT8, eBioscience), CD45RA biotin (clone HI100, eBioscience), Streptavidin Cy3 (Cedarlane Laboratories) and CD27 FITC (clone O323; eBioscience). After washing in PBS, cells were fixed in 1 mM BS^3 (Perbio Science). The reaction was quenched with 50 mM Tris (pH 7,2) in PBS. After washing in PBS followed by hybridization buffer, cells were incubated in 0.75 μg/ml of the protein nucleic acid telomeric probe $(C_3TA_2)_3$ conjugated to Cy5 (Panagene). After being heated for 10 minutes at 82°C, samples were left to hybridize. Samples were washed in post-hybridization buffer followed by PBS and analyzed immediately by flow cytometry. All samples were run in triplicate alongside cryopreserved PBMC with known telomere fluorescence to ensure consistency of results. Kilobase length was determined from mean fluorescence intensity values using a standard curve. The standard curve was constructed using samples of varying telomere length analyzed both by flow-FISH and telomeric restriction fragment analysis [15].

5. Signal-Joint TREC quantification by Real-Time PCR

DNA was purified from 10^6 PBMCs using DNAzol reagent (Gibco Life Technologies). Signal-joint TRECs were quantified by nested Real-Time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) and ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Specific primers and probes were used for sjTRECs and the CD3γ, used as a housekeeping gene for absolute quantification of sjTRECs levels: sj-out5 5'-CTCTCCTATCTCTGCTCTGAA-3'; sj-out3 5'-ACTCACTTTTCCGAGGCTGA-3'; sj-in5 5'-CCTCTGTCAACAAAGGTGAT-3'; sj-in3 5'-GTGCTGGCATCAGAGTGTGT-3'; CD3-out5 5'-ACTGACATGGAACAGGGGAAG-3'; CD3-out3 5'-CCAGCTCTGAAGTAGGGAACATAT-3'; CD3-in5 5'-

GGCTATCATTCTTCTTCAAGGT-3'; CD3-in3 5'-CCTCTCTTCAGCCATTTAA GTA-3'; sj-Probe1 5'-AATAAGTTCAGCCCTCCATGTCACACTf-3'; sj-Probe2 5'-XTGTTTTCCATCCTGGGGAGTGTTTCAp-3'; CD3-Probe1 5'-GGCTGAAGGTTAG GGATACCAATATTCCTGTCTCf-3'; CD3-Probe2 5'-XCTAGTGATGGGCTCTTCC CTTGAGCCCTTCp-3'. pCD3-TREC plasmid was kindly provided by Rémy Cheynier (Institute Pasteur, Paris).

6. TCR-chain CDR3 spectratyping

Total RNA was extracted from 10^5 to 10^6 cells with RNeasy kit (Qiagen) and first strand cDNA synthesized from 1-2μg of RNA with the Superscript III kit (Invitrogen) using an equivolume mixture of random hexamers and oligo (dT). Spectratyping analysis was performed by Dário Ligeiro from Immunogenetics Laboratory, Centro de Histocompatibilidade do Sul – CHSul. Briefly, amplification of the TCRVB CDR3 was performed using primers specific for each Vβ family [16] except for Vβ6 and Vβ21 [17] and a common CB reverse primer [16]; followed by a run-off reaction that extends each different PCR product with a constant CB FAM labelled primer [16]; and a third step, in which each different Vβ PCR labelled fragment is separated on a capillary electrophoresis based DNA automated sequencer. Data was collected and analyzed with GeneMapper v4.0 (Applied Biosystems) for size and fluorescence intensity determination. The results are depicted as peaks and classified as normal polyclonal repertoire, if the CDR3 in-frame transcript distribution has a Gaussian shape with 8 to 10 peaks for each Vβ family, or skewed if there is predominance of a few classes of clonotypes, according with the scoring previously detailed [18,19].

7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). Data are presented as mean plus or minus standard error of mean (SEM). *P* values less than 0.05 were considered significant: * indicates $P < 0.05$; ** indicates $P < 0.001$; *** indicates $P < 0.0001$.

Results

Chapter 1.1

IL-7 sustains CD31 expression in human naive CD4$^+$ T cells and preferentially expands the CD31$^+$ subset in a PI3K-dependent manner

The expression of CD31 has been proposed to identify two subsets within the naive CD4$^+$ T cell pool which have undergone different levels of post-thymic proliferation [9]. The CD31$^+$ naive CD4$^+$ T cell subset has been described as comprising the RTE population [9-12], whereas the CD31$^-$ naive CD4$^+$ T cell subset has been proposed to arise following homeostatic proliferation in the periphery [9,12]. IL-7 is a key modulator of naive T cell homeostasis [20-23], promoting survival and mediating homeostatic proliferation of the naive CD4$^+$ T cell pool [24]. Interestingly, naive T cells, particularly CD31$^+$ naive CD4$^+$ T cells, have been shown to proliferate following IL-7 administration in a clinical trial [25]. Therefore, we sought to investigate potentially distinct effects of IL-7 *in vitro* stimulation of CD31$^+$ and CD31$^-$ naive CD4$^+$ T cell subsets from umbilical cord and adult peripheral blood.

In order to circumvent possible fluctuations in CD31 expression during IL-7 stimulation, we purified the CD31$^+$ and CD31$^-$ subsets within naive CD4$^+$ T cells by FACS sorting prior to culture (Figure 1). A representative gating strategy for the isolation of CD31 subsets from adult and cord samples by selecting the CD31^{bright} and CD31^{low} populations within naive (CD45RA$^+$ CD45RO$^-$) CD4$^+$ T cells is illustrated on Figure 1A. The resulting post-sort populations were highly pure (Figure 1B).

We first investigated the expression of the cell cycle entry marker Ki67 on the CD31 naive CD4$^+$ T cell subsets following a 7 day culture period in the presence of IL-7 (Figure 2A). As previously described [5,6,8], IL-7-induced cycling is more potent in naive CD4$^+$ T cells derived from cord blood than from adult peripheral blood (Figure 2A). Moreover, only 12 out of the 22 adult samples studied entered cell cycle in the presence of IL-7, whereas a substantial proportion of all 12 cord blood samples assessed expressed Ki67 in response to IL-7. The adult IL-7-responders did not significantly differ from non-responders in terms of sex distribution, proportion of naive (CD45RA$^+$) cells within the

$CD4^+$ T cell population, proportion of $CD31^+$ cells within naive or total $CD4^+$ T cell population, nor proportion of IL-7Rα^+ cells within $CD31^+$, $CD31^-$ or total naive $CD4^+$ T cells (data not shown). Of note, the average age of adult IL-7-responders was lower than of non-responders although the age distribution was not significantly different between them (28.9 ± 2.42 years vs 36.4 ±3.41 years, respectively; *P* = .109).

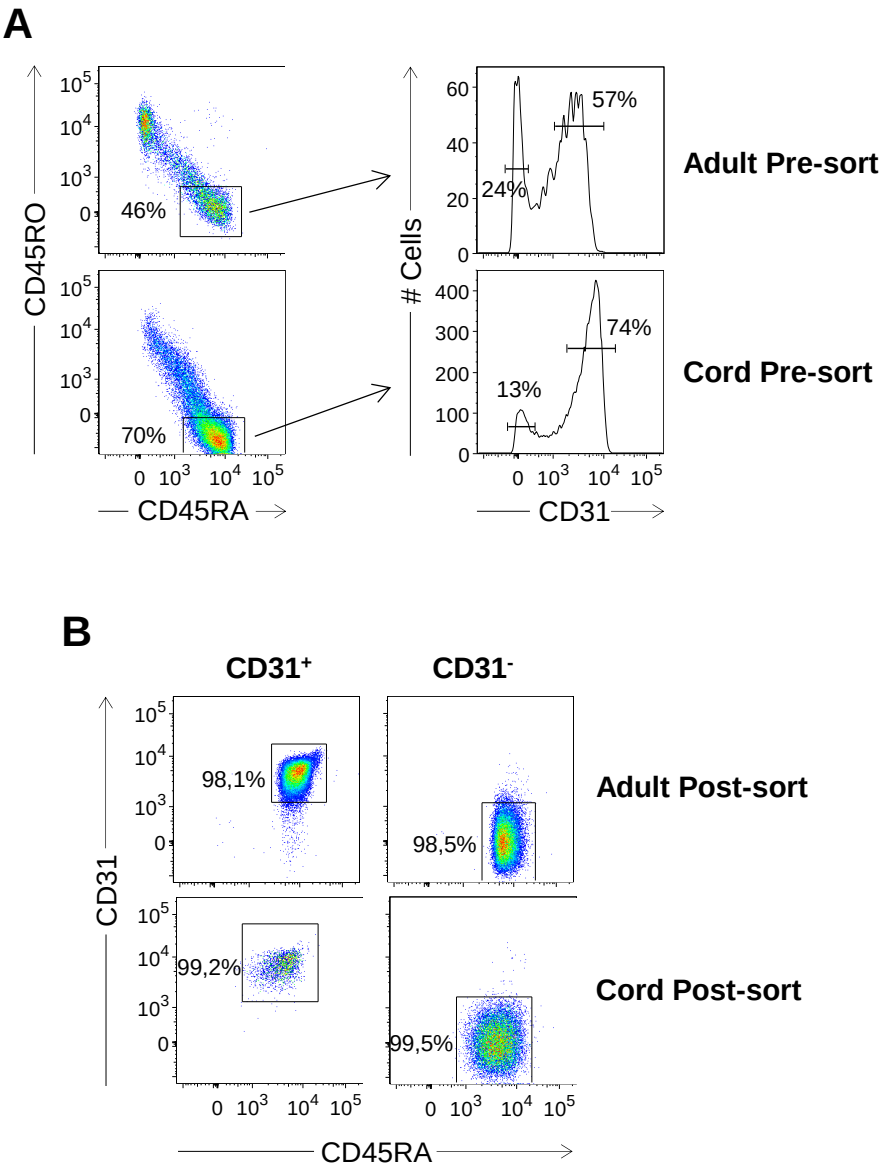

Figure 1: CD31 expression profiles and gating strategy used to purify $CD31^+$ and $CD31^-$ naive $CD4^+$ T cell subsets from adult and cord blood.

$CD4^+$ T cells were negatively selected using the EasySep Human $CD4^+$ T cell Enrichment Kit and stained using monoclonal antibodies for CD4, CD45RO, CD45RA and CD31. A) Representative flow cytometry profiles of $CD4^+$ T cells stained for CD45RO, CD45RA and CD31 are shown for adult and cord blood samples. Also shown is the gating strategy used for FACS sorting. After gating on viable lymphocytes and $CD4^+$ T cells, cells were gated on $CD45RA^+$ and $CD45RO^-$ expression followed by tight gates on $CD31^+$ and $CD31^-$ cells. B) Representative pseudo-colour plots showing the purity of $CD31^+$ and $CD31^-$ subsets isolated by FACS sorting.

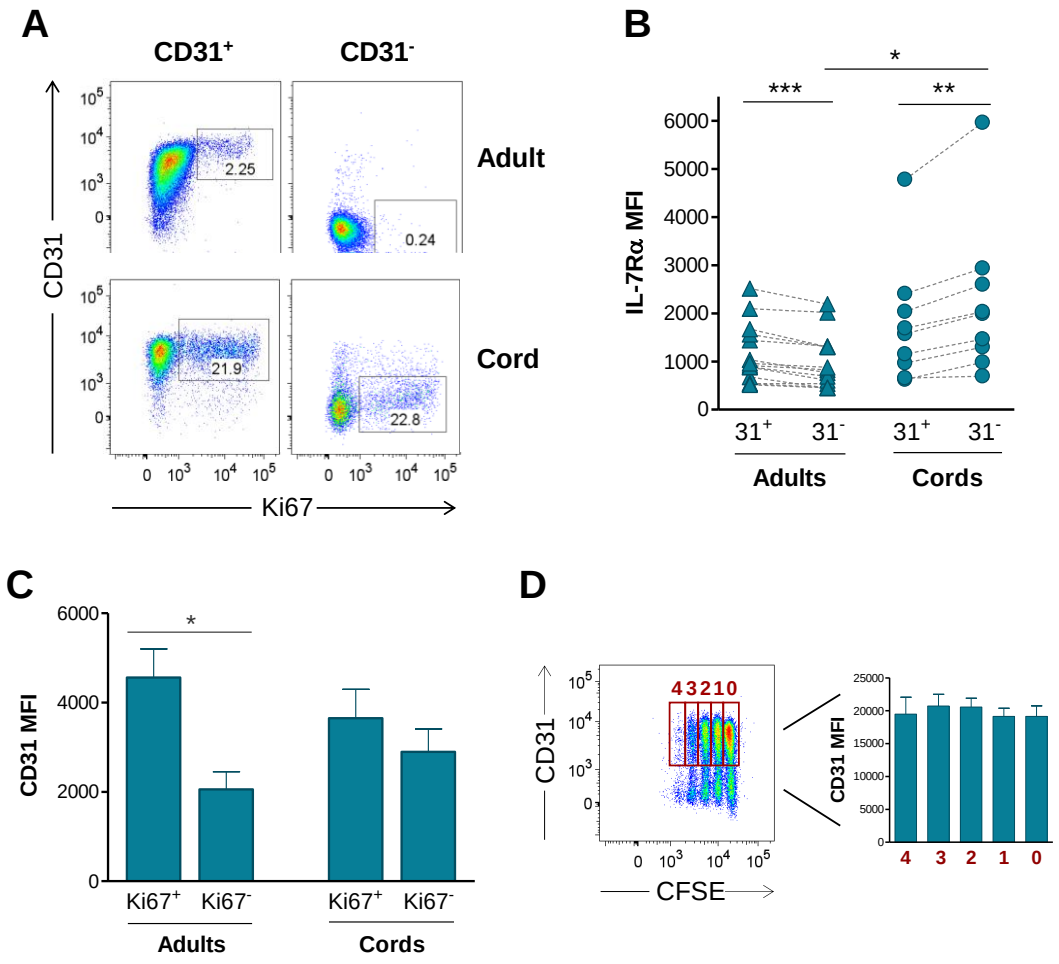

Figure 2: IL-7-induced cycling of adult naive CD4$^+$ T cells is restricted to the CD31$^+$ subset.

A) Representative dot-plots of CD31 and Ki67 flow cytometric analysis of purified CD31$^+$ and CD31$^-$ naive CD4$^+$ T cell subsets from adult (top panel) and cord blood (lower panel) samples cultured in the presence of IL-7 for 7 days. B) *Ex vivo* analysis of IL-7Rα median fluorescence intensity (MFI) on freshly isolated mononuclear cells from adult and cord blood samples. Each symbol represents one individual. C) CD31 MFI was assessed within the purified CD31$^+$ naive subset further gated on Ki67$^+$ or Ki67$^-$ cells after 7 days in culture with IL-7. Three adults and four cord blood samples were studied. D) Representative dot-plot illustrating CFSE labelling of cord blood CD45RA$^+$ CD4$^+$ T cells cultured with IL-7 for 7 days. CD31$^+$ cells were further gated according to the number of cell divisions and bars show CD31 MFI from four experiments. Bars represent mean±SEM. Statistical analysis was performed using paired or unpaired *t* test as appropriate (GraphPad Prism).

Within adult IL-7-responders, only CD31-expressing naive $CD4^+$ T cells were able to enter cell cycle in response to IL-7 (Figure 2A, upper panel), although the proportion of Ki67-expressing cells was significantly lower than within cord blood $CD31^+$ naive $CD4^+$ T cells (2.82% ± 1.11% vs 26.7% ± 3.22% $Ki67^+$ cells, respectively; $P = 0.001$). On the other hand, IL-7 induced cell cycling of both $CD31^+$ and $CD31^-$ naive $CD4^+$ T cells from cord blood (Figure 2A, lower panel). We next investigated if the proliferative responses to IL-7 correlated with the basal levels of IL-7Rα expression (Figure 2B). Although adult $CD31^+$ naive $CD4^+$ T cells expressed significantly higher levels of IL-7Rα than their $CD31^-$ counterparts, the opposite was true for cord blood samples (Figure 2B). Furthermore, the levels of IL-7Rα were significantly higher on cord blood than on adult $CD31^-$ cells, whereas $CD31^+$ cells from cord blood and adult samples expressed similar levels of this marker (Figure 2B). Thus the *ex vivo* levels of IL-7Rα expression did not correlate with the extent of the proliferative responses to IL-7, suggesting that alternative factors other than the basal levels of IL-7Rα expression might influence the ability to undergo IL-7-driven proliferation.

Given that CD31 down-modulation has been proposed to be triggered by homeostatic proliferation, we next assessed if IL-7-driven cycling was associated with decreased CD31 expression on adult and cord blood $CD31^+$ naive $CD4^+$ T cells. As shown on Figure 2C, we found no significant differences on the intensity of CD31 expression between cycling and non-cycling cord blood $CD31^+$ naive $CD4^+$ T cells, whereas cycling adult $CD31^+$ naive $CD4^+$ T cells expressed significantly higher levels of CD31 than non-cycling cells. These results suggest that CD31 expression is not lost upon IL-7-driven cycling. We further confirmed this hypothesis by investigating if the number of cell divisions affected the level of CD31 expression. For that purpose, we performed a CFSE dilution assay on IL-7-stimulated naive $CD4^+$ T cells from cord blood and assessed the intensity of CD31 expression within non-proliferating as well as within each generation of proliferating $CD31^+$ cells (Figure 2D). As previously described [8,26], the low levels of cell cycling within adult naive $CD4^+$ T cells in response to IL-7 precluded the performance of this assay on adult samples. The intensity of CD31 expression remained relatively high throughout the rounds of cell division (Figure 2D). Hence IL-7-driven proliferation of cord blood $CD31^+$ naive $CD4^+$ T cells did not significantly affect CD31 expression levels (paired *t* test comparing all generations; data not shown).

We further assessed if, in addition to maintaining CD31 expression levels on $CD31^+$ naive $CD4^+$ T cells, IL-7 stimulation could induce CD31 re-expression on $CD31^-$ naive $CD4^+$ T cells (Figure 3). Although we observed a significant increase in the intensity of CD31 expression on $CD31^+$ naive $CD4^+$ T cells after a 7 day culture period with IL-7, CD31 expression on both adult and cord blood $CD31^-$ naive $CD4^+$ T cells remained virtually undetectable (Figure 3A). These results were confirmed with a CD31 expression time-course, during which we observed consistently high levels of CD31 within adult $CD31^+$ naive $CD4^+$ T cells for up to 13 days in the presence of IL-7 (Figure 3B). Culture of these cells in medium alone (Control) or in the presence of IL-2 induced a similar reduction in the intensity of CD31 expression (Figure 3B). As for adult $CD31^-$ naive $CD4^+$ T cells, the extremely low levels of CD31 expression quantified immediately after purification (Day 0) were maintained throughout the time-course in all culture conditions tested (Figure 3B). Hence IL-7 stimulation does not induce the loss of CD31 expression on $CD31^+$ nor its re-expression on $CD31^-$ naive $CD4^+$ T cells.

In order to investigate if the failure of adult $CD31^-$ naive $CD4^+$ T cells to proliferate in the presence IL-7 was due to an overall inability to respond to IL-7 stimulation, we assessed several markers associated with IL-7 responsiveness (Figure 4). The expression of IL-7Rα has been described to be down-modulated in the presence of IL-7 [27,28]. Thus, we measured the expression of IL-7Rα before and after 7 days of culture with IL-7, and observed that its levels were dramatically decreased in all the subsets following IL-7 stimulation (Figure 4A). Bcl-2 is an anti-apoptotic protein that is up-regulated by IL-7-induced signalling [29]. Again all subsets responded to IL-7 stimulation by expressing substantially higher levels of Bcl-2 following 7 days in culture with IL-7 (Figure 4B). IL-7-induced Bcl-2 up-regulation is known to involve the activation of the JAK/STAT signalling pathway and the subsequent phosphorylation of STAT-5 [29-31,31]. After a short stimulation with IL-7, STAT-5 phosphorylation was enhanced in comparison with cells left unstimulated (Control) for the same period of time, regardless of the subset studied (Figure 4C). We next assessed the incorporation of the viability dye 7-AAD after a 7 day culture period in the presence or absence (Control) of IL-7 (Figure 4D). As previously described [3-7], cord blood naive $CD4^+$ T cells were highly susceptible to spontaneous apoptosis (Figure 4D). The presence of IL-7 was able to reduce the proportion of non-viable cells to negligible levels within $CD31^+$ and $CD31^-$ naive $CD4^+$ T cells from cord blood as well as from adult samples (Figure 4D). Taken together, these data indicate that

although adult CD31$^-$ naive CD4$^+$ T cells do not proliferate following IL-7 stimulation, they are responsive to IL-7-induced survival signals. Thus, despite inducing distinct proliferative outcomes in CD31$^+$ and CD31$^-$ naive CD4$^+$ T cells, IL-7 promotes the survival of both subsets, in association with STAT-5 phosphorylation and Bcl-2 up-regulation.

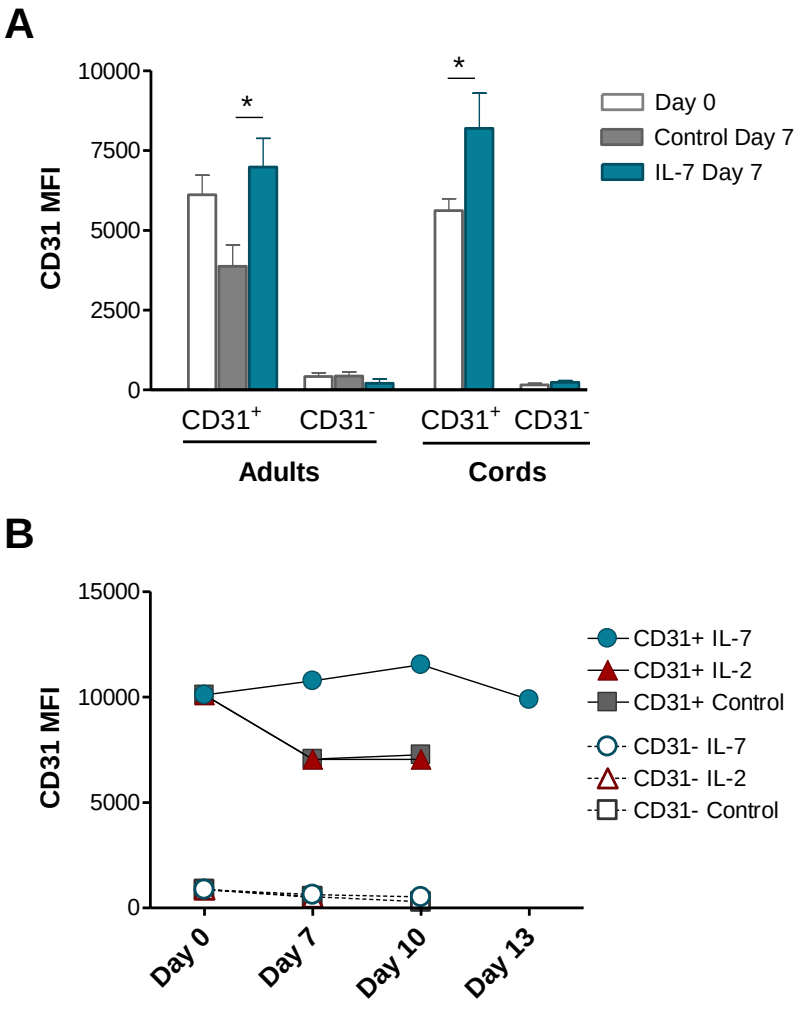

Figure 3: IL-7 promotes the maintenance but not re-expression of CD31 on both adult and cord blood naive CD4$^+$ T cells.

A) Bar graph shows the MFI of CD31 expression on purified CD31$^+$ and CD31$^-$ naive CD4$^+$ T cells from adult (n=13) and cord blood (n=5) samples before (Day 0) and after 7 days in the presence or absence (Control) of IL-7. Analysis of cord blood subsets cultured in the absence of IL-7 was precluded by the high rate of cell death. B) Longitudinal analysis of CD31 MFI of adult naive CD4$^+$ subsets cultured in the presence of IL-7, IL-2 or medium alone (Control) for up to 13 days (data representative of three individuals). Open symbols represent CD31$^+$ purified cells while closed symbols correspond to the CD31$^-$ fraction. Statistical analysis was performed using paired *t* test (GraphPad Prism).

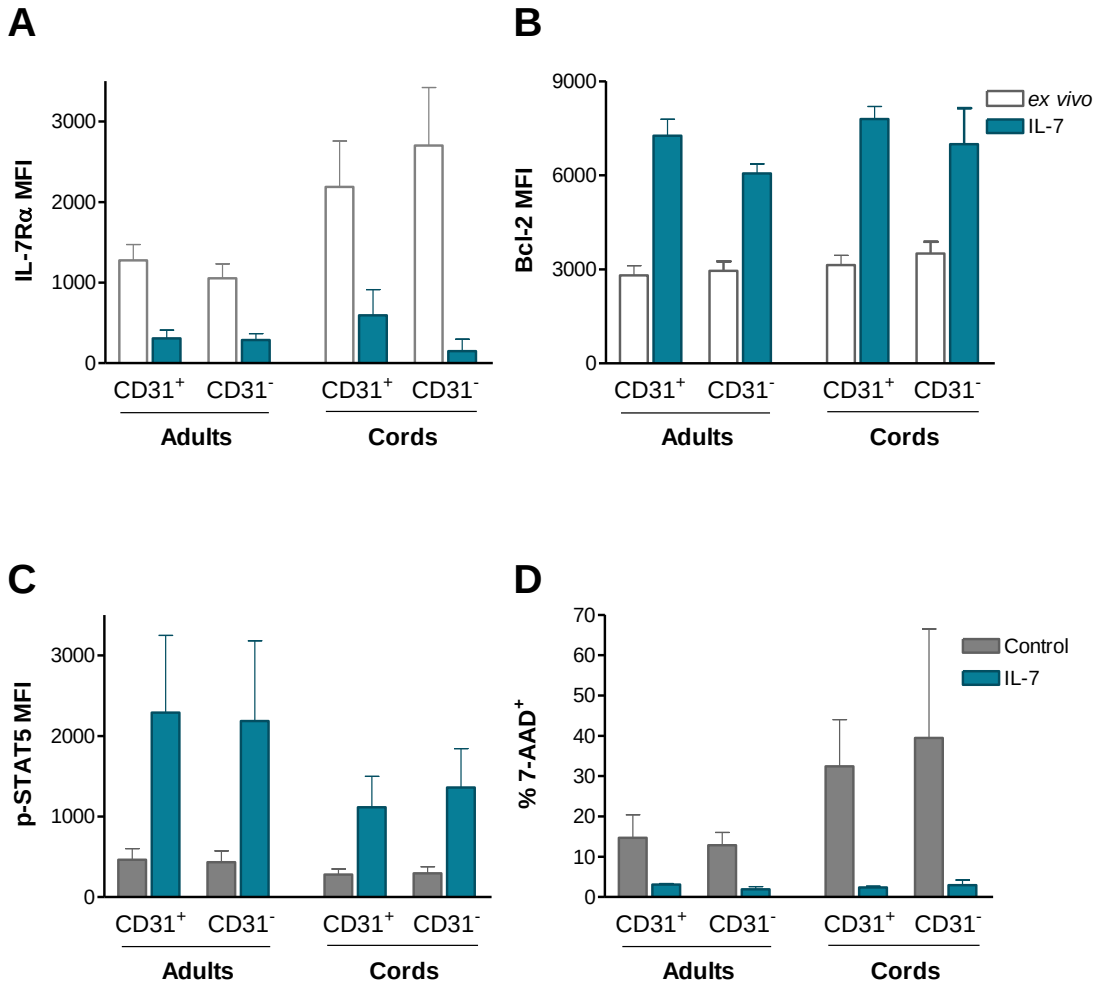

Figure 4: IL-7 stimulation leads to IL-7Rα down-modulation, Bcl-2 up-regulation, STAT5 phosphorylation and rescue from apoptosis in both CD31$^+$ and CD31$^-$ naive CD4$^+$ subsets.

IL-7Rα (A), Bcl-2 (B), and p-STAT5 (C) expression levels as well as 7-AAD incorporation (D) were evaluated by flow cytometry within gated CD31$^+$ and CD31$^-$ naive CD4$^+$ subsets. IL-7Rα and Bcl-2 MFI were evaluated *ex vivo* in adult PBMC (n=6 and n=9, respectively) and cord blood cells (n=4 and n=6, respectively) and in the corresponding purified CD31$^+$ and CD31$^-$ naive subsets cultured in the presence of IL-7 for 7 days. p-STAT5 was assessed on freshly isolated mononuclear cells from adult (n=5) and cord blood (n=3) samples either stimulated with IL-7 for 15 minutes or left unstimulated for the same period of time (Control). 7-AAD incorporation was measured in purified CD31$^+$ and CD31$^-$ subsets after 7 days of culture in the presence or absence (Control) of IL-7. Bars represent mean MFI values ± SEM.

The next important question was which signalling pathways were upstream of the observed IL-7-induced proliferative responses. The PI3K pathway constituted a likely candidate given its suggested role in the IL-7-induced modulation of cell survival, growth, metabolism and proliferation [32]. Interestingly, the PI3K pathway has been shown to be essential for the proliferation but not the survival of cord blood naive $CD4^+$ T cells [3]. The MEK/ERK pathway is a member of the MAPK family which is thought to be mainly activated by growth-promoting mitogenic factors [33] and has been shown to be activated in T-ALL cells by IL-7 [34]. Hence we first assessed the effects of blocking either the PI3K or the MEK/ERK signalling pathways on the cycling and proliferation levels in response to IL-7 stimulation (Figure 5). For this purpose, cells were incubated with cell-permeable specific inhibitors of either the PI3K or MEK/ERK pathways, LY294002 or PD98059 respectively, prior to culture with IL-7. As illustrated in Figure 5, blocking the PI3K pathway abrogated the IL-7-induced cycling of the adult $CD31^+$ subset and proliferation of cord blood naive $CD4^+$ T cells. In contrast, blocking the MEK-ERK pathway had negligible effects on the proliferative responses to IL-7 (Figure 5).

We next assessed if inhibiting the PI3K pathway also impacted the modulation of Bcl-2 and IL-7Rα expression induced by IL-7 (Figure 6). Neither the up-regulation of Bcl-2 (Figure 6A) nor the down-regulation IL-7Rα (Figure 6B) expression was substantially affected by blocking the PI3K pathway. Similar results were obtained when the MEK/ERK pathway was blocked (Figure 6). In order to investigate whether the PI3K pathway was also essential for the IL-7-induced pro-survival effects, we assessed the impact of the PI3K and MEK/ERK inhibitors on the apoptosis levels of IL-7-stimulated naive $CD4^+$ T cell subsets using Annexin V staining and PI incorporation (Figure 7). As illustrated by the representative experiment shown in Figure 7, the proportion of apoptotic (Annexin V^+ PI^-) and dead (Annexin V^+ PI^+) cells was not substantially affected by the inhibition of either the PI3K or the MEK/ERK pathways. However we observed a slight reduction in the viability of adult $CD31^-$ naive $CD4^+$ T cells in the presence of the PI3K inhibitor LY294002 (Figure 7). A previous study has reported a minimal decrease in cell viability of human naive $CD4^+$ T cells in the presence of the PI3K inhibitor LY294002, although this decrease was observed both in the presence and in the absence of IL-7 [3]. Thus the minor impact of the PI3K inhibition on the survival of adult $CD31^-$ naive $CD4^+$ T cells might reflect an IL-7-independent effect of LY294002 itself.

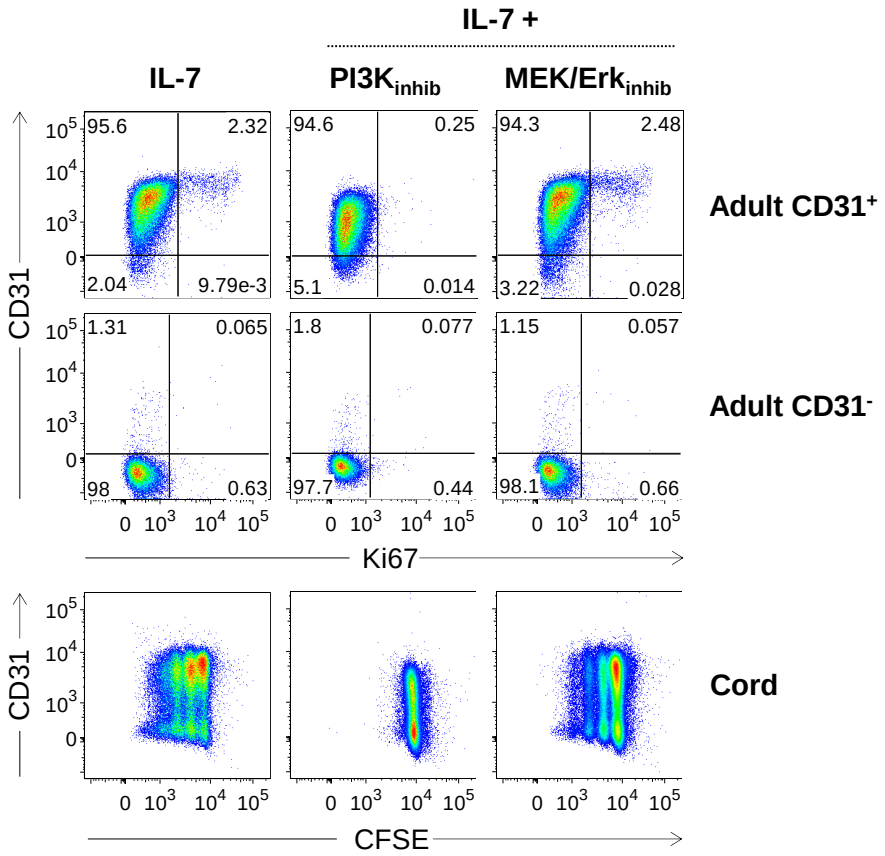

Figure 5: IL-7-induced proliferation of adult CD31$^+$ and cord blood naive CD4$^+$ T cells is dependent on the PI3K pathway.

Purified CD31$^+$ and CD31$^-$ subsets from adult as well as total naive CD4$^+$ T cells from cord blood were cultured in the presence of IL-7 with or without the PI3K inhibitor LY294002 or the MEK/ERK inhibitor PD98059 for 7 days. Proliferation was assessed using Ki67 in adult subsets. CFSE labelling was used to measure proliferation on whole naive CD4$^+$ T cells from cord blood samples. Representative examples out of six adults and four cord blood studied are shown.

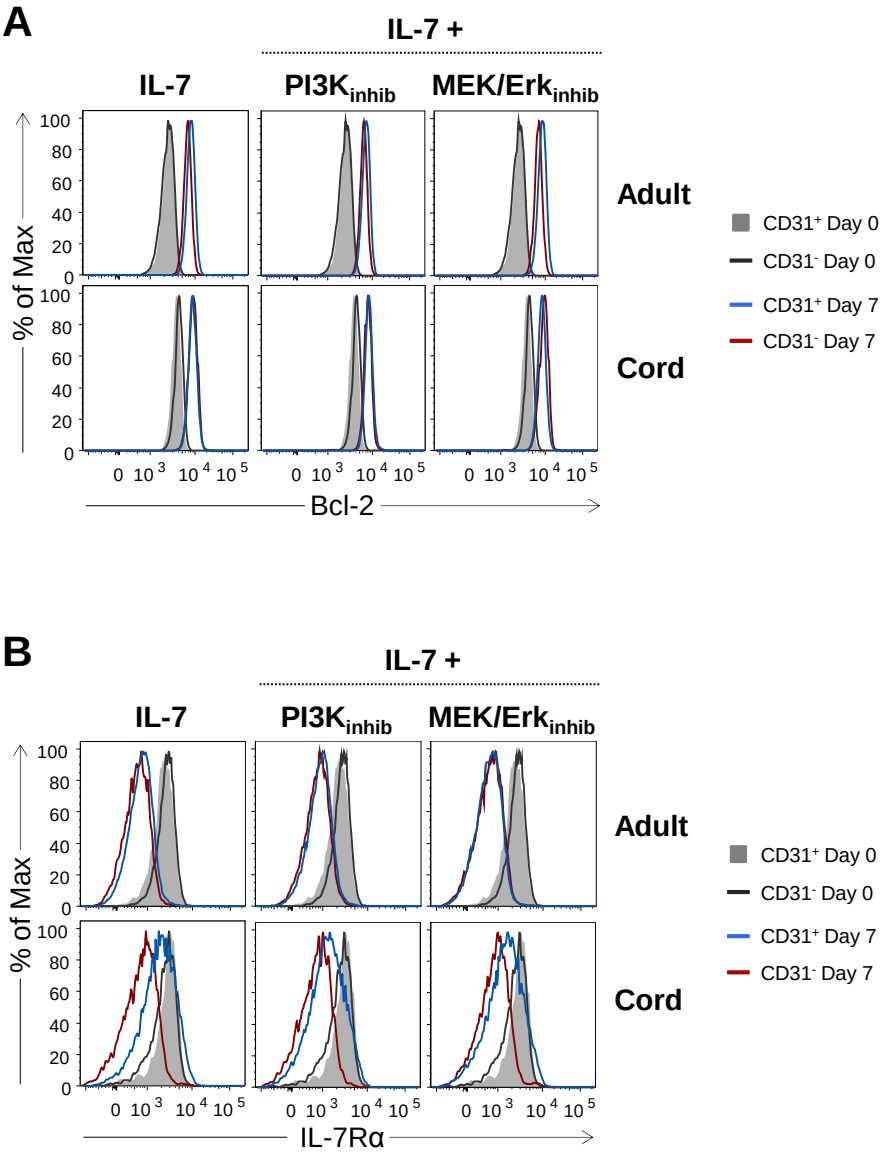

Figure 6: Bcl-2 and IL-7Rα expression on adult CD31$^+$ naive CD4$^+$ T cells is independent of the PI3K pathway.

Purified CD31$^+$ and CD31$^-$ naive CD4$^+$ T cells from adult and cord blood were cultured in the presence of IL-7 with or without the PI3K inhibitor LY294002 or the MEK/ERK inhibitor PD98059 for 7 days. Overlays show Bcl-2 (A) and IL-7Rα (B) expression at day 0 within CD31$^+$ (grey filled) and CD31$^-$ cells (black line), and at day 7 within CD31$^+$ (blue line) and CD31$^-$ (red line) cells. Representative examples out of six adults and four cord blood studied are shown.

Finally, we assessed the impact of PI3K or MEK/ERK inhibition on the expression levels of CD31 and of the CD31 ligand CD38 [35] on IL-7-stimulated CD31$^+$ naive CD4$^+$ T cells from cord blood as well as from adult samples (Figure 8). Blocking the PI3K pathway resulted in significantly lower levels of CD31 expression on both adult and cord blood CD31$^+$ naive CD4$^+$ T cells in comparison with the CD31 levels obtained after culture with IL-7 alone ($P = 0.002$ and $P = 0.009$, respectively, paired *t* test ; Figure 8A). Interestingly, the loss of the IL-7-induced up-regulation of CD31 was observed both in adults with a proliferative response to IL-7 (filled symbols) and in IL-7-non-responders (opens symbols) (Figure 8A), suggesting that the PI3K pathway independently mediates the maintenance of CD31 expression and the proliferative responses induced by IL-7 stimulation. As previously described [4], IL-7 stimulation significantly down-modulated CD38 expression on cord blood naive CD4$^+$ T cells to levels similar to those observed on adult naive CD4$^+$ T cells ($P < 0.001$, paired *t* test; Figure 8B). The levels of CD38 were also significantly decreased on adult naive CD4$^+$ T cells following IL-7 stimulation ($P < 0.001$, paired *t* test; Figure 8B). Blocking the PI3K or MEK/ERK pathways did not significantly affect the levels of CD38 on either adult or cord blood naive CD4$^+$ T cells (Figure 8B). These data suggest that the PI3K pathway is involved in the IL-7-induced modulation of CD31 but not of CD38 expression.

Overall we showed that *in vitro* stimulation with IL-7 alone is able to induce cell cycling and up-regulation of CD31 expression on adult CD31$^+$ naive CD4$^+$ T cells. Moreover, both CD31$^+$ and CD31$^-$ naive CD4$^+$ T cells from cord blood samples proliferated in the presence of IL-7. Finally, we demonstrated that the PI3K pathway plays a major role on the IL-7-induced effects on proliferation and CD31 expression but not on survival of naive CD4$^+$ T cells.

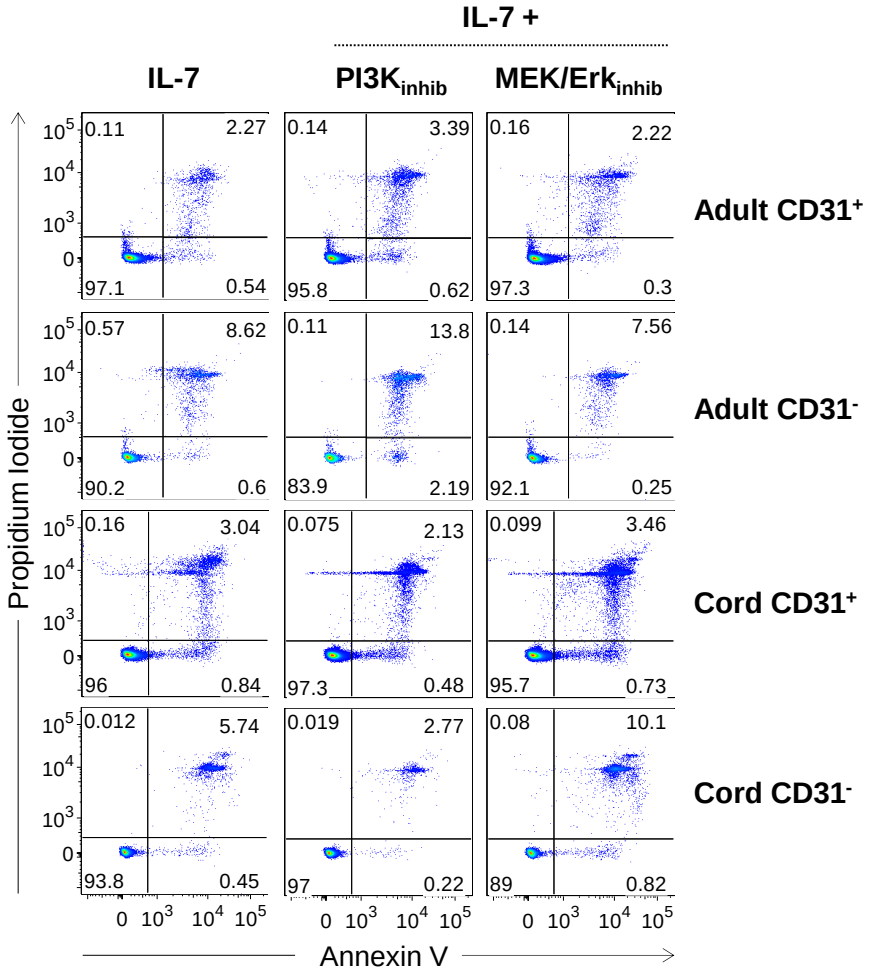

Figure 7: IL-7-mediated survival of naive CD4$^+$ T cell subsets is only minimally affected by PI3K inhibition.

Purified CD31$^+$ and CD31$^-$ naive CD4$^+$ T cells from adult and cord blood were cultured in the presence of IL-7 with or without the PI3K inhibitor LY294002 or the MEK/ERK inhibitor PD98059 for 7 days. Representative pseudo-colour plots are shown illustrating the evaluation of apoptosis by Annexin V and Propidium Iodide (PI) staining out of six adults and four cord blood studied.

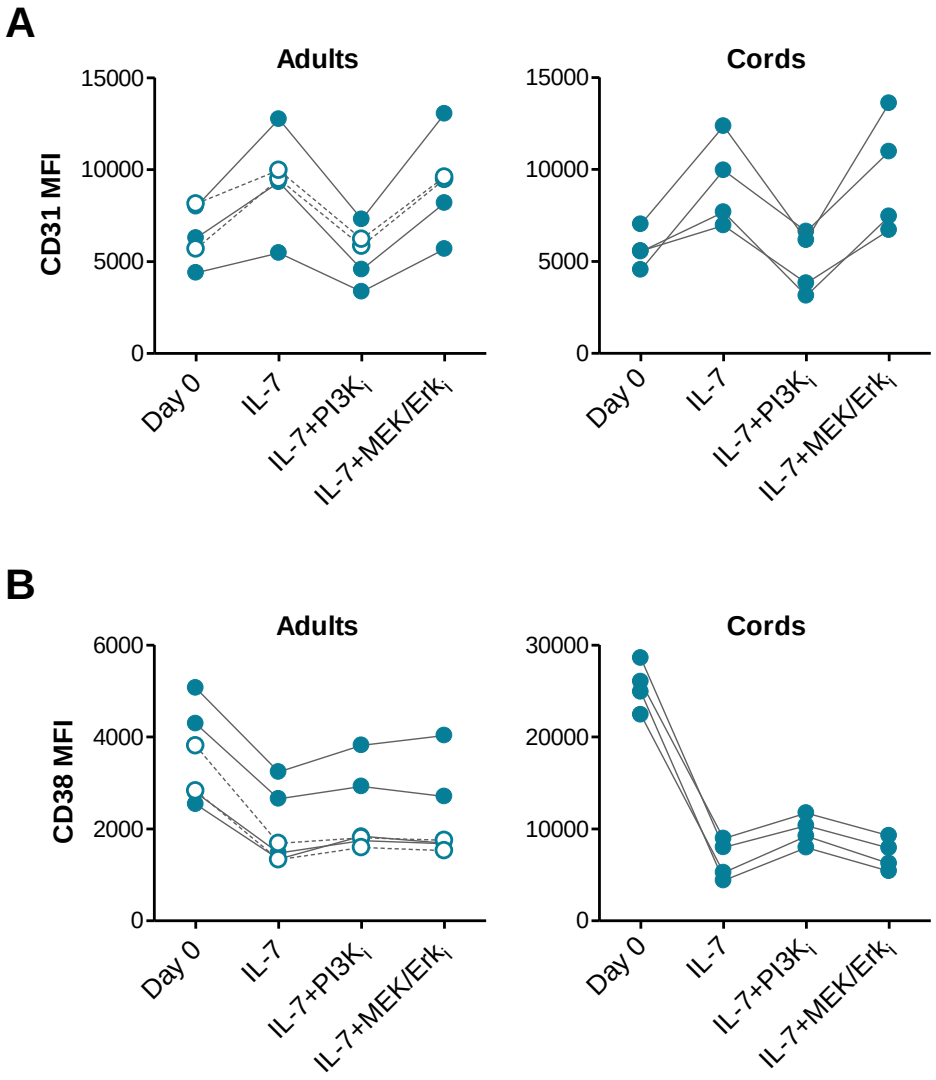

Figure 8: IL-7-mediated CD31 maintenance on both adult and CB naive CD4$^+$ T cells is dependent on the PI3K pathway.

A) CD31 MFI was assessed on purified CD31$^+$ naive CD4$^+$ T cells at day 0 and after 7 days in culture with IL-7 alone or in addition to LY294002 or PD98059. Each symbol represents one individual. B) CD38 MFI is shown in the same culture conditions in adult (n =6) and cord blood (n=4) samples, respectively. Filled symbols refer to individuals with a proliferative response to IL-7 and open symbols to those that did not proliferate.

Chapter 1.2

Long Term Immune Reconstitution Following Haplotype-Mismatched Hematopoietic Stem Cell Transplantation

Haploidentical HSCT is an allogeneic stem cell transplant from a family donor who shares only one haplotype with the recipient [36]. The mismatched $CD34^+$ stem cell graft infused into the patient contains only residual numbers of T cells, which leads to the generation of a donor-derived immune system wherein thymic T cell selection will take place in an HLA-mismatched environment [37]. The use of intensive conditioning regimens, together with the use of T cell depleted grafts, leads to major imbalances in T cell homeostasis and provide a tool to study the mechanisms of *de novo* T cell reconstitution, albeit in a HLA-mismatched environment. Although this therapeutic approach is increasingly adopted in patients with hematological malignancies who lack a suitable related or unrelated HLA-matched donor, there are few studies characterizing immune reconstitution following this type of HSCT [38,39]. In the present study, we performed a detailed evaluation of the composition of naive and memory T cell pools in a group of patients who underwent haploidentical related HSCT after a chemotherapy-alone conditioning regimen for the treatment of high risk leukemia [40]. These patients were at the time of the study four to six years post-transplant and were studied in parallel with the respective donors, who were always one of the parents, and age-matched healthy controls.

We first evaluated the degree of immune reconstitution in the transplant recipients by assessing the absolute numbers of lymphocyte subsets in peripheral blood (Figure 9). Although we observed a tendency for slightly lower numbers of total lymphocytes (Figure 9A) and T cells, as assessed by the expression of CD3 (Figure 9B), in transplant recipients when compared to donors and age-matched controls, the lymphocyte and T cell counts were not statistically different between the three cohorts. Conversely, transplant patients tended to have higher absolute numbers of B cells, identified by the expression of CD19 (Figure 9C), than the other two groups, but again these differences did not reach statistical significance. Similarly, the number of NK cells, identified by the co-expression of CD16 and CD56 (Figure 9D), were not statistically different when recipients, donors and age-matched controls were compared.

Taken together, these data suggest that our cohort of haploidentical HSCT recipients featured similar numbers of basic lymphocyte subsets as compared to age-matched controls, indicating a successful reconstitution of the size of these peripheral lymphocyte pools.

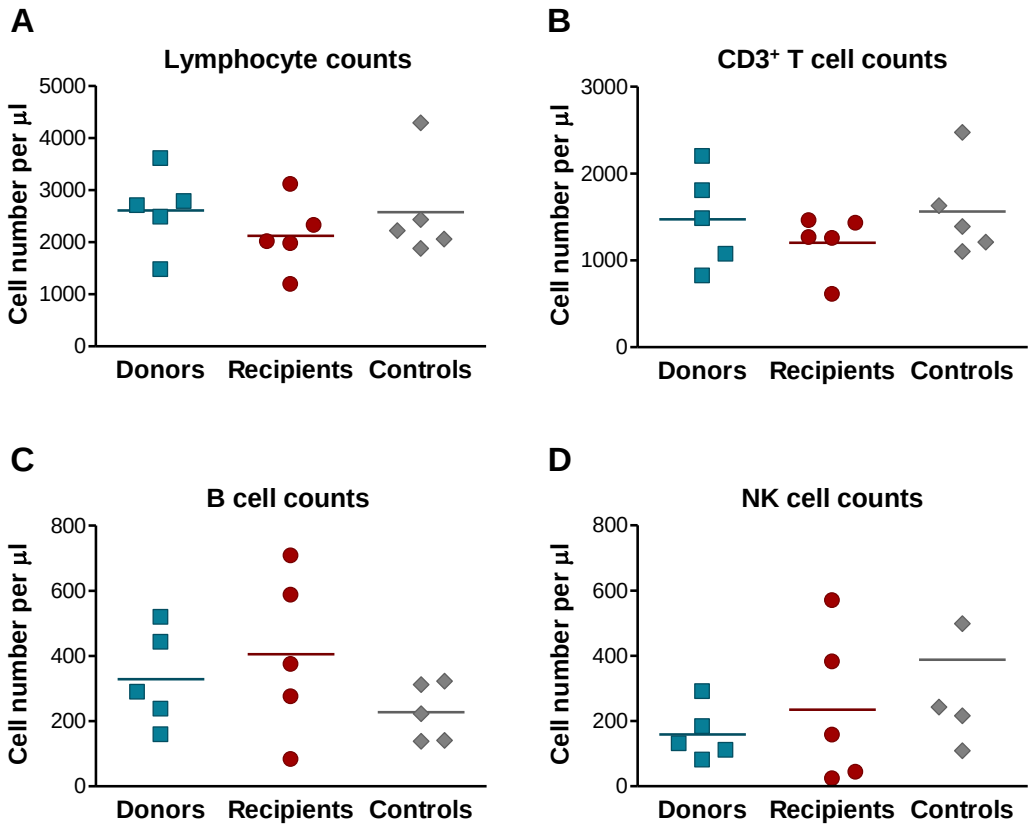

Figure 9: Absolute numbers of basic lymphocyte subsets.

Absolute numbers of basic lymphocyte subsets in peripheral blood were determined in donors, recipients and age-matched controls. Graphs show the cell number per µl of total lymphocytes (A), T cells identified by CD3 expression (B), B cells identified by CD19 expression and natural killer cells identified by the co-expression of CD56 and CD16 (D). Each symbol represents an individual. Mean values are shown as horizontal lines. There was no statistically significant difference in the absolute numbers of the different lymphocyte subsets when the three cohorts were compared. Statistical analysis was performed using the Wilcoxon matched pairs test (GraphPad Prism).

We next assessed the absolute numbers of $CD4^+$ (Figure 10A) and $CD8^+$ (Figure 10B) T cells in the three cohorts. We observed a modest decrease in $CD4^+$ T cell counts in recipients as compared to donors and age-matched controls (Figure 10A). Nevertheless, we found no statistically significant differences in $CD4^+$ (Figure 10A) and $CD8^+$ (Figure 10B) T cell counts between the three cohorts. These results further indicate that the size of the T cell pool has been effectively restored in haploidentical HSCT recipients. The high degree of HLA mismatch between donor and recipient does not seem to hinder long term immune reconstitution in these patients, although it might preferentially impact the restoration of the $CD4^+$ T cell pool.

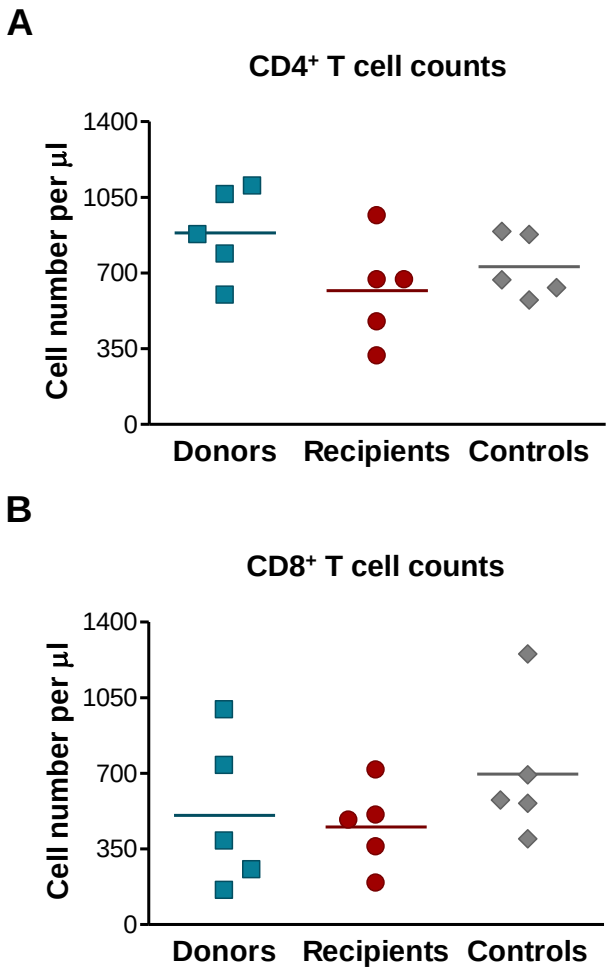

Figure 10: Absolute numbers of $CD4^+$ and $CD8^+$ T cells.

Absolute numbers of $CD4^+$ (A) and $CD8^+$ (B) T cells in peripheral blood were determined in donors, recipients and age-matched controls. The results are expressed as cell number per µl. Each symbol represents an individual. Mean values are shown as horizontal lines. There was no statistically significant difference in the absolute numbers of $CD4^+$ (A) and $CD8^+$ (B) T cells when the three cohorts were compared. Statistical analysis was performed using the Wilcoxon matched pairs test (GraphPad Prism).

In order to investigate whether T cell reconstitution was associated with any imbalances in naive and memory T cell subset distribution, we determined the frequency of naive and memory subsets as defined by the expression of CD45RA and CD27 within $CD4^+$ and $CD8^+$ T lymphocytes (Figure 11). We observed a tendency for a greater proportion of naive $CD45RA^+CD27^+$ $CD4^+$ (Figure 11A) and $CD8^+$ (Figure 11B) T cells in transplant recipients as compared to donors and age-matched controls. On the contrary, recipients showed lower frequencies of the highly differentiated $CD45RA^-CD27^-$ and $CD45RA^+CD27^-$ memory subsets both within $CD4^+$ (Figure 11A) and $CD8^+$ (Figure 11B) T cell populations, particularly when compared to donors. These differences were more striking within $CD8^+$ T cells, although they did not reach statistical significance (Figure 11B). The levels of naive T cells have been shown to decrease, whereas highly differentiated memory T cells increase, during ageing [1,41-47]. Hence the distinct differentiation state profiles observed in recipients and donors are likely due to the age gap between these two cohorts, given that the donors were always one of the parents. Nonetheless, recipients tended to have a "younger" profile than age-matched controls, suggesting that age is not solely responsible for these differences.

We next sought to assess the mechanisms underlying T cell reconstitution in these patients, in particular the relative contribution of thymic output and peripheral expansion. In order to achieve this, we assessed the levels of Recent Thymic Emigrants, as estimated by the expression of CD31 within naive $CD4^+$ T cells and quantification of signal-joint TRECs (sjTRECs), and measured telomere length as an indicator of peripheral expansion (Figures 12, 13). The detrimental effects of ageing, disease and conditioning regimens on thymic function might limit the replenishment of the naive T cell pool with *de novo* generated T cells and thus favour immune reconstitution via peripheral expansion [48-52]. Nevertheless, the increase in TREC content observed within $CD4^+$ T cells in adults following highly active anti-retroviral therapy in HIV-infected patients [53-57], as well as after stem cell transplantation [58,59], suggest that the adult thymus retains the ability to generate new T cells. Several studies have used CD31 expression within naive $CD4^+$ T cells to indirectly assess thymic output following hematopoietic stem cell transplantation [11,60,61]. Although CD31 expression cannot be considered an absolute marker of Recent Thymic Emigrants given that $CD31^+$ naive $CD4^+$ T cells are able to undergo IL-7-driven homeostatic proliferation without losing CD31 expression (Figure 2), it identifies the population that is most enriched in newly generated T cells, as supported by the

observation that practically all *de novo* produced naive $CD4^+$ T cell after autologous stem cell transplantation express CD31 [61].

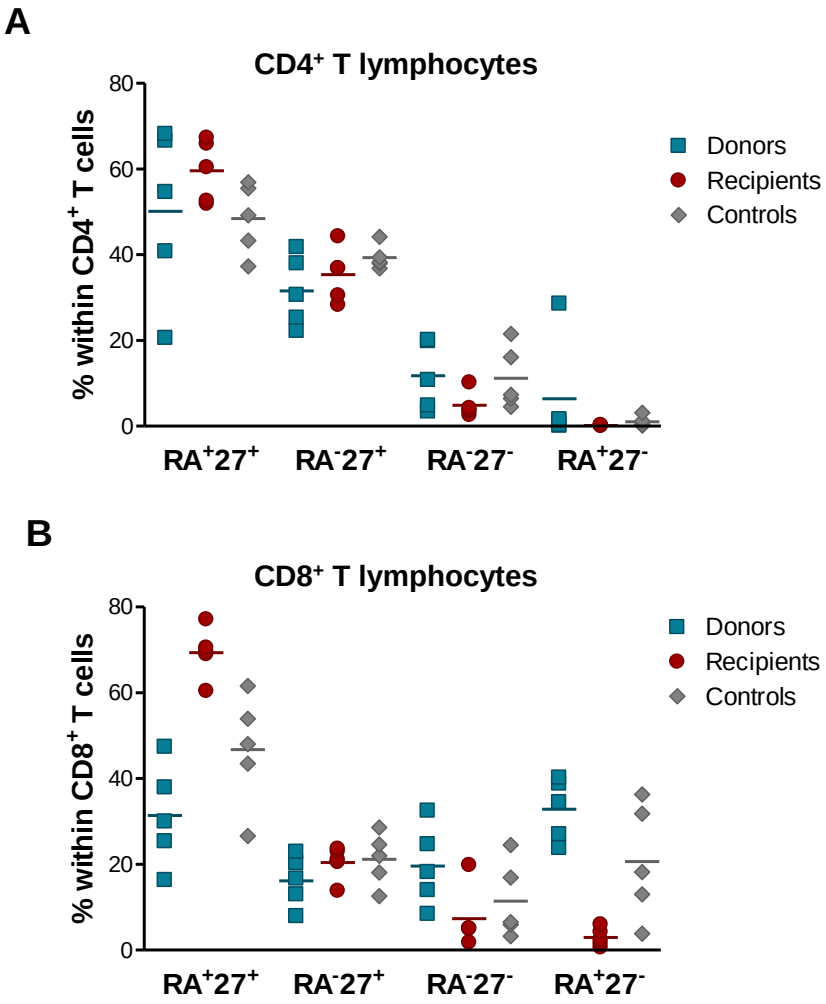

Figure 11: Frequency of naive and memory subsets within the $CD4^+$ and $CD8^+$ T cell pools.

The frequency of the naive and memory subsets, as defined by the expression of CD45RA and CD27, was determined within $CD4^+$ (A) and $CD8^+$ (B) T lymphocytes. A) There were no statistically significant differences on the subset distribution within $CD4^+$ T lymphocytes between the three groups. B) Although recipients tended to have higher frequencies of $CD45RA^+CD27^+$ cells and lower frequencies of $CD45RA^+CD27^-$ cells within the $CD8^+$ T population than donors and age-matched controls, these differences did not reach statistical significance. Mean values are shown as horizontal lines. Statistical analysis was performed using the Wilcoxon matched pairs test (GraphPad Prism).

We thus assessed the proportion of CD31-expressing cells within naive CD45RA$^+$CD62L$^+$ CD4$^+$ T lymphocytes and found that it was significantly higher in recipients than in donors, and similar to that observed in age-matched controls (Figure 12A). In order to further assess the contribution of thymic output to immune reconstitution in our cohort of haploidentical HCST recipients, we quantified the levels of sjTRECs within peripheral blood mononuclear cells (Figure 12B). The assessment of TREC content within purified CD4$^+$ and CD8$^+$ T cell subsets, particularly within naive CD4$^+$ T cells, was precluded by the small number of cells that could be allocated to this assay, given that only a single 50 ml peripheral blood sample was collected from each individual to carry out all the experiments performed in this study. During thymic T cell development, excision of the δ-chain locus during re-arrangement of the T cell receptor α-chain locus produces a signal-joint (sj)-TREC which remains in the nucleus as a non-replicating episomal DNA [62]. Thus, upon cell division, the sjTREC is passed on to only one of the two daughter cells. As the progeny cells undergo further divisions, the sjTREC produced in the mother cell is progressively diluted out. Hence, at a population level, sjTREC content reflects the overall outcome of the TREC-enriching contribution of thymic output and the TREC-diluting effect of peripheral expansion [62]. In agreement with the CD31 expression profile, sjTREC content in recipients tended to be higher than in donors and similar to the levels observed in age-matched controls (Figure 12B). These results point to a substantial contribution of thymic output to the immune reconstitution observed in these patients. The assessment of telomere length within T cell subsets gives an indication of the relative replicative history of these populations. Hence, naive CD4$^+$ T cells have been shown to have longer telomeres than their memory counterparts [63,64]. Given that the CD45RA$^+$ CD4$^+$ T cell population is highly enriched in naive T cells, we expected to observe higher telomere-specific fluorescence intensity in this subset than in CD4$^+$ T cells lacking CD45RA expression. As illustrated in Figure 13A, we did observe a brighter fluorescence resulting from hybridisation with a telomere probe within CD45RA-expressing CD4$^+$ T cells. In all three cohorts, CD45RA$^+$ CD4$^+$ T cells had significantly longer telomeres than their CD45RA$^-$ counterparts (paired *t* test: donors $P = 0.0125$; recipients $P = 0.0364$; controls $P = 0.0004$). When we compared the telomere length within CD45RA$^+$ or CD45RA$^-$ CD4$^+$ T cells between the three cohorts, we observed no statistically significant difference (Figure 13B).

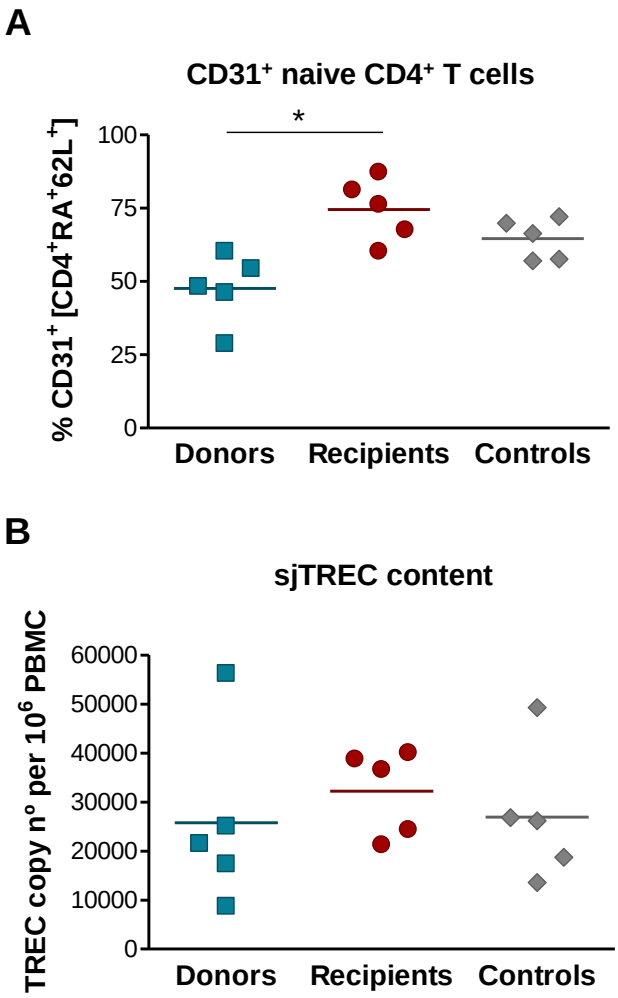

Figure 12: Assessment of relative RTE levels through the expression of CD31 and sjTREC content.

A) CD31 expression was used to identify a population enriched in Recent Thymic Emigrants (RTEs). The proportion of $CD31^+$ cells within naive $CD45RA^+CD62L^+$ $CD4^+$ T lymphocytes was significantly higher in recipients when compared to donors. B) The levels of signal-joint T cell Receptor Excision Circles (sjTRECs) were quantified within PBMCs. Results are expressed as the copy number of sjTRECs per 10^6 PBMCs. In agreement with the proportion of $CD31^+$ cells within naive $CD4^+$ T cells, the sjTREC content tended to be higher in recipients than in donors, although it did not reach statistical significance. Mean values are shown as horizontal lines. Gaussian distribution was confirmed with the Kolmogorov-Smirnov normality test and statistical analysis was performed using paired *t* test (GraphPad Prism).

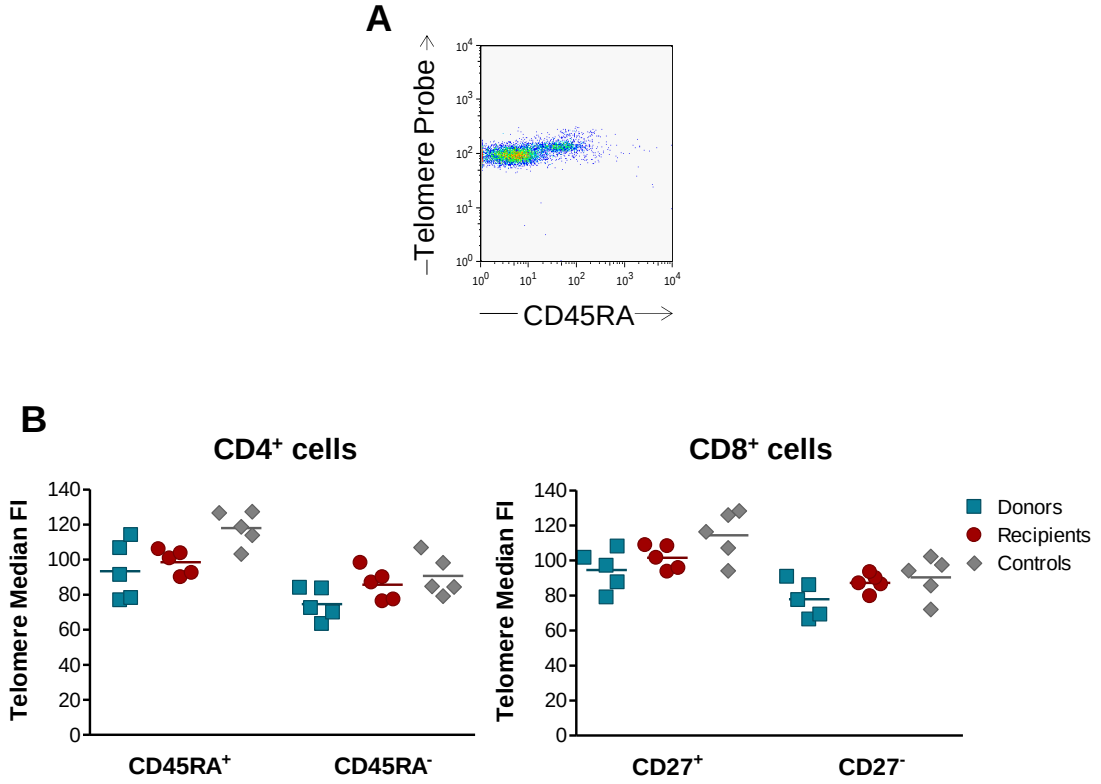

Figure 13: Telomere length measurement within CD4$^+$ and CD8$^+$ T cells.
A) Representative pseudo-colour plot showing telomere probe fluorescence plotted against CD45RA staining within CD4$^+$ T cells. B) Telomere length was measured within CD45RA$^+$ and CD45RA$^-$ CD4$^+$, as well as within CD27$^+$ and CD27$^-$ CD8$^+$ T lymphocytes. In all three cohorts, CD45RA$^+$ CD4$^+$ and CD27$^+$ CD8$^+$ cells had significantly longer telomeres than their respective CD45RA$^-$ CD4$^+$ and CD27$^-$ CD8$^+$ counterparts. Telomere length within each subset was not significantly different when donors, recipients and age-matched controls were compared. Mean values are shown as horizontal lines. Statistical analysis was performed using the Wilcoxon matched pairs test (GraphPad Prism).

For the measurement of telomere length within CD8$^+$ T cells, given that we could only use another surface marker besides CD8, we chose CD27 as it allows the discrimination between a CD27$^+$ population comprising naive CD45RA$^+$CD27$^+$ and early memory CD45RA$^-$CD27$^+$ CD8$^+$ T cells, and a CD27$^-$ population containing highly differentiated memory CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ CD8$^+$ T cells. As expected, CD27$^+$ CD8$^+$ T cells had significantly longer telomeres than their CD27$^-$ counterparts in all three cohorts (paired *t* test: donors P= 0.002; recipients P= 0.0173; controls P= 0.0018). Similarly to the results obtained for CD4$^+$ T cell subsets, the telomere length within CD27$^+$ and CD27$^-$ CD8$^+$ T cells was not statistically different between the three cohorts (Figure 13B). The observation that CD4$^+$ and CD8$^+$ T cell subsets from recipients and age-matched controls have similar telomere lengths further suggests that peripheral expansion was not the major mechanism driving T cell recovery in these patients.

Circulating IL-7 levels have been shown to inversely correlate with peripheral $CD4^+$ T cell counts in lymphopenic hosts, namely in HIV-infected individuals [65,66] and as a result of chemotherapy [65]. In agreement with the observation that the absolute numbers of $CD4^+$ T cells were not significantly decreased in our cohort of transplant recipients in comparison to age-matched controls (Figure 10A), we found that the IL-7 serum levels observed in recipients were no higher than those observed in donors and age-matched controls (data not shown). This result further suggests that these patients have successfully restored the size of the $CD4^+$ T cell pool. Furthermore, persistent TCR activation has been shown to chronically down-modulate IL-7Rα expression [28,67]. Hence, if antigen-driven proliferation was a major mechanism behind the recovery of T cell numbers, we could expect to find significantly lower IL-7Rα levels in these patients. In order to clarify this issue, we assessed the IL-7Rα expression levels within $CD4^+$ and $CD8^+$ T cell subsets (Figure 14). The MFI of IL-7Rα within naive and memory $CD4^+$ T cell subsets as defined by CD45RA and CD27 expression were very similar between the three cohorts (Figure 14A). The same was true for the IL-7Rα levels within CD45RA/CD27 $CD8^+$ T cell subsets (Figure 14C). We further dissected the naive $CD4^+$ T cell population into $CD31^+$ and $CD31^-$ subsets, and found comparable levels of IL-7Rα expression between the three cohorts (Figure 14B). The expression of IL-7Rα levels comparable to the ones observed in healthy age-matched controls suggests that TCR-driven homeostatic proliferation was probably not the major mechanism underlying immune reconstitution in these patients.

Finally, we sought to investigate if the restoration of T cell numbers was accompanied by maintenance of a diverse TCR repertoire. For this purpose, we performed a spectratyping analysis within $CD4^+$ and $CD8^+$ T cells (Figures 15-19). This analysis allows us to assess the complementarity-determining region 3 (CDR3) length distribution within each Vβ family (Figures 15-18). The gene segments encoding T cell receptor α- and β-chains must be re-arranged to produce a functional gene [68]. This process involves the stochastic re-arrangement of gene segments from the variable (V), diversity (D) in the case of the TCR β chain, and joining (J) libraries [68]. The diversity of each TCR chain is concentrated in the CDR3, comprising the junction between V and J or V, D, and J segments, which plays a key role in antigen recognition [68]. In the TCR β-chain, the CDR3 region of any Vβ-Jβ combination may vary in length by as many as six to eight amino acids [69,70].

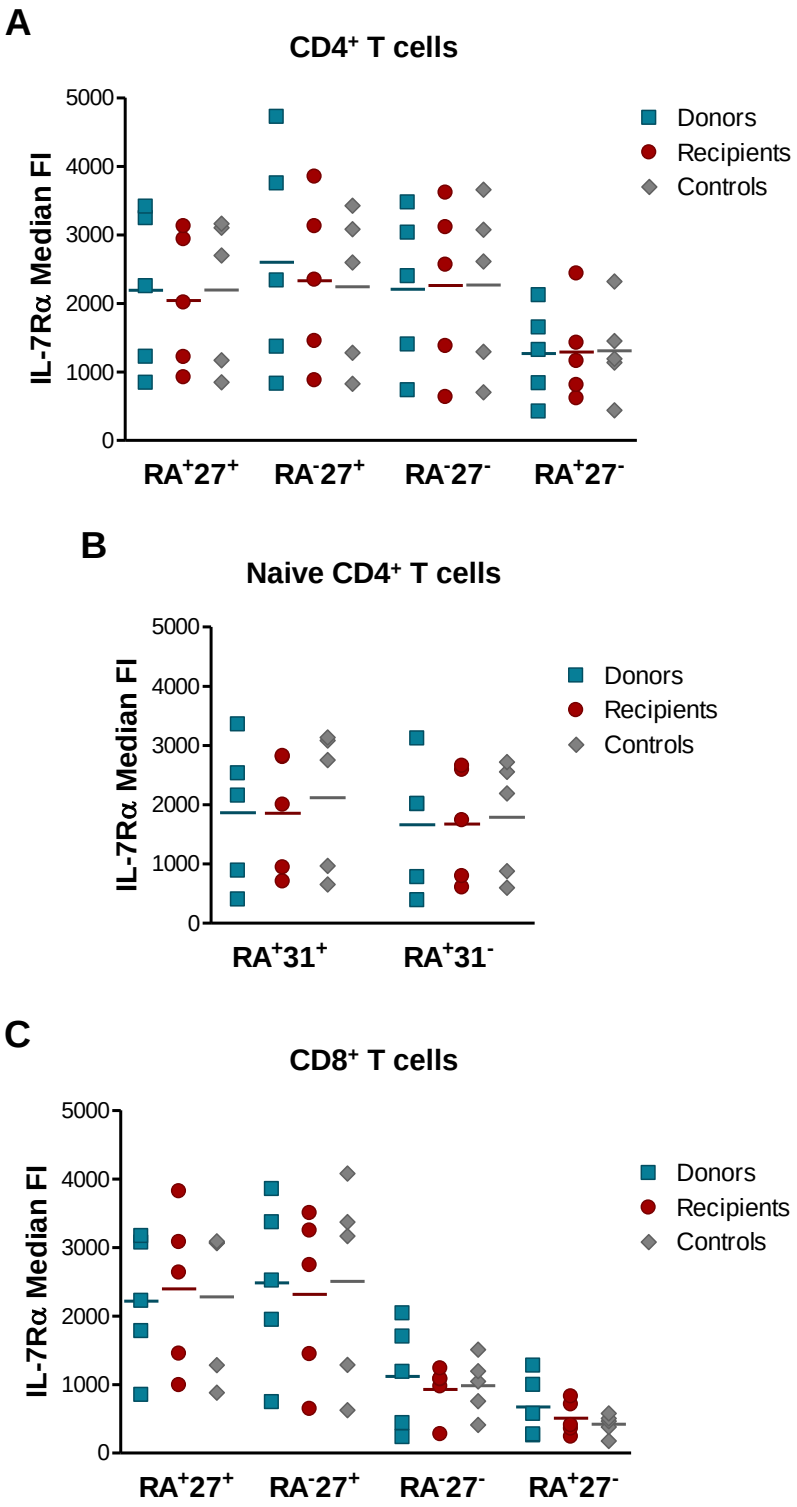

Figure 14: IL-7Rα expression within CD4$^+$ and CD8$^+$ T cell subsets.

IL-7Rα expression was assessed within naive and memory CD4$^+$ and CD8$^+$ T cell subsets. Graphs show the median fluorescence intensity of IL-7Rα within CD4$^+$ (A) and CD8$^+$ (C) T cell subsets defined by CD45RA and CD27 expression. IL-7Rα expression levels within each CD45RA/CD27 CD4$^+$ and CD8$^+$ subset were not significantly different when donors, recipients and age-matched controls were compared. B) Graph shows the MFI of IL-7Rα expression within CD31$^+$ and CD31$^-$ naive CD4$^+$ T cell subsets. IL-7Rα levels within each CD31 naive CD4$^+$ subset were not significantly different between the three cohorts. Mean values are shown as horizontal lines. Statistical analysis was performed using the Wilcoxon matched pairs test (GraphPad Prism).

The progress of T cell repertoire recovery following stem cell transplantation can be monitored by spectratype analysis, which provides a measure of diversity at the level of CDR3 length, reflecting the overall sequence heterogeneity [71,72]. The CDR3 region of the re-arranged TCR β-chain variable region is amplified by PCR, followed by size-based separation and quantification by a multicapillary electrophoresis based Genetic Analyser [73]. Primers specific for each TCR Vβ family are used to provide independent spectratypes, which are classically presented as histograms of the number of T cells bearing receptors plotted against receptor length for each of the TCR Vβ family [73]. T cell pools comprising a diverse polyclonal TCR repertoire present a Gaussian distribution of CDR3 length. If the TCR repertoire is skewed, the distribution of CDR3 lengths is not Gaussian, showing a reduction in the number of peaks or even comprising a single peak in case of clonal dominance [69,74]. We present our results as the proportion of individuals in a given cohort presenting a polyclonal Gaussian, polyclonal skewed, oligoclonal or monoclonal distribution of CDR3 length for each Vβ family within $CD4^+$ (Figure 15) and $CD8^+$ (Figure 17) T cells. We also show representative spectratypes of $CD4^+$ (Figure 16) and $CD8^+$ (Figure 18) T cells from a transplant recipient together with the respective donor and age-matched control.

As illustrated in Figure 15B, all the recipients displayed a polyclonal distribution of CDR3 length within the $CD4^+$ T cell pool, except for a recipient who had an oligoclonal distribution of the Vβ13 and Vβ22 families. This markedly polyclonal spectratype profile closely resembled the one observed in $CD4^+$ T cells from age-matched controls (Figure 15C). As previously described [75-80], we observed more perturbations in TCR Vβ repertoire diversity in $CD8^+$ T cells (Figure 17) than in $CD4^+$ T cells (Figure 15). This may be due to the more robust and prolonged proliferative response upon antigen encounter observed in $CD8^+$ compared to $CD4^+$ T cells [78]. Although $CD8^+$ T cells from one recipient displayed a monoclonal distribution within the Vβ9 and Vβ11 families, while another recipient had a monoclonal distribution within the Vβ22 family, the CD8 TCRBV repertoire was largely polyclonal (Figure 17B). Furthermore, we determined the overall complexity of the TCR Vβ repertoire by assessing the number of discrete peaks detected per Vβ family and scoring each family accordingly, as previously described [19]. The overall spectratype complexity score was calculated as the sum of the scores for each subfamily in each individual. Results are expressed as the average complexity score for $CD4^+$ and $CD8^+$ T cells within each cohort (Figure 19).

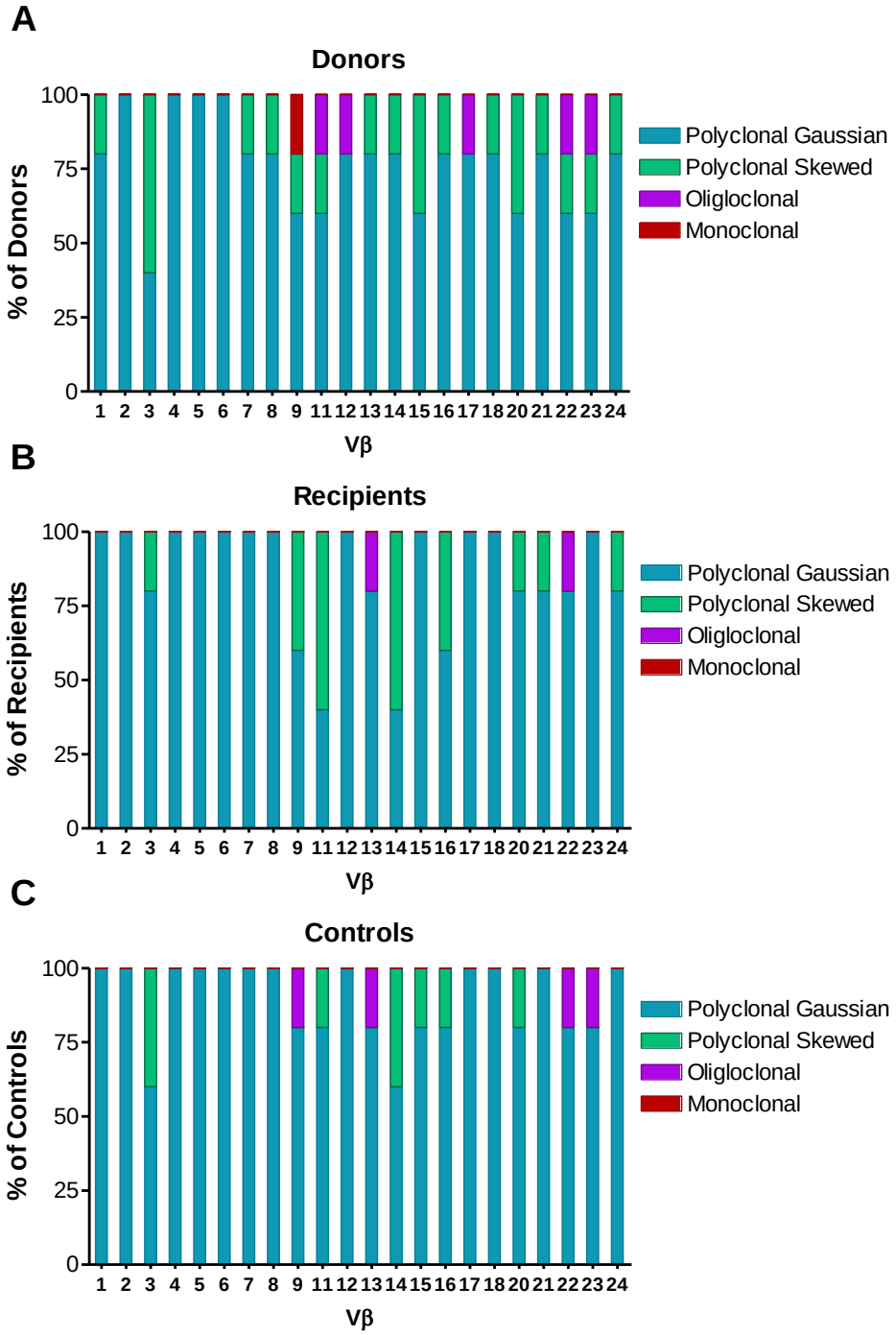

Figure 15: Assessment of TCR repertoire by spectratyping analysis of the CDR3 Vβ regions of CD4$^+$ T cells.

Spectratyping analysis was performed in RNA isolated from purified CD4$^+$ T cells from donors (A), recipients (B) and age-matched controls (C), producing histograms of the number of T cells displaying a given receptor length for each TCR Vβ family. The distribution of each Vβ family was classified as: polyclonal Gaussian when 8 to 10 peaks were present with a dominant peak at the center of the distribution; polyclonal skewed when one of the peaks represented over 40% of the total area or when two dominat peaks represented 70% of the total area for that Vβ; oligoclonal when only two peaks were present or monoclonal when one Vβ peak comprised an area corresponding to over 90% of the total Vβ families. Representation of the peaks were calculated according to the formula % VBn = (peak area VBn_1/ Σ peaks area $VBn_{1\text{-}10}$) x 100. Results are expressed as the percentage of individuals who displayed each of the above described distributions for each Vβ family, within each cohort. No major imbalances were observed in recipients as compared to donors and age-matched controls.

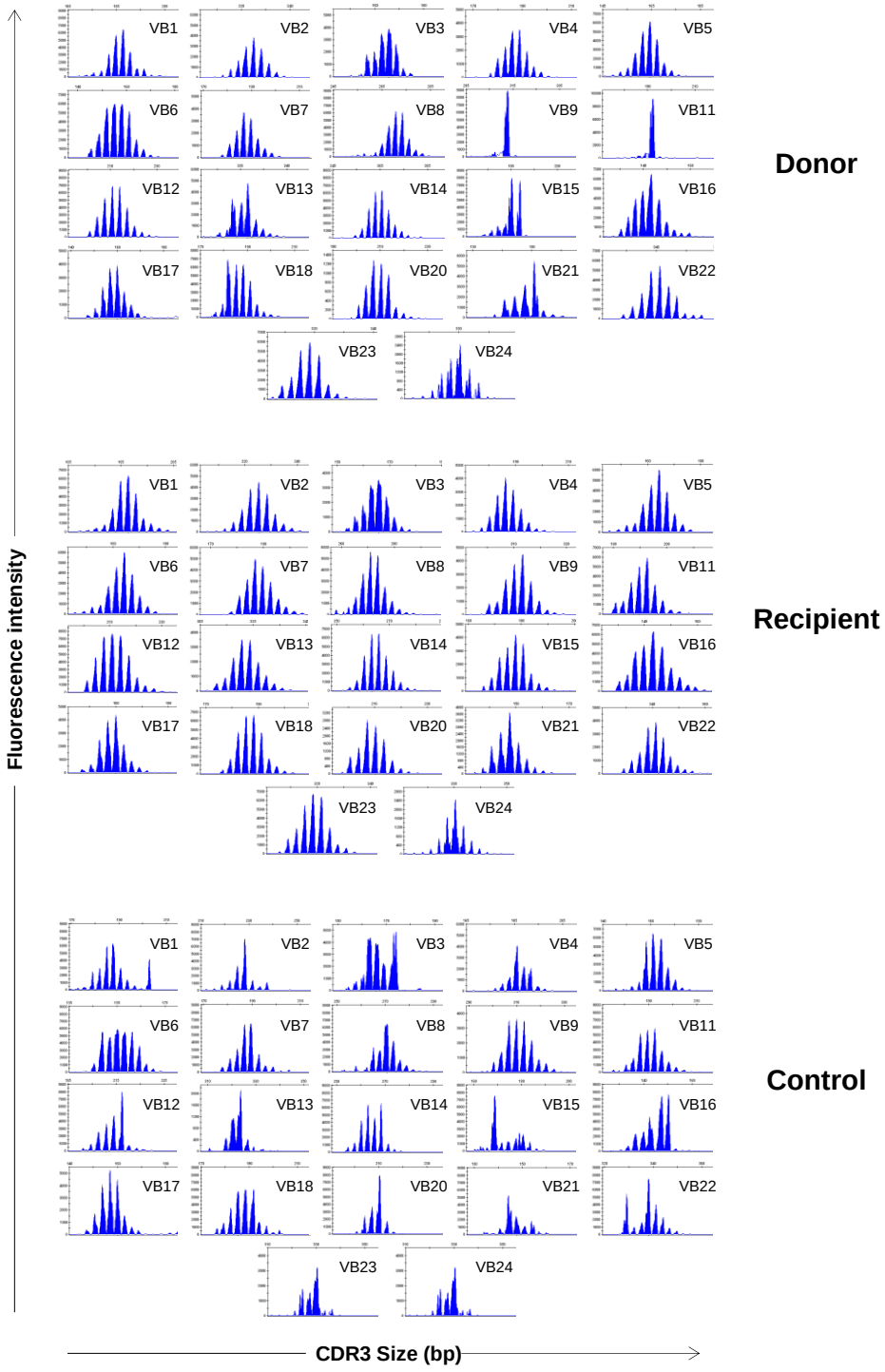

Figure 16: Spectratyping analysis of the CDR3 Vβ regions of CD4$^+$ T cells from a representative recipient together with the respective donor and age-matched control.

The spectratypes obtained for each Vβ family on CD4$^+$ T cells from a representative recipient, the respective donor and age-matched control are shown.

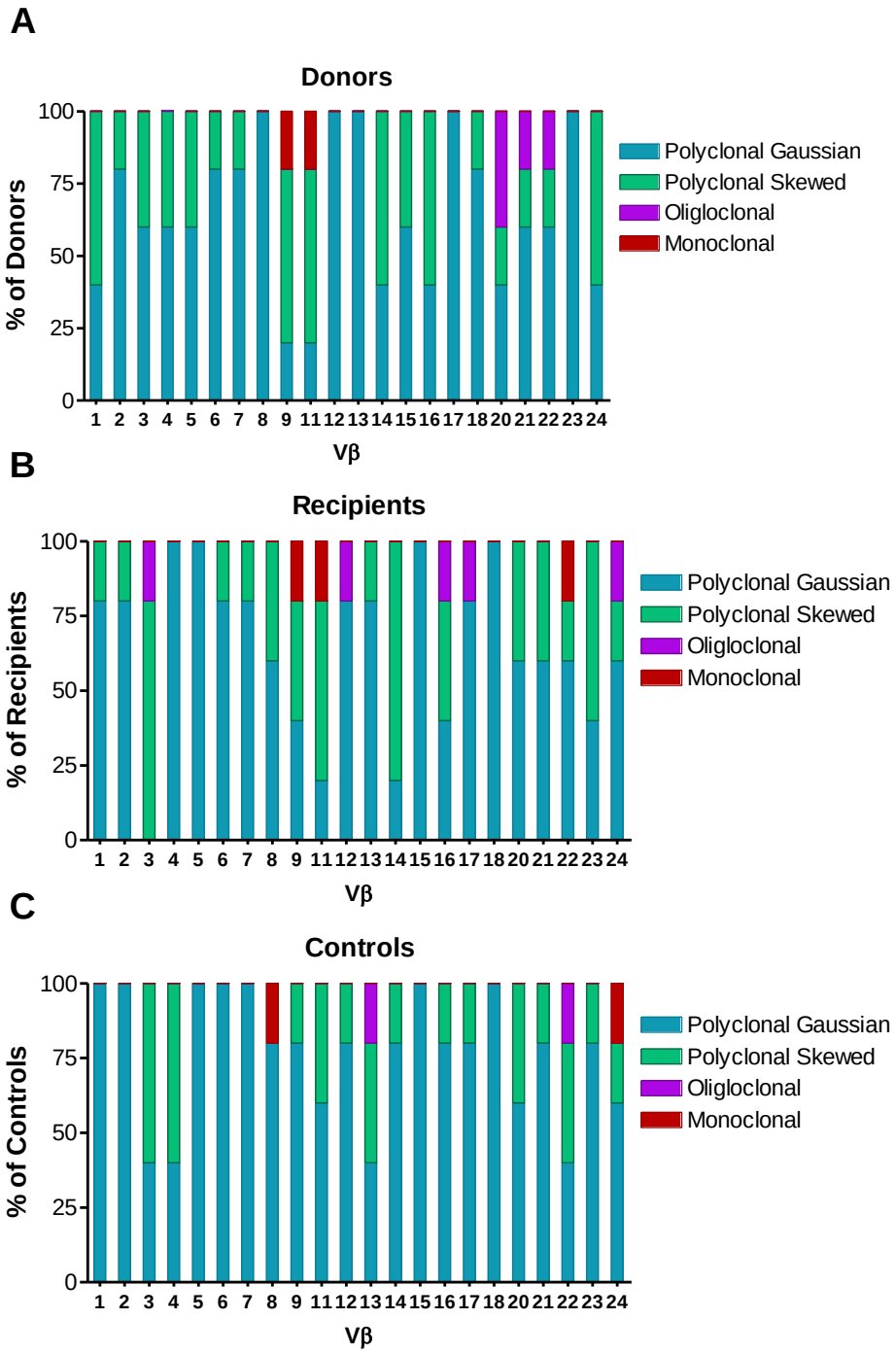

Figure 17: Assessment of TCR repertoire by spectratyping analysis of the CDR3 Vβ regions of CD8$^+$ T cells.

Spectratyping analysis was performed in RNA isolated from purified CD8$^+$ T cells from donors (A), recipients (B) and age-matched controls (C). The distribution of each Vβ family was classified as described in Figure 15. Results are expressed as the percentage of individuals who displayed polyclonal Gaussian, polyclonal skewed, oligoclonal or monoclonal distributions for each Vβ family, within each cohort. No major imbalances were observed in recipients when compared to donors and age-matched controls.

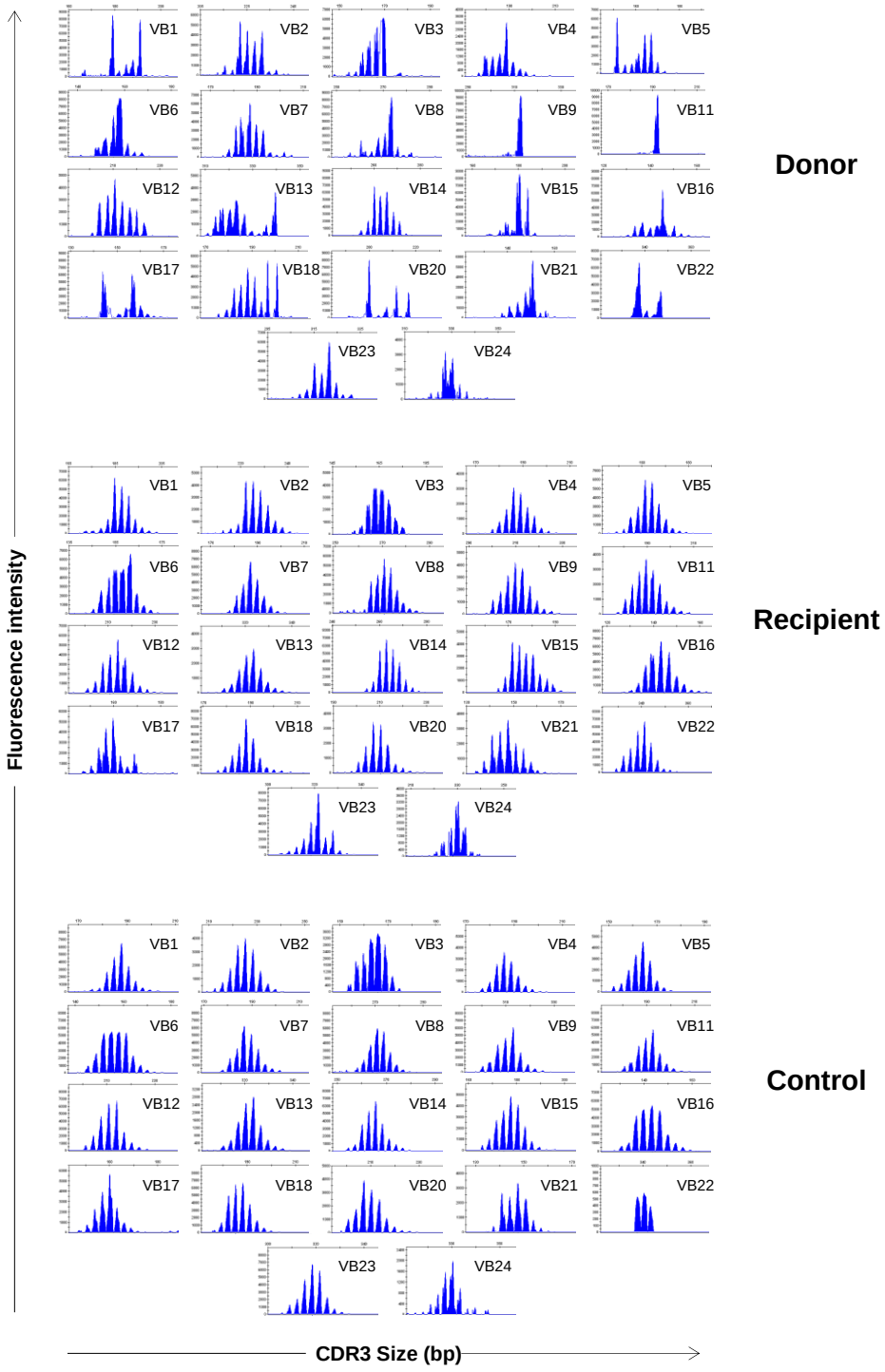

Figure 18: Spectratyping analysis of the CDR3 Vβ regions of CD8$^+$ T cells from a representative recipient together with the respective donor and age-matched control.

The spectratypes obtained for each Vβ family on CD8$^+$ T cells from a representative recipient, the respective donor and age-matched control are shown.

The complexity score within CD4+ and CD8+ T cells was very similar between recipients and age-matched controls, suggesting the maintenance of a broad T cell repertoire (Figure 19). Hence, the spectratyping analysis revealed a largely polyclonal TCRBV repertoire both within the CD4+ and CD8+ T cell pools, suggesting that immune reconstitution was accomplished in these patients whilst maintaining a diverse repertoire.

Overall, our data show that, once the initial post-transplantation period is successfully overcome, full immune reconstitution can be achieved following haploidentical HSCT, pointing to a substantial contribution of thymic output to immune reconstitution, despite the high degree of HLA-mismatch between donor and recipient in this setting.

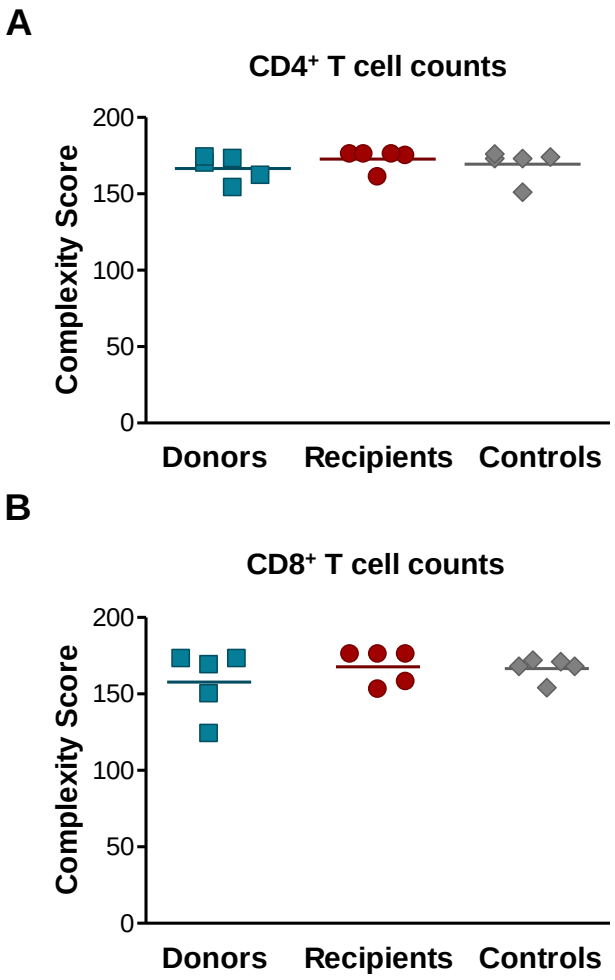

Figure 19: Complexity score within CD4+ and CD8+ T cells.

Spectratype histograms for each Vβ family were given a complexity score depending on the number of peaks obtained (adapated from Wu *et al.* [19]), whereby the appearence of 8 to 10 peaks gives a score of 8 and then onwards, to a minimum score of 1 when only one peak can be observed. The maximum overall complexity score that can be achieved is 176 that would originate from all 22 Vβ families having spectratypes with 8 peaks. Graphs show the overall complexity score within CD4+ (A) and CD8+ T cells (B) from each donor, recipient and age-matched control. Mean values are shown as horizontal lines.

Discussion

We demonstrated that IL-7-induced cycling of naive $CD4^+$ T cells from adult peripheral blood is restricted to the CD31-expressing subset. Furthermore, IL-7 stimulation was associated with maintained or even increased levels of CD31 expression, thus demonstrating that $CD31^+$ naive $CD4^+$ T cells are able to proliferate without losing CD31 expression. IL-7-induced proliferation and CD31 preservation were both dependent on the PI3K pathway, likely contributing to the homeostastic maintenance of the $CD31^+$ naive $CD4^+$ T cell pool. Although our results suggest that adult $CD31^-$ naive $CD4^+$ T cells require other triggers to undergo homeostatic proliferation, IL-7-induced cell survival is likely to play a key role in the maintenance of both $CD31^+$ and $CD31^-$ naive $CD4^+$ T cell subsets.

Thymic involution leads to a decreased output of *de novo* generated naive T cells into the periphery throughout adulthood [1]. Hence the maintenance of the naive T cell pool has to be achieved through a combination of residual thymic output and homeostatic proliferation in the periphery, sustaining naive T cell numbers whilst preserving a diverse repertoire as well as naive phenotypic and functional hallmarks. In particular, the maintenance of the $CD31^+$ naive $CD4^+$ T cell subset during ageing requires homeostatic cues which will induce proliferation without down-modulating CD31 expression. The $CD31^+$ naive $CD4^+$ T cell subset was initially described to comprise cells that had not yet undergone post-thymic proliferation, contrary to the $CD31^-$ subset which was proposed to be generated upon homeostatic prolifertation in the periphery given their lower TREC content compared to $CD31^+$ naive $CD4^+$ T cells [9,81]. However, thymic output alone has been suggested to be insufficient to achieve the $CD31^+$ naive $CD4^+$ T cell numbers observed during ageing, implying a contribution from peripheral expansion to the maintenance of the $CD31^+$ naive $CD4^+$ T cell subset [62]. Moreover, TREC levels within $CD31^+$ naive $CD4^+$ T cells, despite being consistently higher than within the $CD31^-$ subset, have also been shown to decrease, albeit modestly, during aging, further suggesting that $CD31^+$ naive $CD4^+$ T cells undergo at least some level of post-thymic proliferation [10]. Interestingly, a clinical trial in cancer patients has reported that IL-7 administration leads to an age-independent increase in absolute numbers of $CD31^+$ naive $CD4^+$ T cells [25]. Our results point to IL-7 as a potential homeostatic cue with the capacity to induce proliferation of $CD31^+$ naive $CD4^+$ T cells in the periphery whilst preserving

CD31 expression, ensuring the maintenance of the size as well as diversity of the naive T cell pool.

These data imply that CD31 expression is not sufficient to identify RTEs given that the CD31$^+$ naive CD4$^+$ T cell subset might include cells that have already undergone post-thymic proliferation. Nonetheless, CD31 is a relevant marker which distinguishs a naive CD4$^+$ T cell population highly enriched in RTEs, as demonstrated by the high TREC content and the age-dependent decrease in the size of the CD31$^+$ naive CD4$^+$ T cell pool [9-12].

On the other hand, our results show that IL-7-induced proliferation of CD31$^+$ naive CD4$^+$ T cells does not result in the appearance of a CD31$^-$ sub-population, suggesting that other homeostatic mechanisms are implicated in the generation of the CD31$^-$ naive CD4$^+$ T cell subset. TCR triggering with low-affinity antigens, namely self-MHC/peptide complexes involved in naive T cell homeostasis, has been proposed as a likely candidate [9,12], supported by evidence of recent TCR engagement in these cells [12], although a putative co-stimulatory role for IL-7 in this process cannot be precluded.

CD31 expression might be associated with sensitivity to TCR-mediated stimuli, given that CD31 engagement has been shown to inhibit TCR-mediated signal transduction via its cytoplasmic ITIMs [82]. We can thus speculate that the CD31$^-$ naive CD4$^+$ T cell subset might undergo homeostatic proliferation upon TCR engagement with self-MHC/peptide complexes [2], whereas CD31$^+$ naive CD4$^+$ T cells might be impervious to this proliferative trigger. Furthermore, the involvement of CD31 in transendothelial migration of neutrophils and monocytes [83] points to a putative role for this marker in the migration of CD31$^+$ naive T cells into secondary lymphoid organs [9], where they might encounter IL-7 and consequently undergo homeostatic proliferation.

The distinct responses to IL-7 observed in adult CD31$^+$ and CD31$^-$ naive CD4$^+$ T cells did not correlate with the basal levels of IL-7Rα expression. This observation is in agreement with another *in vitro* study where the responsiveness of human naive CD4$^+$ T cells to IL-7 has been found to not correlate with IL-7Rα expression levels [28].

In order to investigate which signalling pathways mediate the IL-7-induced effects on naive CD4$^+$ T cell subsets, we used inhibitors to specifically block the MEK/ERK and PI3K pathways. We found that IL-7-induced proliferation of adult CD31$^+$, as well as of both CD31$^+$ and CD31$^-$ naive CD4$^+$ T cells from cord blood, required PI3K activation, as previously described for total naive CD4$^+$ T cells from cord blood [3]. Furthermore, the

effects of IL-7 on the maintenance or increase of CD31 expression levels were also dependent on the PI3K pathway. Conversely, the down-modulation of the CD31 ligand CD38 following IL-7 stimulation was not reversed upon PI3K inhibition. Although IL-7-induced proliferation was PI3K-dependent, IL-7 stimulation led to IL-7Rα down-modulation, Bcl-2 up-regulation and protection against apoptosis in both $CD31^+$ and $CD31^-$ naive $CD4^+$ T cells even in the presence of the PI3K inhibitor. Thus the observation that IL-7-induced proliferation of adult naive $CD4^+$ T cells was restricted to the $CD31^+$ subset might be due to a selective block in the activation of the PI3K pathway in adult $CD31^-$ naive $CD4^+$ T cells in response to IL-7.

Overall, our data suggests that CD31 expression identifies a naive $CD4^+$ T cell population enriched in RTEs that is preferentially expanded upon IL-7 stimulation via a PI3K-dependent pathway. Hence therapeutic administration of IL-7 might benefit the maintenance of a diverse T cell repertoire by promoting the survival and homeostatic proliferation of the $CD31^+$ naive $CD4^+$ T cell subset in different settings, namely during ageing and following stem cell transplantation.

In the case of the latter scenario, recovery of a $CD31^+$ naive $CD4^+$ T cell pool might be hindered by age-related thymic involution as well as by conditioning regimens that further disrupt the IL-7-rich thymic micro-environment [84]. On the other hand, $CD31^+$ naive $CD4^+$ T cell numbers may also be recovered through homeostatic proliferation in the periphery [85], which according to our results might be driven by IL-7. In order to assess if naive T cell homeostasis was restored following haploidentical HSCT and the potential mechanisms underlying this recovery, we performed an evaluation of immune reconstitution in a group of five patients who were four to six years post-transplant at the time of the study. We found that transplant recipients displayed $CD4^+$ and $CD8^+$ T cell counts, as well as a naive and memory profile, comparable to age-matched controls. In particular, the proportion of $CD31^+$ cells within the naive $CD4^+$ T cell population in recipients was similar to that observed in age-matched controls and significantly higher when compared to the respective donors. A study in mice has shown that RTEs have a survival advantage over resident naive T cells in the periphery, being preferentially incorporated in the naive T cell pool [86]. Hence the substantial proportion of $CD31^+$ cells within the naive $CD4^+$ T cell pool suggests that thymic output might have contributed to the replenishment of the naive T cell pool. We sought to further investigate the relative contribution of thymic output and peripheral expansion to the replenishment of the T cell

pool in these patients. The relatively high TREC content within PBMCs and the presence of similarly long telomeres in CD4$^+$ and CD8$^+$ T cell subsets from recipients when compared to age-matched controls indicate that thymic output must have contributed at least partly to immune reconstitution.

The levels of IL-7Rα expression within naive and memory CD4$^+$ and CD8$^+$ T cells, particularly within CD31 naive CD4$^+$ subsets, were very similar when the three cohorts were compared, suggesting that homeostatic stimuli rather than TCR activation, which has been shown to persistently down-modulate IL-7Rα expression [28,67], were probably the major triggers for peripheral T cell expansion in these patients. Moreover, the presence of a broad and largely polyclonal T cell repertoire in transplant recipients, comparable to the one observed in age-matched controls, supports the view that immune reconstitution was likely driven by thymic output together with homeostatic proliferation of peripheral T cells. The contribution of thymic output to immune reconstitution might decrease the risk of GVHD through negative selection of self-reactive T cells during *de novo* T cell generation [87]. Pre-clinical studies in animal models have reported that IL-7 therapy boosts thymic function and homeostatic proliferation in the periphery following stem cell transplantation [88-93]. As mentioned above, a rhIL-7 clinical trial in cancer patients has shown that IL-7 administration leads to increased numbers of CD31$^+$ naive T cells [25]. In light of our results, we can speculate that this outcome is at least partly due to the preferential expansion of CD31$^+$ naive T cells driven by IL-7.

Overall, our data demonstrate that IL-7 induces proliferation and maintenance of CD31 expression on CD31$^+$ naive CD4$^+$ T cells through a PI3K-dependent mechanism, possibly contributing to the homeostatic maintenance of this subset throughout adulthood. Furthermore, our results suggest that T cell homeostasis, in particular the CD31$^+$ naive CD4$^+$ T cell pool, was successfully restored following haploidentical HSCT, a process which appears to have relied on both thymus-dependent and -independent mechanisms.

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CHAPTER 2

Characterization of human CD45RA$^+$CD27$^-$ CD4$^+$ T cells

Introduction

Human CD4$^+$ T cell subsets can be identified according to the expression of CD45RA and CD27: CD45RA$^+$CD27$^+$, CD45RA$^-$CD27$^+$, CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$. The characterisation of these subsets based on surface receptor expression, functional properties, TREC content and telomere length has established that the CD45RA$^+$CD27$^+$ population comprises naive cells, the CD45RA$^-$CD27$^+$ subset encompasses cells at an early stage of differentiation, whereas both CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ subsets consist of highly differentiated CD4$^+$ T cells [1,2]. The latter population has been described in human CD8$^+$ T cells as the most differentiated type of memory cells, which is supported by their low proliferative capacity, high susceptibility to apoptosis and loss of CD28, CD27, and CCR7 expression [1,3-5]. CD45RA$^+$27$^-$ CD8$^+$ T cells have been shown to accumulate during ageing [6,7] and chronic viral infections [8-12], comprising large clonal expansions of virus-specific cells [4,10]. On the other hand, CD45RA$^+$27$^-$ CD4$^+$ T cells are only present at very low frequencies and, although these cells also accumulate with ageing and, more strikingly, with CMV infection [2], this subset remains poorly characterized. While the origin of these cells remains to be elucidated, it has been proposed that CD45RA re-expression only occurs in the absence of antigen [5,13,14].

The aim of this work was to perform a detailed study of the CD45RA$^+$27$^-$ CD4$^+$ T cell subset in order to understand the relevance and the impact of the accumulation of this rare and under-characterized subset. In the first part of this chapter, we report a detailed characterization of the CD4$^+$ T cell subsets defined by the expression of CD45RA and CD27 in terms of their degree of differentiation, functionality, ability to proliferate, survive and trigger relevant signalling pathways following activation. We also sought to uncover the potential mechanism responsible for the re-expression of CD45RA on

memory $CD4^+$ T cells. In the second part, our aim was to elucidate whether the $CD45RA^+27^-$ $CD4^+$ T cell subset is truly quiescent, as was previously reported for CD45RA re-expressing $CD8^+$ T cells [12], or if this subset was actually close to senescence. Cellular senescence differs from quiescence in that the growth arrest state is permanent, whilst quiescent cells may re-enter the cell cycle upon appropriate stimulation [15]. For this purpose, we assessed several senescence-associated markers, such as γ-H2AX, telomere length and telomerase activity, and the impact of the p38 pathway on the expression of these markers in $CD45RA^+27^-$ $CD4^+$ T cells.

Methods

1. Blood samples

Heparinized peripheral blood was collected from healthy volunteers between the ages of 26 and 60 (median age 39). All donors provided written informed consent and the work was approved by the Ethics Committee of the Royal Free Hospital.

2. Purification of Lymphocyte Subsets

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). $CD4^+$ T cells were purified by positive selection using the VARIOMACS system (Miltenyi Biotec) according to the manufacturer's instructions. In some experiments, $CD4^+$ T cells were further sorted into CD45RA/CD27 subsets using a FACSAria flow cytometer (BD Biosciences, San Jose, CA) after staining with CD45RA and CD27 antibodies for 30 minutes at 4°C in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma-Aldrich).

3. *In vitro* Cell Culture

Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 mg/mL streptomycin, 50 µg/ml gentamicin and 2 mM L-glutamine (all from Invitrogen) at 37°C in a humidified 5% CO_2 incubator. Purified $CD4^+$ subsets were activated in the presence of anti-CD3 antibody (purified OKT3, 0.5 µg/ml), together with rhIL-2 (5 ng/ml; R&D Systems) or autologous PBMC irradiated with 40 Gy γ-radiation, as a source of multiple co-stimulatory ligands provided

by B cells, dendritic cells, and macrophages found in these populations. In other experiments, cells were cultured in the presence of rhIL-2 (5 ng/ml), rhIL-7 (10 ng/ml) or rhIL-15 (5 ng/ml) (all from R&D Systems). Cytokines were added at the beginning of the cell culture and were not replenished. Cells were harvested at different times for phenotypic and functional analyses. In some experiments, the p38 inhibitor BIRB796 was added to the culture. BIRB796 was obtained from David Kipling already dissolved in DMSO at the concentration of 50 mM. It was diluted in 0.1% DMSO and used at a final concentration of 500 nM. Cells were pretreated with the inhibitor for 30 minutes. A solution of 0.1% DMSO was used as a vehicle control.

4. Proliferation assessment by [^{3}H]Thymidine Incorporation

Purified CD45RA/CD27 CD4$^+$ T cell subsets were stimulated with anti-CD3 (purified OKT3, 0.5 μg/mL) and irradiated APCs in a 1:1 ratio on 96-well round-bottomed tissue culture plates (Falcon, BD). The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 4 days before adding tritiated thymidine ([^{3}H]thymidine) (GE Healthcare) and incubating for a further 7 hour period before placing the plates at -20ºC. Proliferation was expressed as the mean [^{3}H]thymidine incorporation, quantified as counts per minute (cpm), of triplicate wells.

5. Flow Cytometric Analysis

5.1. Surface staining

Cells resuspended in PBS containing 1% BSA and 0.1% sodium azide (Sigma-Aldrich) were stained for 10 minutes at room temperature with the following anti–human monoclonal antibodies: CD45RA FITC (clone HI100; BD Pharmingen) or APC (clone MEM-56; Caltag); CD45RO PE (clone UCHL1); CD4 PerCP (clone SK3) or PE-Cy7 (clone SK3); CD27 PE (clone M-T271); CD28 FITC (clone CD28.2); CD127 PE (clone hIL-7R-M21); CCR7 PE-Cy7 (clone 3D12) (all from BD Pharmingen); CD57 PE (clone TB03; Miltenyi Biotec). Samples were acquired on a BD BD LSR II flow-cytometer (BD Biosciences) after fixation with 1% formaldehyde (Sigma-Aldrich). Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

5.2. Intracellular staining

Intracellular staining for Granzyme B PE (clone GB11; eBioscience), Perforin FITC (clone δG9; BD Pharmingen), Bcl-2 FITC (clone 124; Dako) or PE (clone Bcl-2/100; BD Pharmingen), Ki67 FITC (clone B56; BD Biosciences) and total p38 Alexa Fluor 488 (rabbit anti-p38, Cell Signaling; Alexa Fluor 488 goat anti-rabbit Ig, Invitrogen) was performed using the Foxp3 Staining Buffer Set (Miltenyi Biotec) according to the manufacturer's instructions. Samples were acquired on a BD FACS Calibur 2 flow-cytometer (BD Biosciences) after fixation with 1% formaldehyde (Sigma-Aldrich). Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

5.3. Measurement of Cytokine Production.

PBMCs were activated with anti-CD3 (purified OKT3, 0.5 μg/ml) and rhIL-2 (5 ng/ml; R&D Systems) at 37°C in a humidified 5% CO_2 incubator. Unstimulated controls were also included. After 2 hours, Brefeldin A (5 μg/ml, Sigma-Aldrich) was added, and cells were incubated overnight at 37°C. Cells were subsequently washed and stained for surface CD4, CD45RA and CD27, followed by staining for intracellular TNF-α PE (clone MAb11; BD Pharmingen) using the Foxp3 Staining Buffer Set (Miltenyi Biotec) according to the manufacturer's instructions. Samples were acquired on a BD FACS Calibur 2 flow-cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

5.4. Assessment of Apoptosis

Apoptosis was assessed using an Annexin V/ Propidium Iodide (PI) detection kit (BD Biosciences). Cells resuspended in 1x Binding Buffer were incubated with Annexin V antibody and PI for 15 minutes at room temperature. Samples were immediately acquired on a BD FACS Calibur 2 flow-cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

5.5. CFSE dilution assay

Proliferation was assessed by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay. Cells were labeled with 0.5 μM CFSE (Molecular Probes-Invitrogen, Carlsbad, CA) at 37°C for 15 minutes in the dark, quenched with ice-cold culture medium at 4°C for 5 minutes, and washed 3 times before culture in the presence of 50 ng/ml rhIL-7. Samples were acquired on a BD FACS Calibur 2 flow-cytometer (BD Biosciences)

after fixation with 1% formaldehyde (Sigma-Aldrich). Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

5.6. Phosphorylation state analysis by Phosflow

For the detection of pAkt(Ser473), PBMCs were rested overnight in RPMI (Sigma-Aldrich) with 1% human AB serum (Sigma-Aldrich), and then starved in serum-free RPMI for 2 hours prior to stimulation. The analysis of p38 (pT180/pY182) was performed directly *ex vivo* or after stimulation with 25ng/ml of TNF-α for 10 minutes. Following surface staining for CD45RA, CD27 and CD4, cells were activated with anti-CD3 (purified OKT3, 1 μg/ml) on ice for 20 minutes. Primary mAbs were cross-linked with anti-mouse IgG F(Ab′)2 (20 μg/ml, Jackson ImmunoResearch) by incubating on ice for 20 minutes. Cells were then stimulated at 37°C (5 minutes for pAkt; 20 minutes for phospho-p38). The unstimulated control cells underwent the same manipulations but without addition of anti-CD3 and cross-linker. Activation was arrested by fixing the cells with warm Cytofix Buffer (BD Biosciences) at 37°C for 10 minutes. Cells were permeabilized with ice-cold Perm Buffer III (BD Biosciences) at 4°C for 30 minutes and then incubated with PE mouse anti-Akt (pS473) or with Alexa Fluor 488 anti-p38 (pT180/pY182) (both from BD Biosciences) for 30 minutes at room temperature. Cells were washed in Stain Buffer (BD Pharmingen) before acquisition.

γ-H2AX (pSer139) (Alexa 488; clone 2F3; Biolegend) expression was assessed directly *ex vivo* or after short-term activation (30 min, 1h, 24h) of total CD4$^+$ T cells with 0.5 μg/ml of immobilized anti-CD3 and 5 ng/ml of rhIL-2, following surface staining for CD45RA and CD27. In other experiments, purified CD45RA/CD27 CD4$^+$ subsets were activated in the same conditions for 4 days. As a positive control, total CD4$^+$ T cells were irradiated with with 40 Gy γ-radiation. Intracellular staining for γ-H2AX was performed using the BD Phosflow buffers as described above.

Samples were acquired on a BD LSR II flow cytometer (BD Biosciences) and analysed using FlowJo software (TreeStar, Ashland, OR).

5.7. Telomere length measurement by Flow-FISH

MACS-sorted CD4$^+$ T were surface stained using CD45RA biotin (clone HI100; eBioscience), Streptavidin Cy3 (Cedarlane Laboratories) and CD27 FITC (clone M-T271; BD Pharmingen). Telomere length of cell populations defined by expression of CD45RA

and CD27 was measured using the Flow-FISH protocol as described on section 4.6 of the Methods in Chapter 1.

6. Measurement of Telomerase Activity by TRAP assay

Telomerase activity was determined using a modified version of the telomeric repeat amplification protocol (Oncor, Gaithersburg, MD) as previously described [16]. Purified subsets were activated with anti-CD3 (purified OKT3, 0.5 μg/ml) and irradiated APCs for 4 days. Cell extracts from equivalent numbers of $Ki67^+$ cells were used for telomeric elongation, using a [γ-^{33}P] ATP-end-labeled telomerase substrate (TS) primer. These samples were then amplified by PCR amplification, using 25 to 28 cycles of 30 seconds at 94°C and 30 seconds at 59°C. The PCR products were run on a 12% poly-acrylamide gel (Sigma-Aldrich) which was then exposed to an autoradiography film (Hyperfilm MP, Amersham). Telomerase activity was calculated as a ratio between the optical density of the telomeric repeat bands and of the internal standard band (IS). As a negative control lysis buffer was used in place of cell extract. A control template containing the same sequence as the TS primer plus 8 telomeric repeats was used as a PCR positive control.

7. Real-Time quantitative PCR (RT-qPCR)

The mRNA levels of the transcription factors Blimp-1, T-bet and Eomes were measured in purified $CD4^+$ CD45RA/CD27 before (*ex vivo*) and after a 3 day culture period in the presence of rhIL-7 (5, 10, 25, 50 ng/ml). Expression of Bcl-2 mRNA was analyzed in $CD4^+$ cells cultured with anti-CD3 (purified OKT3, 0.5 μg/ml) and rhIL-2 (5 ng/ml) in the presence or absence of BIRB796 for 3 days. Total RNA was purified with RNeasy columns (Qiagen). Reverse transcriptions were performed with random primers using the MuLVRT reverse transcriptase (Invitrogen). The mRNA levels of Blimp-1, T-bet, Eomes and Bcl2 were determined by real-time quantitative PCR (RT-qPCR) on an ABI PRISM 7500 with SYBR® Green PCR Master Mix according to the protocol provided by the manufacturer (both from Applied Biosystems) with the following primers: Bcl-2 forward 5'-TTG CTT TAC GTG GCC TGT TTC-3', Bcl-2 reverse 5'-GAA GAC CCT GAA GGA CAG CCAT-3'; T-bet forward 5'-GGT CGC GCT CAA CAA CCA CCT-3', T-bet reverse 5'-CAT CCG CCG TCC CTG CTT GG-3'; Eomes forward 5'-GGC AAA GCC GAC AAT AAC AT-3', Eomes reverse 5'-TTC CCG AAT GAA ATC TCC TG-3'; Blimp-1 forward 5'-CTT ATC CCG GAG AGC TGA CA-3', Blimp-1

reverse 5'-GCT CGG TTG CTT TAG ACT GC-3'. The housekeeping 18S mRNA, used as an external standard, was amplified from the same cDNA reaction mixture using the primers: forward 5'-GGA GAG GGA GCC TGA GAA AC-3', reverse 5'- TCG GGA GTG GGT AAT TTG C-3'. Each sample was run in triplicate and target mRNA level was expressed as a ratio to the level of 18S to control for differing levels of cDNA in each sample.

8. Western blot analysis

CD4$^+$ T cells were activated with PMA (0.5 μg/ml, Sigma-Aldrich) and ionomycin (0.5 μg/ml, Sigma-Aldrich) in the presence or absence of BIRB796. Cells were harvested after 30 minutes of stimulation and lysates were obtained by sonicating cells in 50 mM Tris-HCl (pH 7.5), 2 mM EGTA, 0.1% Triton X-100 buffer. Lysates from 2 x10^6 cells were fractionated on SDS-polyacrylamide electrophoresis gels and analyzed by immunoblotting with either anti-phospho-p38 (pThr180/pTyr182, Cell Signaling), anti-pJNK (pThr183/pTyr185, Cell Signaling) or anti-β-actin (Abcam) using the ECL Advanced Western Blotting Detection kit (Amersham Biosciences), according to the protocol provided by the manufacturer.

9. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). Data are presented as mean plus or minus standard error of mean (SEM). *P* values less than 0.05 were considered significant: * indicates $P < 0.05$; ** indicates $P < 0.001$; *** indicates $P < 0.0001$.

Results

Chapter 2.1

IL-7-driven homeostatic mechanism induces CD45RA re-expression on CD45RA$^-$CD27$^+$ CD4$^+$ T cells

The CD45RA$^+$CD27$^-$ CD8$^+$ T cell subset has been described as terminally differentiated with limited capacity for self-renewal [1,17]. The small numbers of CD45RA$^+$CD27$^-$ cells within the CD4$^+$ T cell population has thus far precluded the extensive study of this subset in healthy donors. Therefore we sought to purify the four subsets defined by the expression of CD45RA and CD27 by FACS sorting in order to efficiently isolate and characterise the CD45RA$^+$CD27$^-$ CD4$^+$ T cell subset. We observed that the percentage of CD4$^+$ CD45RA$^+$CD27$^-$ cells is higher in older individuals, with a significant positive correlation with CMV infection, and that these cells have a highly differentiated phenotype (low IL-7Rα, CD28 and CCR7 expression) [2]. We further characterised the CD4$^+$ CD45RA$^+$CD27$^-$ subset in healthy donors by assessing their cytotoxic potential and the expression of activation markers (Figure 1). We analysed the expression of the apoptotic marker Fas (CD95) and of CD57, a marker known to be expressed on late stage effector CD8$^+$ T cells [18], by gating within each of the CD45RA/CD27 CD4$^+$ subsets directly *ex vivo* (Figure 1B, C). As expected, the expression of CD57 was practically undetectable within CD45RA$^+$CD27$^+$ cells (Figure 1B). The CD45RA$^+$CD27$^-$ subset expressed significantly higher levels of CD57 than any of the other subsets, indicating that this is indeed a highly differentiated population (Figure 1B). In contrast, the expression of CD95 was significantly lower on the CD45RA$^+$CD27$^-$ subset compared to the CD45RA$^-$CD27$^+$ and CD45RA$^-$CD27$^-$ subsets (Figure 1C), as was previously described for CD45RA$^+$CD27$^-$ CD8$^+$ T cells [3]. We also investigated the functional properties of the CD45RA/CD27 CD4$^+$ T cell subsets by determining the expression of cytolytic molecules granzyme B and perforin, which was similarly low in CD45RA$^+$CD27$^+$ and CD45RA$^-$CD27$^+$ CD4$^+$ T cells (Figure 1D, E). In contrast, CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ CD4$^+$ T cells expressed both markers, the levels of which were significantly higher in the latter population (Figure 1D, E).

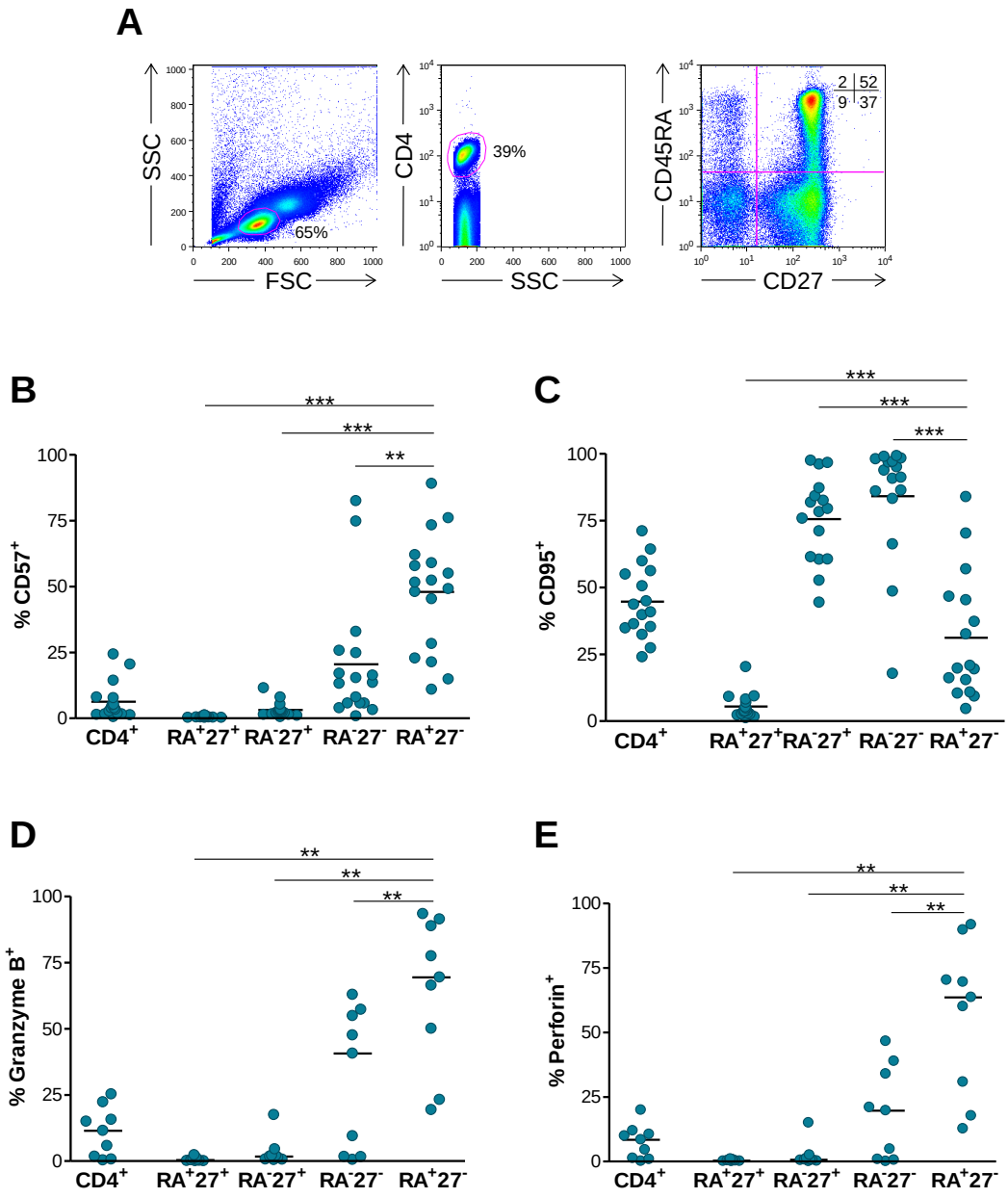

Figure 1: CD4$^+$ CD45RA$^+$ CD27$^-$ cells express high levels of differentiation markers and of cytolytic molecules.

A) Phenotypic analysis of CD45RA/CD27 expression on CD4$^+$ T cells. PBMCs stained for CD4, CD45RA and CD27 were analysed by flow cytometry. Representative pseudo-color plots are shown. B-E) CD4$^+$ T cells were purified using Magnetic Cell Sorting and surface stained for CD45RA, CD27, CD57 (B) and CD95 (C), and intracellularly for Granzyme B (D) and Perforin (E). The percentage of cells expressing each marker was analysed gating within total CD4$^+$ cells and within each of the CD45RA/CD27 subsets. Horizontal lines depict median values. Statistical analysis was performed using the Wilcoxon matched pairs test (GraphPad Prism).

These data indicate that although CD45RA$^+$CD27$^-$ CD4$^+$ T cells display phenotypic characteristics of highly differentiated T cells, they are potentially able to perform cytotoxic effector functions.

In addition to their cytotoxic potential, CD45RA$^+$CD27$^-$ CD4$^+$ T cells are also multifunctional as assessed by multiparameter flow cytometric analysis of IFN-γ, IL-2, TNF-α and CD40 ligand co-expression [2]. Another crucial indicator of CD4$^+$ T cell functionality is the ability to proliferate and survive following TCR activation. In order to address this issue, we evaluated the ability of purified subsets defined by CD45RA and CD27 expression to accumulate in culture following activation with anti-CD3 and irradiated autologous APCs (Figure 2). We observed that, after an initial slight increase in cell number, CD45RA$^+$CD27$^-$ cells underwent a steeper decline than the other subsets (Figure 2A). CD45RA$^+$CD27$^-$ cells consistently showed the lowest cell recovery, failing to accumulate in culture after activation (Figure 2B). To clarify the contribution of reduced proliferation and/or increased cell death to the decreasing numbers of CD45RA$^+$CD27$^-$ cells after *in vitro* activation, we first assessed the proliferative ability of the CD45RA/CD27 CD4$^+$ T cell subsets (Figure 3). The expression of the cell cycle-related nuclear protein Ki67 was quantified before (*ex vivo*) and after TCR activation (Figure 3A). Within the freshly isolated CD45RA/CD27 CD4$^+$ T cell subsets, only CD45RA$^-$CD27$^+$ and CD45RA$^-$CD27$^-$ cells appear to express an appreciable amount of Ki67 (Figure 3A upper panel), suggesting that the CD45RA$^+$CD27$^-$ CD4$^+$ T cell subset is comprised of mostly resting cells as was described for CD45RA re-expressing CD8$^+$ T cells [12]. Following *in vitro* activation with anti-CD3 and irradiated autologous APCs, CD45RA$^+$CD27$^-$ CD4$^+$ T cells consistently expressed high levels of Ki67 (Figure 3A lower panel, B). Proliferation was also assessed by tritiated thymidine incorporation (Figure 3C) which confirmed the results obtained by Ki67 staining, with CD45RA$^+$CD27$^-$ CD4$^+$ T cells showing proliferative ability following activation. These results indicate that the CD45RA$^+$CD27$^-$ CD4$^+$ T cell subset is not exhausted and suggest that the inability to accumulate in culture might be due to a high susceptibility to apoptosis following activation. To confirm this hypothesis, we performed an apoptosis time-course by monitoring Annexin V staining and PI incorporation during activation (Figure 4). The percentage of live cells (Annexin V$^-$ PI$^-$) observed in each time point is shown (Figure 4B). By day 3, the proportion of live cells within the CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ subsets was reduced to less than 50%.

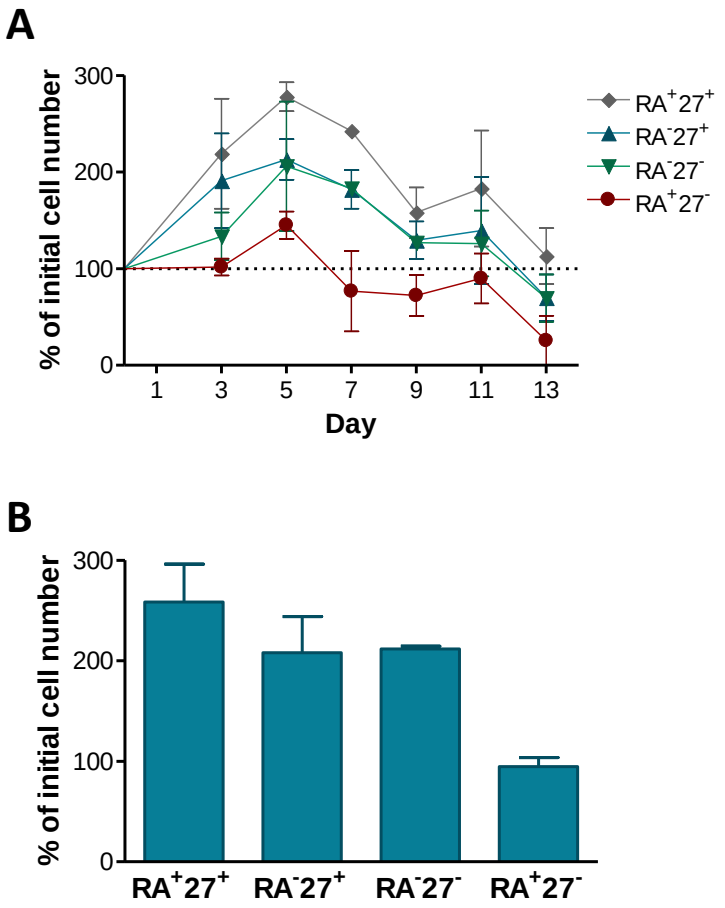

Figure 2: CD4$^+$ CD45RA$^+$CD427$^-$ cells do not accumulate in culture following activation.

A) Purified CD45RA/CD27 CD4$^+$ T cell subsets were activated with anti-CD3 and irradiated APCs. On the indicated time-points, the cell number was determined using a hemocytometer. Results are expressed as a percentage of the initial number of cells placed in culture. Error bars represent the SE from the mean of two separate experiments. B) Purified CD45RA/CD27 CD4$^+$ T cell subsets were activated with anti-CD3 and IL-2 for 4 days. Bar graph shows the cell numbers recovered as a percentage of the initial number of cells placed in culture. Error bars represent the SE from the mean of three separate experiments (GraphPad Prism).

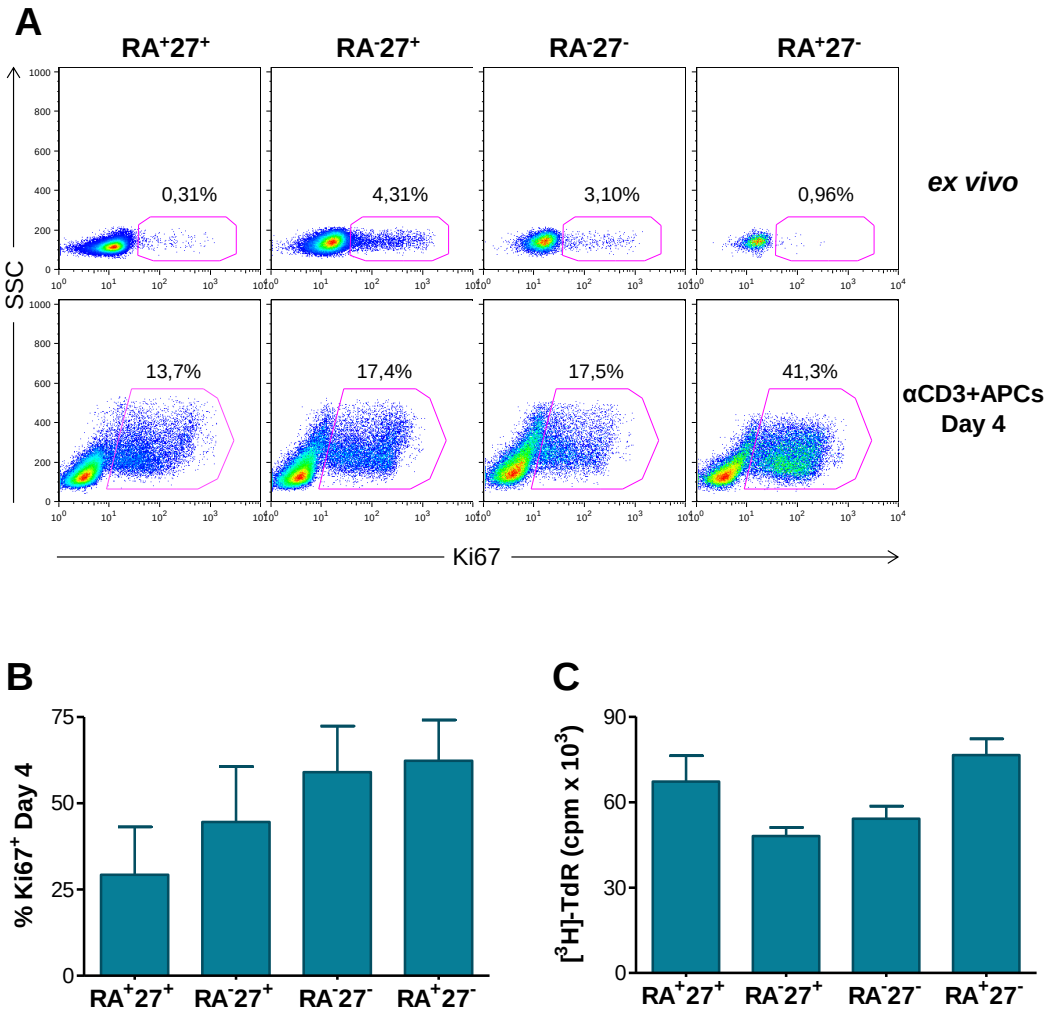

Figure 3: CD4$^+$ CD45RA$^+$ CD27$^-$ cells display slow turnover *ex vivo* but are able to proliferate following activation.

A, upper panel) CD4$^+$ T cells were stained *ex vivo* and analysed by flow cytometry. The percentage of cells expressing Ki67 was determined by gating within total CD4$^+$ cells and within each of the CD45RA/CD27 subsets. Pseudo-colour plots from a representative experiment out of two performed are shown. A, lower panel, B, C) Purified CD45RA/CD27 CD4$^+$ T cell subsets were activated with anti-CD3 and irradiated APCs for 4 days. A, lower panel) Ki67 expression was assessed within each subset. Representative pseudo-colour plots are shown. B) Bar graph shows the percentage of Ki67 positive cells within each subset. Error bars represent the SE from the mean of five separate experiments. C) Proliferation was also assessed by tritiated thymidine incorporation under the same culture conditions. Results are expressed as counts per minute (cpm). Error bars represent the SE from the mean of three separate experiments.

The susceptibility to apoptosis following activation was more pronounced within the $CD45RA^+CD27^-$ population, which was extinct by day 15 (Figure 4B). Moreover, we observed that $CD4^+$ $CD45RA^+CD27^-$ cells expressed significantly lower levels of the anti-apoptotic protein Bcl-2, measured by intracellular staining of $CD4^+$ T cell subsets directly *ex vivo*, compared to all the other subsets [2]. Taken together, these data indicate that pro-survival pathways are defective in $CD45RA^+CD27^-$ $CD4^+$ T cells making them susceptible to apoptosis, as has been described for $CD8^+$ T cells [5,19].

The PI3K/Akt pathway plays a critical role in T cell survival by blocking pro-apoptotic proteins and promoting the function of pro-survival components, in particular several members of the Bcl-2 family [20-23]. Akt can be phosphorylated on two residues, serine 473 and threonine 308 [24]. Previous studies have shown that there is defective phosphorylation of Akt(Ser473) but not Akt(Thr308) in highly differentiated $CD27^-CD28^-$ $CD8^+$ T cells [25,26]. The $CD27^-CD28^-$ subset is heterogeneous and comprises both $CD45RA^-CD27^-$ and $CD45RA^+CD27^-$ T cells [1]. We sought to determine whether $CD45RA^+CD27^-$ $CD4^+$ T cells also had impaired Akt(Ser473) phosphorylation. In order to achieve this goal, we proceeded with the optimization of pAkt(Ser473) detection by flow cytometry (Figure 5). The detection of pAkt with traditional methods for analysing intracellular signalling pathways, such as Western Blot, was precluded by the extremely low percentages of the $CD45RA^+CD27^-$ subset within $CD4^+$ T cells (less than 1% in most healthy donors). The flow cytometric approach allowed us to work with total $CD4^+$ T cells and analyse the expression of phospho-proteins at a single cell level by gating within each CD45RA/CD27 $CD4^+$ subset. As described for other $CD4^+$ T cell populations [27,28], the background levels of Akt phosphorylation may hinder the detection of pAkt(Ser473) up-regulation upon activation. To overcome this issue, $CD4^+$ T cells were rested over-night in medium containing 1% human serum, followed by a further 2 hour starvation in serum-free medium. This approach was effective in lowering the pAkt(Ser473) background levels, allowing a maximal increase in phosphorylation upon activation. We opted to optimize the technique using anti-CD3 and anti-CD28 as an optimal stimulation of total $CD4^+$ T cells (Figure 5). As predicted, the levels of Akt(Set473) phosphorylation upon activation correlated with the expression levels of the co-stimulatory molecule CD28: the subsets that expressed high levels of CD28, i.e. $CD45RA^+CD27^+$ and $CD45RA^-CD27^+$ [2], had the highest levels of pAkt(Set473), whilst cells that expressed low or negligible levels

of CD28, i.e. CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ [2], were unable to phosphorylate Akt(Ser473) upon activation (Figure 5).

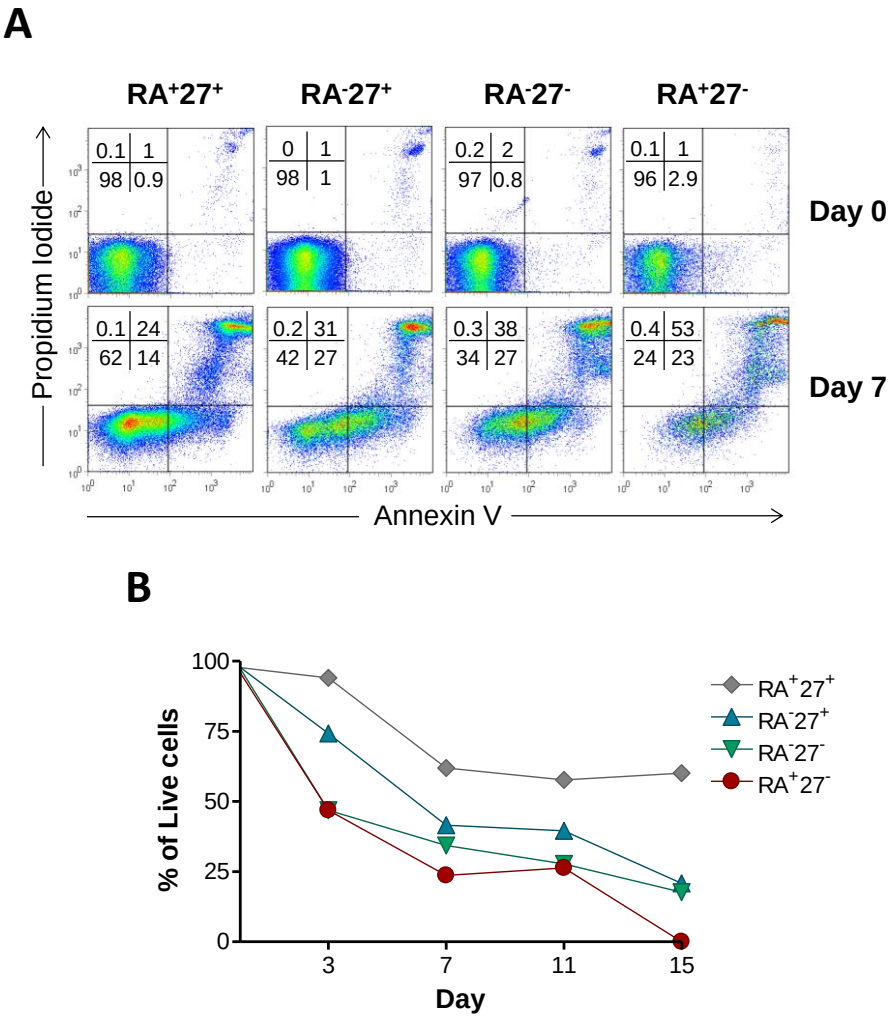

Figure 4: CD4$^+$ CD45RA$^+$CD427$^-$ cells have impaired cell survival following activation.

Purified CD45RA/CD27 CD4$^+$ T cell subsets were activated with anti-CD3 and irradiated APCs. Apoptosis was assessed by Annexin V staining and PI incorporation. A) Pseudo-colour plots show the Annexin V/PI profile of each subset *ex vivo* (Day 0) and after 7 days in culture with anti-CD3 and irradiated APCs. The results shown are representative of four experiments performed. B) The percentage of live cells (Annexin V$^-$ PI$^-$) was assessed within each subset in the indicated days of culture with anti-CD3 and irradiated APCs.

In order to ensure that the pAkt(Ser473) staining was specific, the PI3K/Akt pathway was blocked, as a negative control, by incubating the cells with the PI3K inhibitor LY294002 prior to activation, which abrogated Akt(Ser473) phosphorylation in CD45RA$^+$CD27$^+$ and CD45RA$^-$CD27$^+$ cells (Figure 5). As a vehicle control to exclude any impact from the DMSO present in the PI3K inhibitor solution, activated cells were pre-incubated with an equivalent concentration of DMSO.

Once the technique was validated, we were then able to assess Akt(Ser473) phosphorylation within the CD45RA/CD27 CD4$^+$ subsets by activating total PBMCs (Figure 6). By removing the requirement for purified CD4$^+$ T cells, autologous APCs were retained as a source of multiple co-stimulatory ligands to enable the activation of all the subsets, including the ones that express low levels of CD28. Nevertheless, despite the presence of alternative co-stimulatory signals provided by these APCs, CD45RA$^+$CD27$^-$ CD4$^+$ T cells still showed impaired Akt(Ser473) phosphorylation (Figure 6). The results thus far portray the CD45RA$^+$CD27$^-$ CD4$^+$ T cell subset as a potentially effective cytotoxic population with proliferative potential. On the other hand, this subset is prone to apoptosis following activation, a characteristic associated with low levels of Bcl-2 expression and Akt(Ser473) phosphorylation.

Although the presence of CD45RA$^+$CD27$^-$ CD4$^+$ T cells has been previously described [29], the mechanism by which they are generated is not known. While it has been shown that IL-7 and IL-15 can induce CD45RA re-expression on CD8$^+$ T cells [5], it remains unclear if either CD45RA$^-$CD27$^+$ or CD45RA$^-$CD27$^-$ CD4$^+$ T cells are able to re-express CD45RA and, if they are, which stimulatory signal could drive this process. It has been proposed that memory CD8$^+$ T cells progressively re-express CD45RA in the absence of antigenic stimulation and hence this process would appear to be indicative of a resting or quiescent state [12,14]. Moreover, virus-specific CD45RA re-expressing CD8$^+$ T cells activated with peptide Ag *in vitro* have been shown to down-modulate CD45RA, while concomitantly up-regulating CD45RO [10,12,30,31]. We also observed that purified CD45RA$^+$CD27$^-$ CD4$^+$ T cells lose CD45RA expression and progressively acquire a CD45RA$^-$CD45RO$^+$ phenotype following activation with anti-CD3 and IL-2 (data not shown). This result is in agreement with the view that CD45RA re-expression, which is observed in the presence of homeostatic cytokines, is prevented by TCR stimulation [5]. As mentioned above, previous studies have shown that γ_C cytokines, which drive homeostatic proliferation, can also induce CD45RA re-expression on CD8$^+$ T cells [5,32,33].

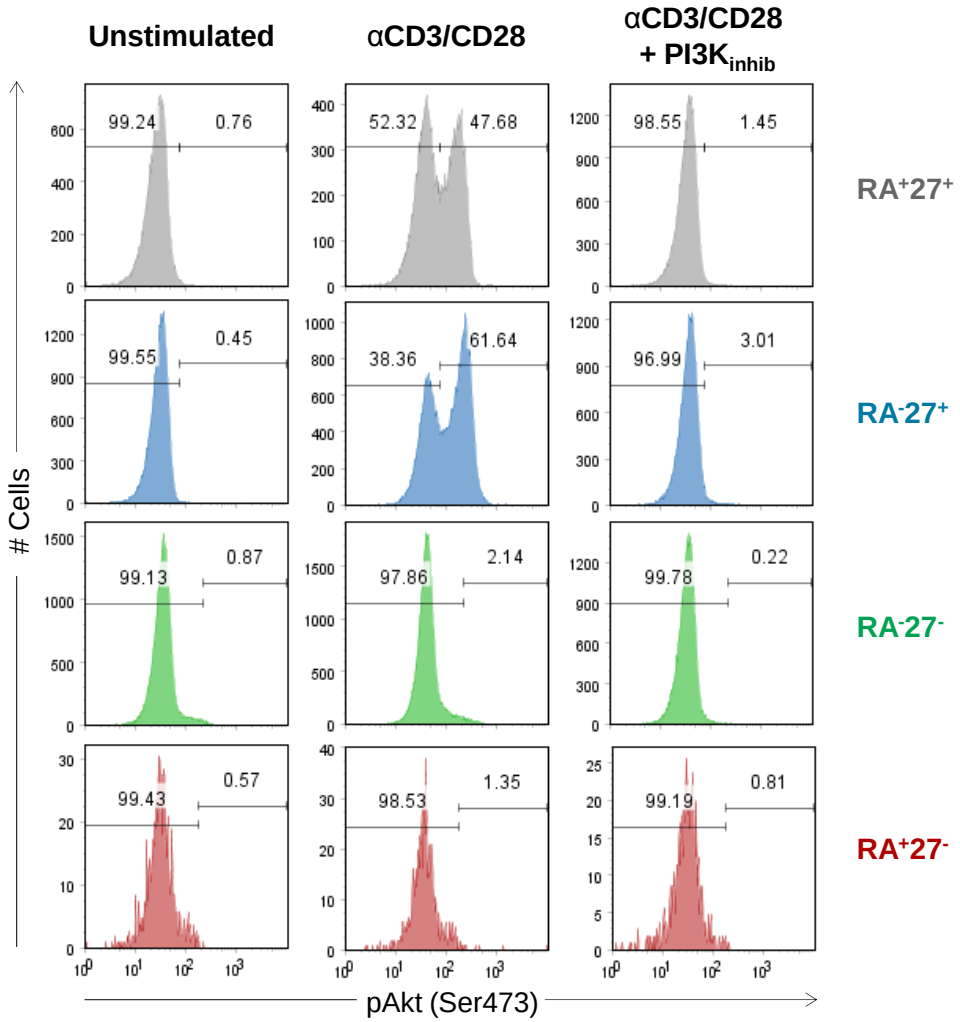

Figure 5: Detection of pAkt(Ser473) phosphorylation by flow cytometry.

Total $CD4^+$ T cells were starved over-night in 1% human serum. Prior to stimulation, cells were starved in serum-free medium for 2 hours. After surface staining for CD45RA and CD27, cells were stimulated with 1µg/ml of anti-CD3 and 1µg/ml of anti-CD28 for 10 minutes at 37°C. Activated cells were immediately fixed with 2% formaldehyde. Cells were permeabilized with 90% methanol and then incubated with anti-pAkt(Ser473) antibody. Samples were immediately analysed by flow cytometry. Pre-incubation with the PI3K inhibitor LY294002 was used as a negative control. DMSO was used as a vehicle control.

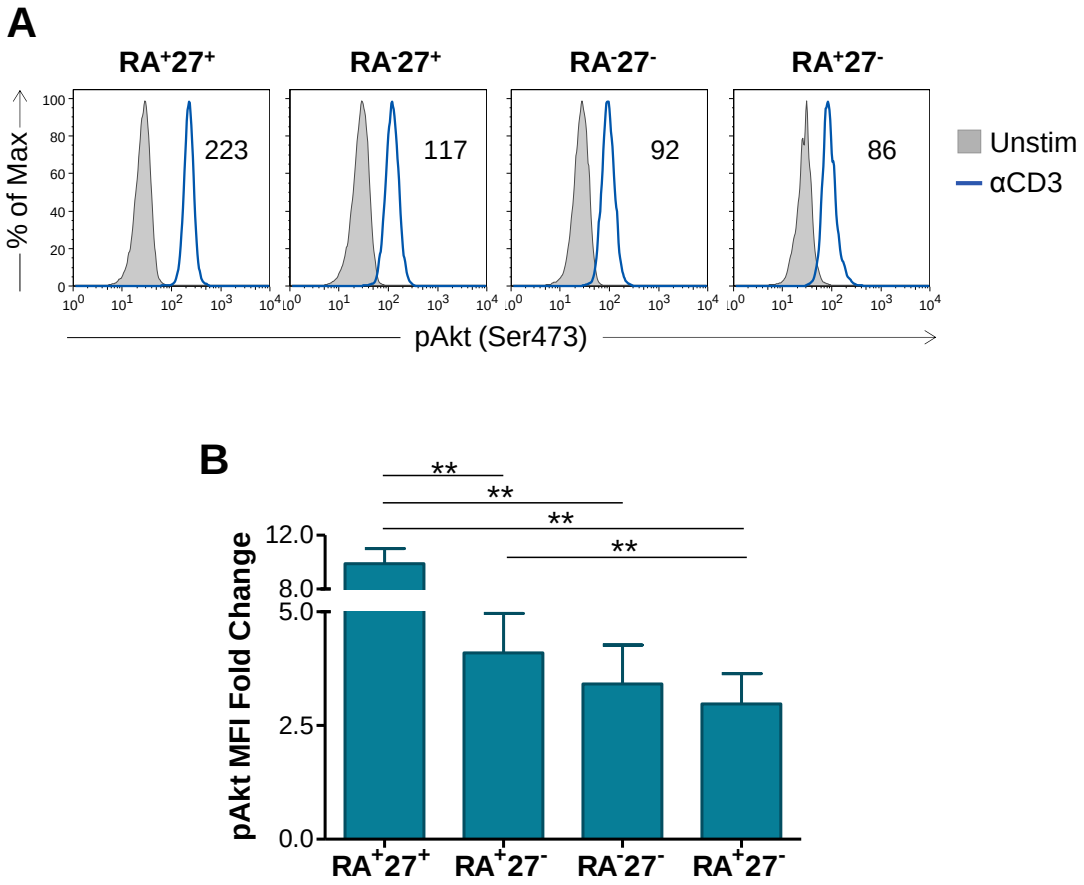

Figure 6: CD4$^+$ CD45RA$^+$CD27$^-$ cells have impaired Akt(Ser473) phosphorylation.

A) Representative overlays of pAkt(Ser473) expression within CD4$^+$ CD45RA/ CD27 subsets are shown. PBMCs were activated with anti-CD3 (solid line) or left unstimulated whilst undergoing the same protocol in the absence of anti-CD3 (grey histogram). The values represent the median fluorescent intensity of pAkt(Ser473) within each subset following activation. B) Bar graph represents the fold change in pAkt(Ser473) MFI after activation relative to the MFI observed in unstimulated cells within the respective subset. Error bars represent the SE from the mean of five separate experiments. Statistical analysis was performed using paired t test (GraphPad Prism).

In order to elucidate whether CD45RA re-expression on $CD4^+$ T cells is driven by a homeostatic mechanism mediated by γ_C cytokines, we cultured purified $CD45RA^-CD27^+$ and $CD45RA^-CD27^-$ cells in the presence of IL-2, IL-7 or IL-15 in the absence of TCR stimulation (Figures 7-8). IL-7 is known to induce the proliferation of $CD45RA^+$ $CD4^+$ T cells without inducing CD45RO expression [34,35]. We first investigated whether this cytokine could induce CD45RA re-expression on $CD45RA^-CD27^+$ $CD4^+$ T cells. As illustrated on Figure 7A, a population re-expressing CD45RA and down-modulating CD45RO emerged from the $CD45RA^-CD27^+$ subset cultured in the presence of IL-7. TCR stimulation alone did not induce CD45RA re-expression neither did the other cytokines tested, such as TGF-β, IL-10 and IFN-α (data not shown). In order to clarify whether CD45RA re-expression is accompanied by IL-7-driven proliferation, we performed a CFSE dilution assay on $CD45RA^-CD27^+$ cells in the presence of IL-7. The $CD45RA^+$ population showed a higher rate of proliferation than the cells that did not re-express CD45RA (Figure 7B), indicating that CD45RA re-expression is accompanied by IL-7-driven proliferation. We next determined whether the CD45RA re-expressing cells that were generated *in vitro* phenotypically resembled those that are found *in vivo*. In order to achieve this, $CD45RA^-CD27^+$ $CD4^+$ T cells were cultured *in vitro* in the presence of IL-7 and the expression of Bcl-2 and IL-7Rα was monitored at different time points (Figure 7C). The population that did not re-express CD45RA ($CD45RA^-$) showed high levels of Bcl-2 throughout the culture period (Figure 7C). As for IL-7Rα expression, the $CD45RA^-$ population displayed the normal kinetics associated with the presence of IL-7 [36,37], that is an initial down-modulation of IL-7Rα, followed by a recovery of the original levels (Figure 7C). In contrast, the progressive down-regulation of both Bcl-2 and IL7-Rα on the population that re-expressed CD45RA ($CD45RA^+$) was not transient (Figure 7C). The CD45RA re-expressing $CD4^+$ T cells generated *in vitro* by IL-7 thus closely resemble the naturally occurring $CD45RA^+CD27^-$ cells in terms of Bcl-2 and IL-7Rα expression. These results suggest that IL-7-driven homeostatic proliferation could induce the re-expression of CD45RA on a sub-population of $CD45RA^-CD27^+$ $CD4^+$ T cells. We also investigated if CD45RA re-expression could be induced on $CD45RA^-CD27^+$ cells by other γ_C cytokines. Although a low level of CD45RA expression was observed in a small proportion of $CD45RA^-CD27^+$ $CD4^+$ T cells that were cultured with IL-2 or IL-15 (Figure 8A), this was considerably lower than that induced by IL-7 (Figure 7A).

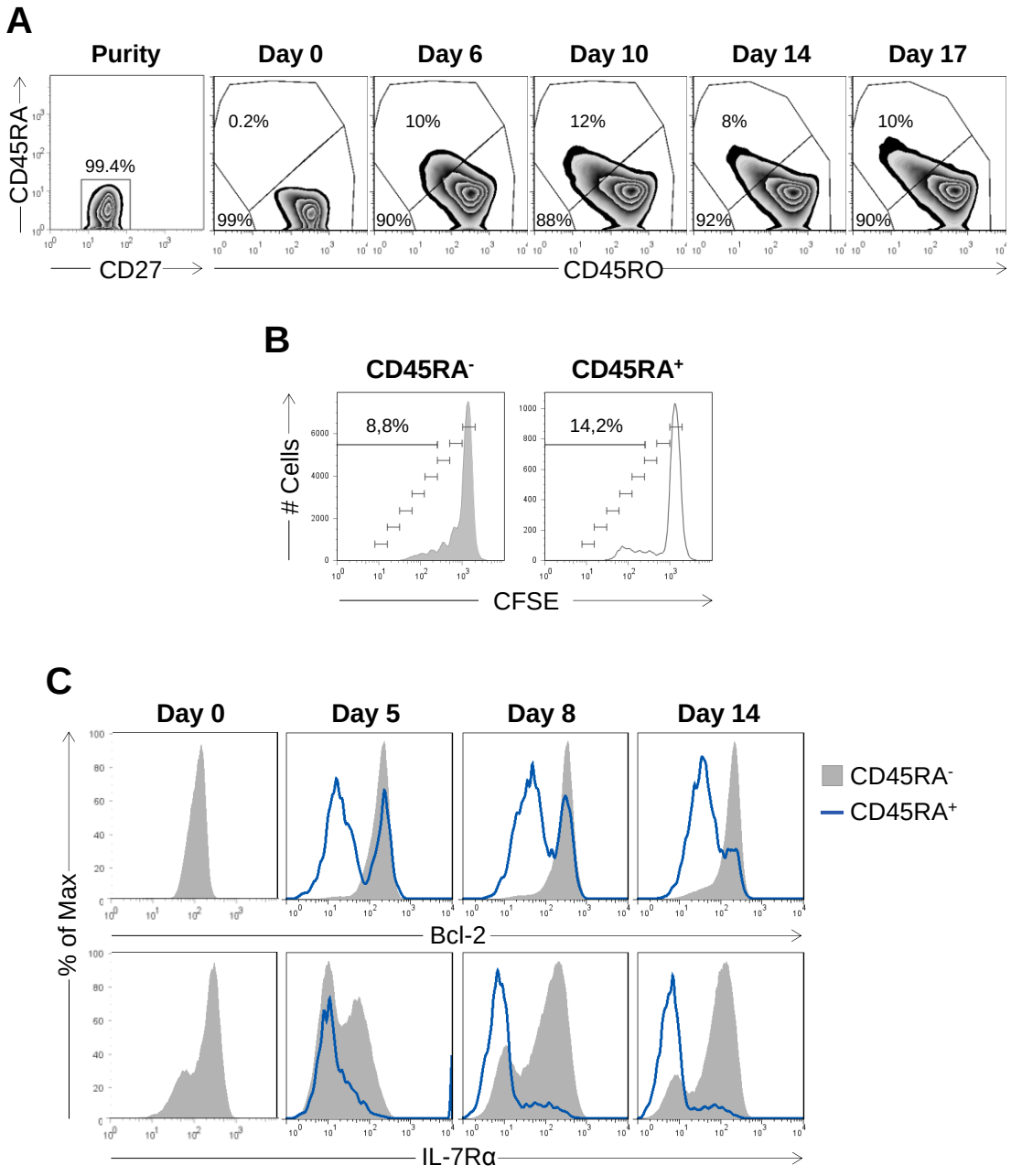

Figure 7: CD4$^+$ CD45RA$^-$CD27$^+$ cells stably re-express CD45RA following IL-7-driven proliferation.
CD4$^+$CD45RA$^-$CD27$^+$ cells were purified by FACS sorting and analysed for the expression of CD45RA and CD45RO prior to culture. A) Cells were stimulated with IL-7 and CD45RA/CD45RO expression was assessed by flow cytometry at the indicated time-points. The results shown are representative of twelve experiments. B) CFSE dilution was assessed in the cells that re-expressed CD45RA (grey line) and in the population that remained CD45RA$^-$ (grey histogram) following 14 days of culture in the presence of IL-7. Values represent the percentage of cells that underwent over 2 rounds of cell division. Histograms from a representative experiment out of two performed are shown. C) Overlays represent Bcl-2 and IL-7Rα expression before and during culture in the presence of IL-7. Expression of these markers was assessed in the cells that re-expressed CD45RA (blue line) and in the population that remained CD45RA$^-$(grey histogram). Histograms from a representative experiment out of three performed are shown.

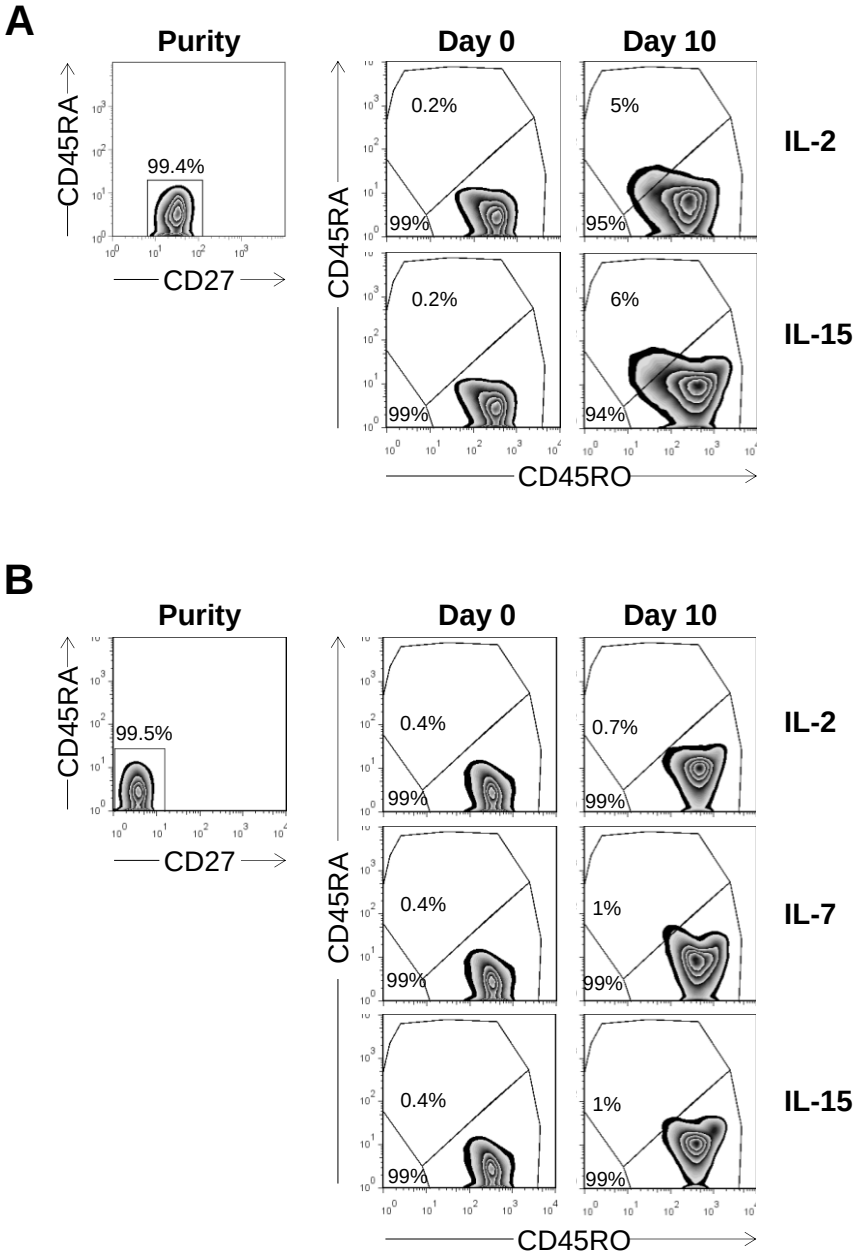

Figure 8: $CD4^+$ $CD45RA^-CD27^+$ cells do not re-express CD45RA when stimulated with other γ_C cytokines nor do $CD45RA^-CD27^-$.

$CD4^+$ $CD45RA^-CD27^+$ (A) and $CD45RA^-CD27^-$ (B) cells were purified by FACS sorting and analysed for the expression of CD45RA and CD45RO prior to culture. Cells were stimulated with IL-2, IL-7 or IL-15 and CD45RA/CD45RO expression was assessed by flow cytometry at the indicated time-points. The results shown are representative of three experiments.

Finally, we assessed if the $CD45RA^-CD27^-$ subset cultured in the same experimental conditions would re-express CD45RA. We observed that, regardless of the γ_C cytokine tested, the cells remained $CD45RO^+$ throughout the culture period (Figure 8B). These results suggest that IL-7-driven homeostatic proliferation can induce the CD45RA re-expression on $CD45RA^-CD27^+$ but not on $CD45RA^-CD27^-$ $CD4^+$ T cells to generate a $CD45RA^+$ memory population.

The induction of CD45RA re-expression is most likely prompted by changes in the transcriptional program. We next assessed which transcription factors could be responsible for the switch to CD45RA re-expression on a sub-population of $CD45RA^-$ $CD27^-$ $CD4^+$ T cells. We looked for potential candidates known to be involved in T cell differentiation, such as the T-box transcription factors T-bet and Eomes, and the transcriptional repressor Blimp-1. In order to assess if these transcription factors were differentially expressed in CD45RA/CD27 $CD4^+$ T cell subsets, we measured the *ex vivo* mRNA levels of Blimp-1, T-bet and Eomes in these purified subsets by reverse transcription PCR (Figure 9A). All three transcription factors were present in relatively negligible levels in $CD45RA^+CD27^+$ cells, peaking within the $CD45RA^-CD27^-$ and $CD45RA^+CD27^-$ subsets (Figure 9A). T-bet and Eomes expression was markedly higher in $CD45RA^+CD27^-$ cells compared to all the other subsets (Figure 9A). Of note, $CD45RA^-CD27^+$ cells expressed relatively low levels of both these transcription factors (Figure 9A). The next key question was whether IL-7-induced CD45RA re-expression was associated with the up-regulation of any of these transcription factors on $CD45RA^-$ $CD27^+$ cells. Thus we measured mRNA levels of these same transcription factors were measured in purified $CD4^+$ $CD45RA^-CD27^+$ cells before (*ex vivo*) and after a 3 day culture period in the presence of increasing concentrations of IL-7 (Figure 9B). Blimp-1 mRNA levels did not change after culture with IL-7 (Figure 9B). As for Eomes, even though we didn't measure the *ex vivo* mRNA levels in this particular experiment, the results shown in Figure 9A indicate that $CD45RA^-CD27^+$ cells express low levels of this transcription factor *ex vivo*. Nevertheless, Eomes mRNA levels did not show a dose response to IL-7 (Figure 9B), suggesting that the expression of this transcription factor is probably not induced in $CD45RA^-CD27^+$ in the presence of IL-7. Only T-bet appeared to be induced by IL-7 in $CD45RA^-CD27^+$ cells, peaking following stimulation with the same IL-7 concentration that induced CD45RA re-expression on these cells (10ng/ml) (Figure 9B). Interestingly, a recent study investigating the impact of CMV infection on the

transcriptional program of human $CD8^+$ T cell subsets has shown that $CD45RA^+CD27^-$ cells express significantly higher levels of T-bet when compared to $CD45RA^-CD27^+$ $CD8^+$ T cells [38].

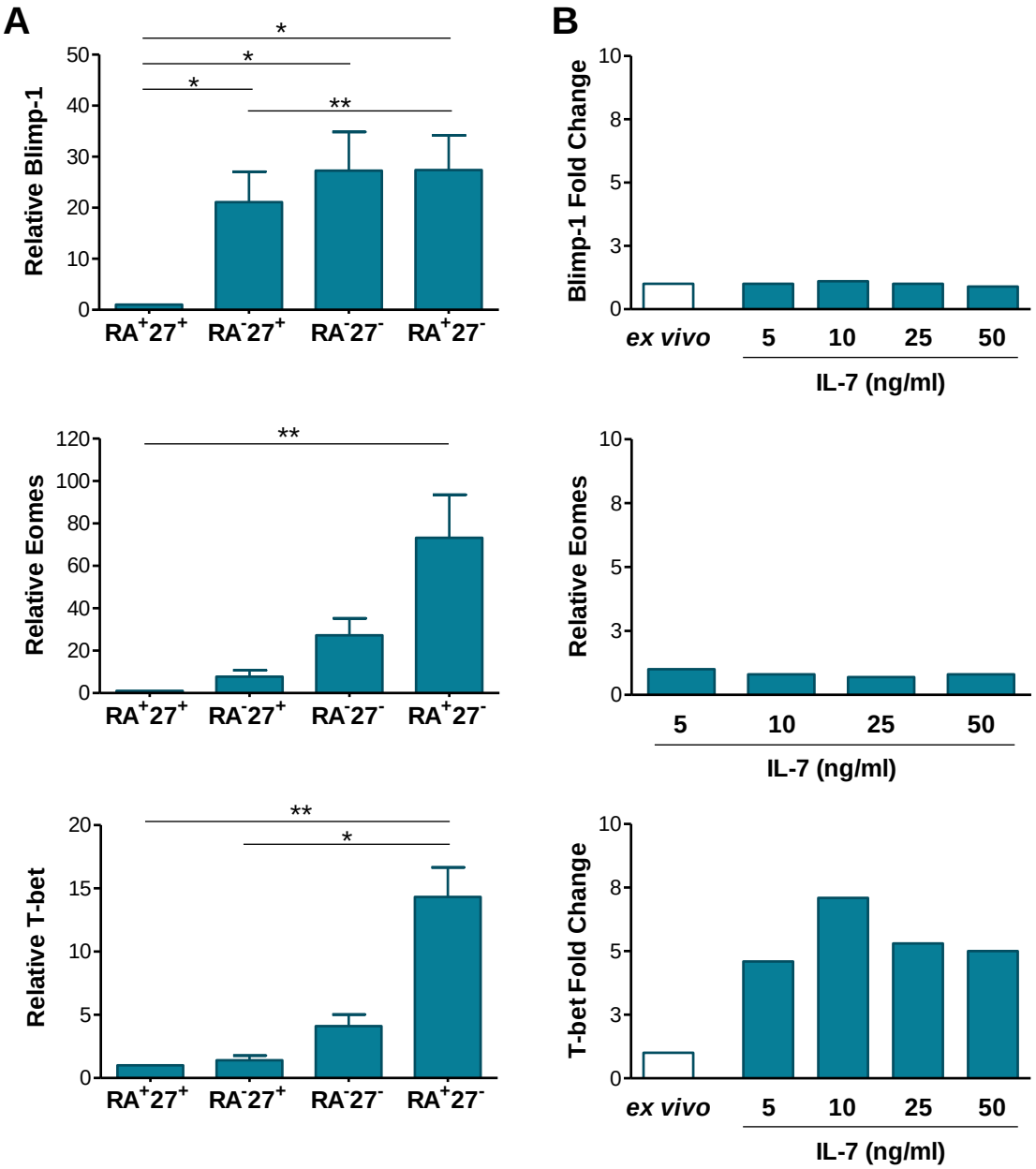

Figure 9: Transcription factors involved in T cell differentiation are highly expressed in $CD4^+$ $CD45RA^+CD27^-$ cells but only T-bet is induced by IL-7.

A) The mRNA levels of the transcription factors Blimp-1, T-bet and Eomes were measured *ex vivo* in purified CD45RA/CD27 $CD4^+$ subsets by reverse transcription PCR. Bar graph represents the mRNA levels in each subset normalized for the levels observed in the $CD45RA^+CD27^+$ subset. Error bars represent the SE from the mean of three separate experiments. B) The mRNA levels of the same transcription factors were measured in purified $CD4^+$ $CD45RA^-CD27^+$ cells before (*ex vivo*) and after a 3 day culture in the presence of different concentrations of IL-7. Bar graph represents the fold change in mRNA levels following IL-7 culture relative to the levels observed *ex vivo*.

Moreover, T-bet is known to repress IL-7Rα expression on $CD8^+$ T cells [39,40] which raises the possibility that T-bet might be a good candidate for the mediator of the phenotypic changes we observed upon CD45RA re-expression (Figure 7C). Although these results are preliminary, they point to a possible role for T-bet in the transcriptional program that is activated in the population of $CD45RA^-CD27^+$ cells induced to re-express CD45RA in the presence of IL-7.

The data gathered so far indicate that $CD45RA^+CD27^-$ $CD4^+$ T cells are not exhausted, although they appear to be prone to cell death following activation. We can hypothesise that this population might be replenished *in vivo* by CD45RA re-expressing cells originating from the $CD45RA^-CD27^+$ $CD4^+$ T cell pool through a homeostatic process driven by IL-7.

Chapter 2.2

CD45RA$^+$CD27$^-$ CD4$^+$ T cells exhibit p38 MAPK-regulated telomere-independent senescence

The CD45RA$^+$CD27$^-$ CD4$^+$ T cell subset displays several characteristics of a senescence-prone population, including expression of high levels of the senescence markers CD57 (Figure 2) and KLRG1 (Di Mitri *et al.*, submitted for publication), and susceptibility to apoptosis following activation (Figure 4). Although CD45RA$^+$CD27$^-$ cells retain proliferative ability (Figure 3), these cells might only be able to undergo a small number of cell divisions before reaching critically short telomeres, which in turn could trigger apoptosis or telomere-dependent senescence. Telomere erosion is a common feature of cells approaching senescence, usually associated with failure to induce telomerase activity upon repeated stimulation [7,41-43]. In order to investigate if CD45RA$^+$CD27$^-$ CD4$^+$ T cells show signs of telomere erosion, we assessed the telomere length on the CD4$^+$ T cell subsets defined by the expression of CD45RA and CD27 (Figure 10A). As previously described for naive CD4$^+$ T cells [44,45], CD45RA$^+$CD27$^+$ cells had significantly longer telomeres in comparison to any of the memory subsets (Figure 10A). We also found that, despite their differentiated phenotype, CD45RA$^+$CD27$^-$ cells had significantly longer telomeres than the other memory subsets (Figure 10A), suggesting that these cells are not undergoing telomere-dependent senescence. Although telomere length may give an indication of residual replicative capacity of T cell subsets, the modulating effects of telomerase, an enzyme able to add back telomeric sequences, must be taken into consideration. Hence we measured telomerase activity following TCR stimulation in all the CD45RA/CD27 subsets and we observed that it was impaired in CD45RA$^+$CD27$^-$ cells (Figure 10B). Taken together, these data suggest that CD45RA$^+$CD27$^-$ cells do not constitute a subset that has reached end-stage differentiation, since they have relatively long telomeres. However these cells may have limited replicative potential, seeing that they lack the ability to turn on telomerase upon activation and will thus not be able to restore any telomere loss resulting from cell division.

The relatively long telomere length observed on CD4$^+$ CD45RA$^+$CD27$^-$ cells suggests that the senescent traits displayed by this subset are not driven by telomere-dependent senescence, whereas their defective telomerase activity implies the opposite.

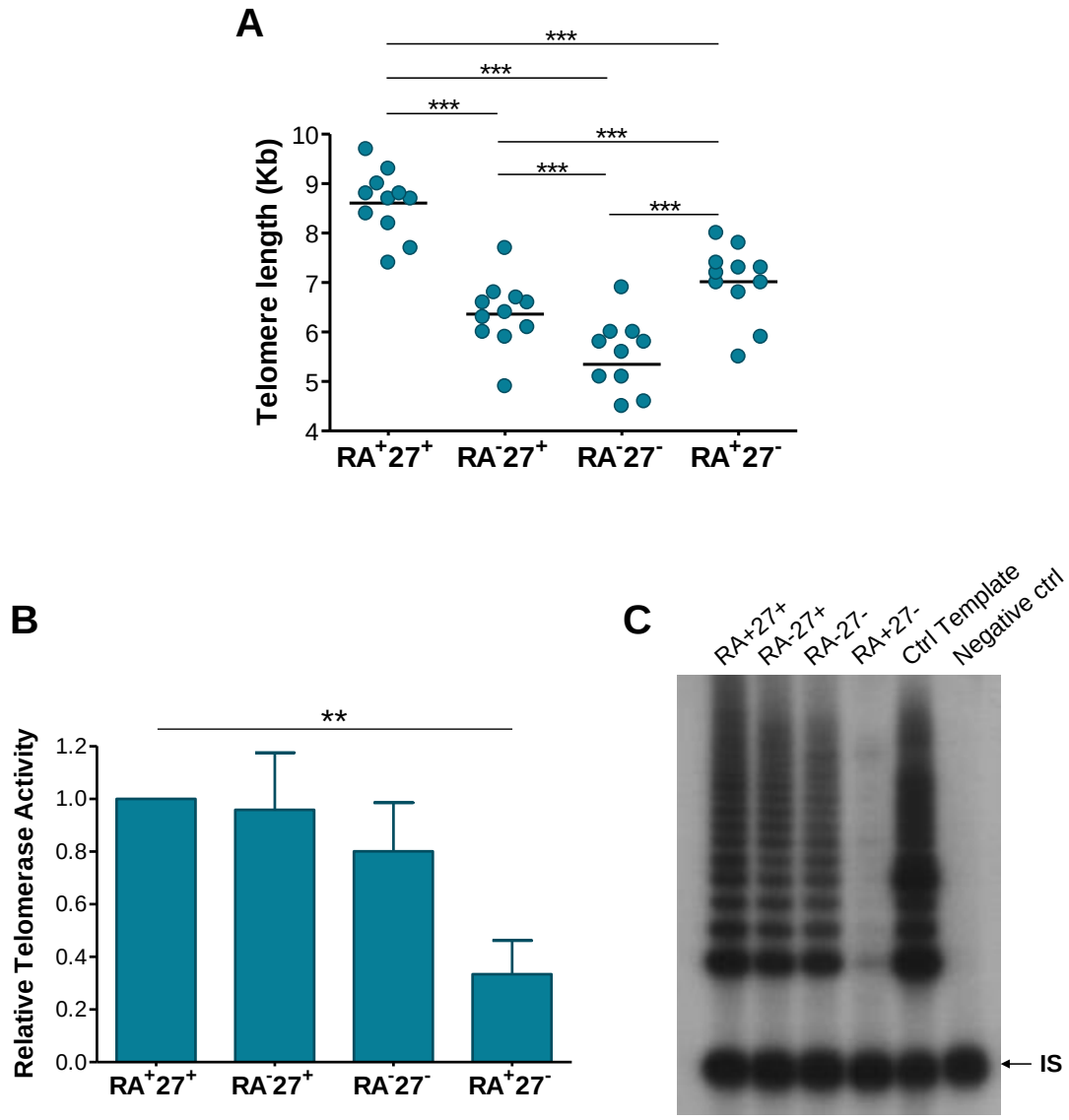

Figure 10: CD4$^+$ CD45RA$^+$CD27$^-$ cells do not have the shortest telomeres but have impaired telomerase activity.

(A) Telomere length was determined by Flow-FISH. Each circle represents one individual with the mean telomere length shown as a horizontal bar. Statistical analysis was performed using the Wilcoxon matched pairs test (GraphPad Prism). (B) Telomerase activity was determined by telomeric repeat amplification protocol assay. Purified subsets were activated with anti-CD3 and irradiated APCs for 4 days. Cell extracts from equivalent numbers of Ki67$^+$ cells were used to determine telomerase activity, calculated as a ratio between the optical density of the telomeric repeat bands and of the internal standard band (IS). Graph represents telomerase activity normalized for the activity observed in the CD45RA$^+$CD27$^+$ subset. Error bars represent the SE from the mean of five separate experiments. Statistical analysis was performed using the Wilcoxon matched pairs test (GraphPad Prism). C) Autoradiography of a TRAP assay acrylamide gel from a representative experiment is shown. Control template consists of PCR mix and telomeric template with no cell extract added. As a negative control, lysis buffer was used instead of cell extract.

In order to clarify these apparently contradictory results, we sought to determine if $CD4^+$ $CD45RA^+CD27^-$ cells expressed the cellular senescence marker γ-H2AX, the phosphorylated form of the histone H2AX. Human senescent cells can be identified by the presence of senescence-associated DNA-damage foci (SDFs) [46], which consist of proteins that are associated with DNA damage, such as γ-H2AX [15]. The local induction of γ-H2AX allows microscopic detection of distinct foci that most likely represent a single DSB [47]. Flow cytometric approaches can also be used to detect γ-H2AX [48,49]. Several studies report a good correlation between the levels of γ-H2AX detected by flow cytometry and the number of DSBs [50-52]. We first optimized the detection of γ-H2AX by flow cytometry (Figure 11). Immuno-fluorescence studies have shown that ionizing radiation (IR) induces the formation of γ-H2AX nuclear foci at the sites of IR-induced DSBs [53-56]. Therefore we used irradiated $CD4^+$ T cells as a positive control for γ-H2AX staining (Figure 11A). We performed a time-course of γ-H2AX staining on total $CD4^+$ T cells activated with anti-CD3 and IL-2 also stained for CD45RA/CD27 (Figure 11B). γ-H2AX-expressing cells are virtually undetectable *ex vivo* in any of the subsets (Figure 11B). Following activation, small percentages of γ-H2AX positive cells are seen, especially after over-night stimulation (Figure 11B). However, the proportion of γ-H2AX-expressing cells was very low following short-term activation, regardless of the subset we gated on (Figure 11B). Activation of total $CD4^+$ T cells for more than 24 hours may lead to changes in the CD45RA/CD27 profile and hence hinder the identification of the original subsets. Therefore we decided to purify the CD45RA/CD27 $CD4^+$ subsets by FACS sorting in order to activate the cells for longer periods of time. The purified subsets were activated with anti-CD3 and IL-2 for 4 days before staining for γ-H2AX (Figure 12). As illustrated in Figure 12A, the longer activation period revealed different profiles of γ-H2AX expression between the subsets. $CD45RA^+CD27^+$ and $CD45RA^-CD27^+$ cells had relatively low levels of γ-H2AX, while the $CD45RA^-CD27^-$ and $CD45RA^+CD27^-$ subsets expressed similarly high levels of γ-H2AX (Figure 12). These data raise the hypothesis that $CD45RA^+CD27^-$ cells might be prone to enter a state of senescence independently of telomere shortening. This type of cellular senescence is called stress-induced premature senescence and has been described to be induced by stressful stimuli, such as DNA damage [57,58], over-expression of oncogenes [59] or oxidative stress [60]. The characterisation of telomere-independent senescence has been mostly performed in fibroblasts [46], whereas its occurrence in human T cells remains to be elucidated.

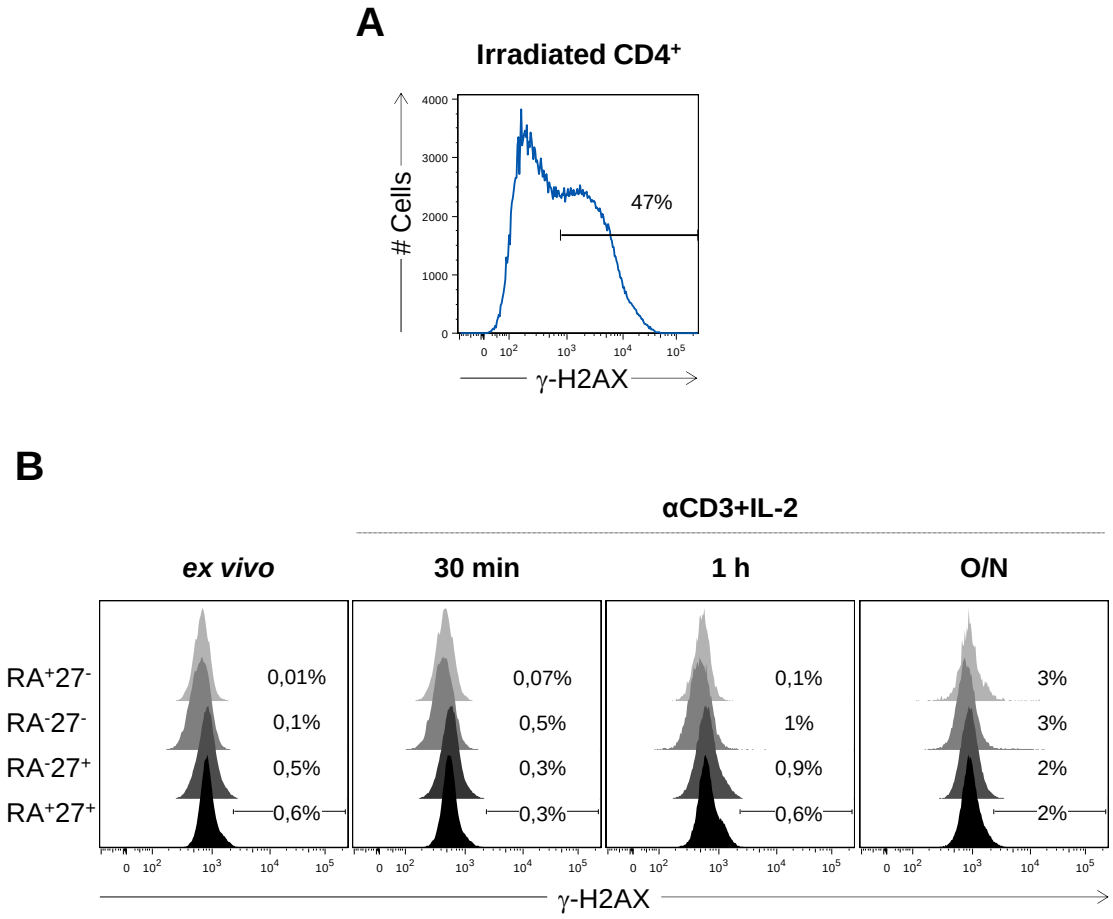

Figure 11: Assessment of γ-H2AX expression by flow cytometry.

Detection of the DNA damage marker phosphorylated histone H2A variant X (γ-H2AX) by flow cytometry was optimized on $CD4^+$ T cells. A) As a positive control, cells were irradiated with 40 Gy γ-radiation. B) $CD4^+$ T cells were stained for CD45RA, CD27 and γ-H2AX *ex vivo* and at the indicated time-points following stimulation with anti-CD3 and IL-2. Histogram overlays from a representative experiment out of three performed are shown. The values represent the percentage of γ-H2AX-positive cells within each subset.

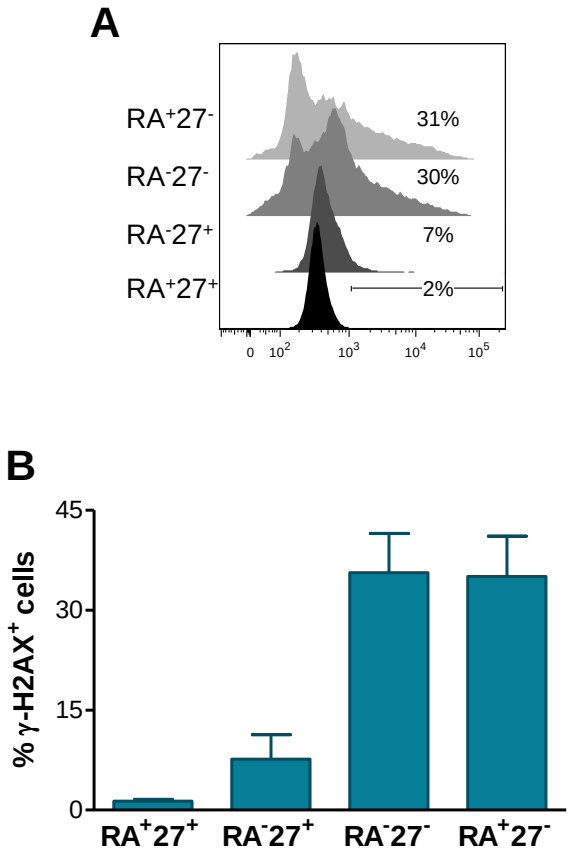

Figure 12: CD4$^+$ CD45RA$^+$CD27$^-$ cells express high levels of γ-H2AX following activation.

Purified CD45RA/CD27 CD4$^+$ T cell subsets were activated with anti-CD3 and IL-2 for 4 days. γ-H2AX expression was assessed by flow cytometry. A) Representative histogram overlays are shown. The values represent the percentage of γ-H2AX-positive cells within each subset. B) Bar graph shows the percentage of γ-H2AX positive cells within each subset. Error bars represent the SE from the mean of three separate experiments.

Besides its well-known functions in inflammation and other types of stress, the p38 MAPK pathway also plays crucial roles in telomere-dependent and -independent senescence [61-63]. In addition, p38 has been found to directly phosphorylate H2AX [64,65]. We addressed the hypothesis that the p38 MAPK pathway may be upstream of the senescence markers observed on CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ cells by assessing the levels of p38 in each CD45RA/CD27 CD4$^+$ subset (Figure 13). The MFI of both total (Figure 13A) and phosphorylated p38 (Figure 13B) was significantly higher in CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ cells compared to the other subsets. The highest levels of both total (Figure 13A) and phosphorylated p38 (Figure 13B) were consistently observed within the CD45RA$^+$CD27$^-$ subset.

Interestingly, when we measured the levels of total p38 following culture of CD45RA$^-$CD27$^+$ cells in the presence of IL-7, we saw that its expression was only detectable within the CD45RA re-expressing population (data not shown). In order to further test the hypothesis that the p38 pathway was involved in the senescent-like phenotype of CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ cells, we assessed the impact of p38 inhibition on these subsets. We first ascertained the efficacy and specificity of the p38 inhibitor BIRB796 by testing its effects on p38 and JNK phosphorylation. As illustrated in Figure 14A, the p38 inhibitor BIRB796 specifically inhibited p38 phosphorylation, whilst having no effect on the phosphorylation of JNK. We next assessed its influence on γ-H2AX levels (Figure 14B). As expected, p38 inhibition had no considerable effect on the already low levels of γ-H2AX observed within the CD45RA$^+$CD27$^+$ and CD45RA$^-$CD27$^+$ subsets (Figure 14B). We observed that γ-H2AX levels within CD45RA$^-$CD27$^-$ cells were similar in the presence or absence of p38 inhibitor, but p38 inhibition induced a considerable decrease on the percentage of γ-H2AX positive cells within the CD45RA$^+$CD27$^-$ subset (Figure 14B). However, the γ-H2AX levels remained relatively high, indicating that other pathways may contribute to γ-H2AX expression on CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ cells.

The p38/H2AX pathway has been shown to be required for stress-induced apoptosis in murine fibroblasts [64] and cancer cell lines [65]. We assessed wether p38 inhibition had an effect on cell recovery by activating purified CD45RA/CD27 CD4$^+$ subsets with anti-CD3 and IL-2 in the presence of the p38 inhibitor BIRB796 (Figure 15A).

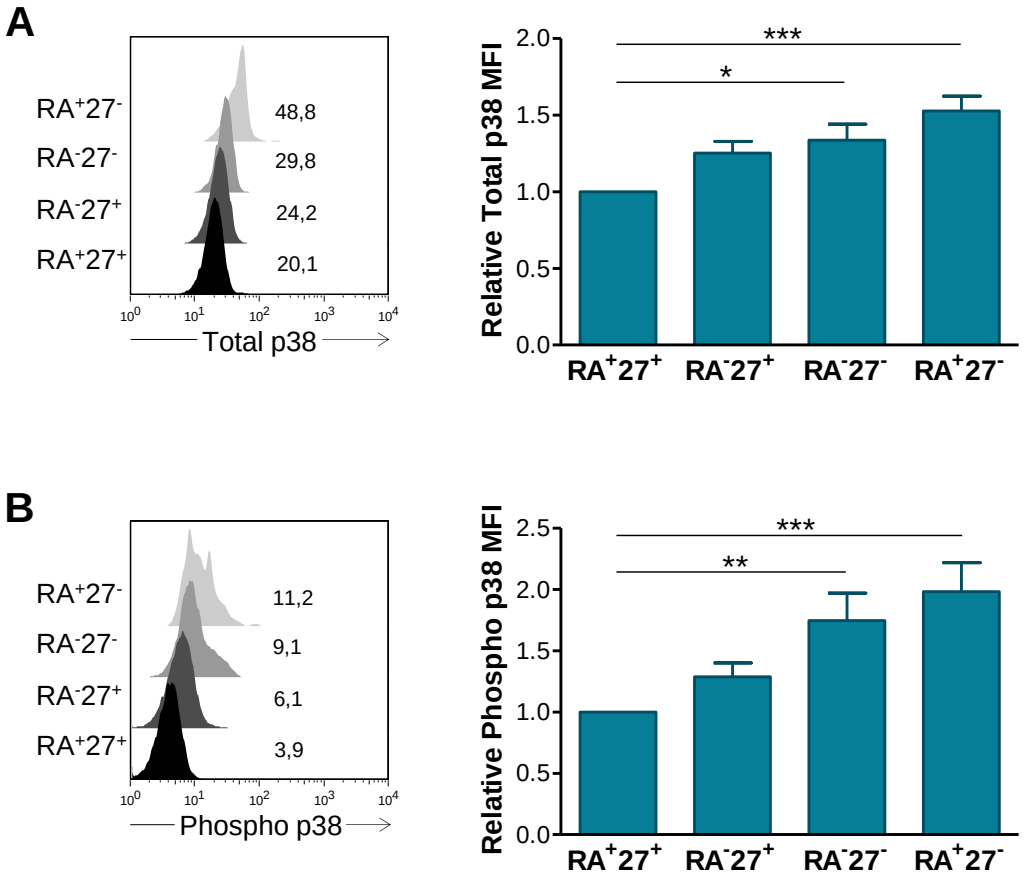

Figure 13: CD4$^+$ CD45RA$^+$CD27$^-$ cells express higher levels of total and phosphorylated p38.

Expression of total and of phosphorylated p38 was assessed *ex vivo* in PBMCs by gating within total CD4$^+$ T cells and within each of the CD45RA/CD27 subsets. Representative histogram overlays of total p38 (A) and of phospho-p38 (B) are shown. The values represent the median fluorescent intensity of p38 within each subset. Bar graphs show the *ex vivo* mean fluorescence intensity of total (A) and phospho-p38 (B). Error bars represent the SE (A n=7, B n=10). Statistical analysis was performed using Dunn's Multiple Comparison Test (GraphPad Prism).

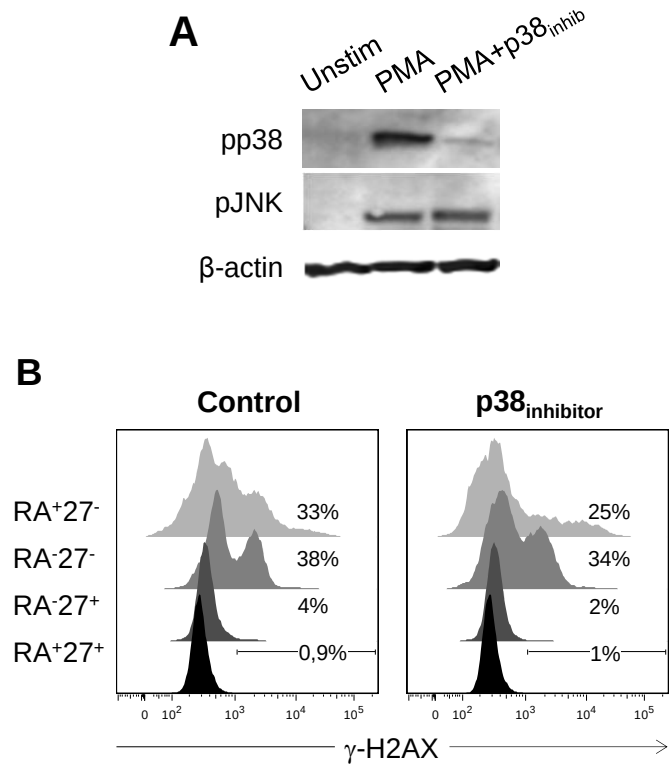

Figure 14: CD4$^+$ CD45RA$^+$CD27$^-$ cells express lower levels of γ-H2AX when the p38 pathway is inhibited.

A) Western blot showing the effects of the p38 inhibitor BIRB796 on p38 and JNK phosphorylation on CD4$^+$ T cells. β-actin was used as a loading control. B) Purified CD45RA/CD27 CD4$^+$ T cell subsets were activated with anti-CD3 and IL-2 for 4 days in the presence or absence (control) of the p38 inhibitor BIRB796. γ-H2AX expression was assessed by flow cytometry. Histogram overlays from a representative experiment out of two performed are shown. The values represent the percentage of γ-H2AX-positive cells within each subset.

As could be anticipated, the cell recovery of $CD45RA^{+}CD27^{+}$ and $CD45RA^{-}CD27^{+}$ cells was not affected by p38 inhibition (Figure 15A). Although p38 inhibition induced only a marginal increase in the cell recovery of $CD45RA^{-}CD27^{-}$ cells, it led to approximately a 2-fold increase in the cell recovery of $CD45RA^{+}CD27^{-}$ cells (Figure 15A). This observation may result from increased cell proliferation and/or decreased cell death within the $CD45RA^{+}CD27^{-}$ subset. To clarify this issue, we determined the expression of Ki67 following activation in the presence of the p38 inhibitor BIRB796 (Figure 15B). We did observe a slight increase in the percentage of Ki67 positive cells within the $CD45RA^{-}CD27^{+}$ and $CD45RA^{-}CD27^{-}$ subsets, yet p38 inhibition had the opposite effect on the $CD45RA^{+}CD27^{-}$ subset (Figure 15B). The decrease in Ki67-expressing cells within the $CD45RA^{+}CD27^{-}$ subset in the presence of the p38 inhibitor BIRB796 points to a preferential effect on cell survival. To verify this hypothesis we assessed the impact of p38 inhibition on Annexin V staining and PI incorporation following activation with anti-CD3 and IL-2 (Figure 15C). Again, p38 inhibition did not greatly affect the $CD45RA^{+}CD27^{+}$ and $CD45RA^{-}CD27^{+}$ subsets, but both the $CD45RA^{-}CD27^{-}$ and $CD45RA^{+}CD27^{-}$ subsets had considerably less apoptotic cells in the presence of the p38 inhibitor BIRB796 (Figure 15C). The inhibition of apoptosis by blocking the p38 pathway was most striking in the $CD45RA^{+}CD27^{-}$ subset (Figure 15C), with an average of 70% less apoptotic cells.

We next sought to investigate the mechanism by which p38 inhibition was promoting cell survival of $CD45RA^{-}CD27^{-}$ and $CD45RA^{+}CD27^{-}$ cells. The p38 pathway has been shown to reduce Bcl-2 levels [66]. As described above, the $CD45RA^{+}CD27^{-}$ subset is defective for Akt(Ser473) phosphorylation (Figure 6) and expresses low levels of Bcl-2 *ex vivo* [2]. We assessed the levels of Bcl-2 expression and of Akt(Ser473) phosphorylation on $CD4^{+}$ T lymphocytes activated in the presence or absence (control) of the p38 inhibitor BIRB796 (Figure 16). Bcl-2 expression was increased at the protein (Figure 16A) and mRNA (Figure 16B) levels as a result of p38 inhibition. In contrast, the levels of pAkt(Ser473) were not increased by p38 inhibition in any of the CD45RA/CD27 subsets (Figure 16C), ruling out any cross-reactivity of the p38 inhibitor BIRB796 with the PI3K/Akt pathway. These data suggest that the p38 pathway may be partly responsible for the susceptibility to apoptosis following activation of $CD45RA^{+}CD27^{-}$ cells through down-modulation of Bcl-2 expression.

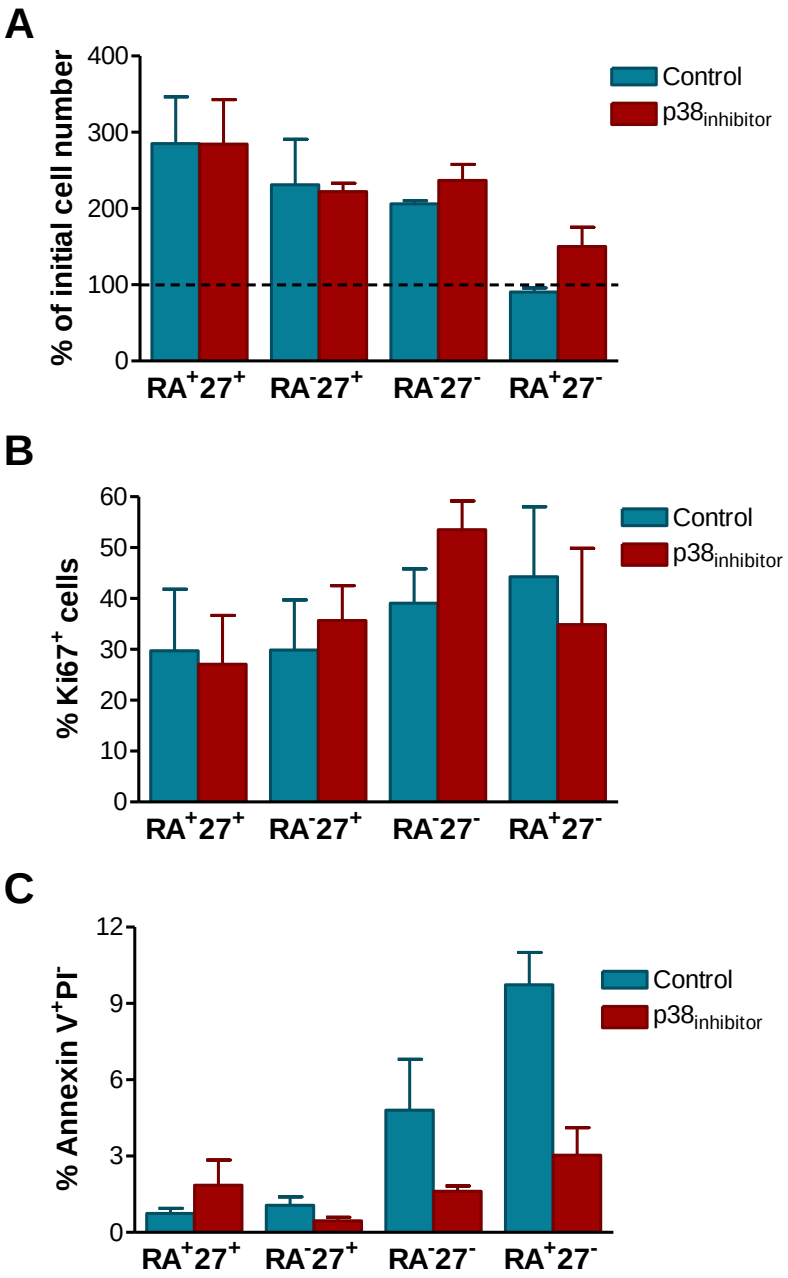

Figure 15: p38 inhibition improves cell recovery and survival but not proliferation of CD45RA$^+$CD27$^-$ cells.

Purified CD45RA/CD27 CD4$^+$ T cell subsets were activated with anti-CD3 and IL-2 (A, C) or irradiated APCs (B) for 4 days in the presence or absence of the p38 inhibitor BIRB796. A) Cell numbers were determined on a hemocytometer. Results are expressed as a percentage of the initial number of cells placed in culture. Error bars represent SE from three separate experiments. B) Ki67 expression was determined as a marker of cell proliferation. Bar graph shows the percentage of Ki67 positive cells. Error bars represent SE from four separate experiments. C) Apoptosis was assessed by Annexin V staining and PI incorporation. Bar graph shows the percentage of apoptotic cells (Annexin V$^+$ PI$^-$) within each subset in the presence or absence of the p38 inhibitor. Error bars represent SE from three separate experiments.

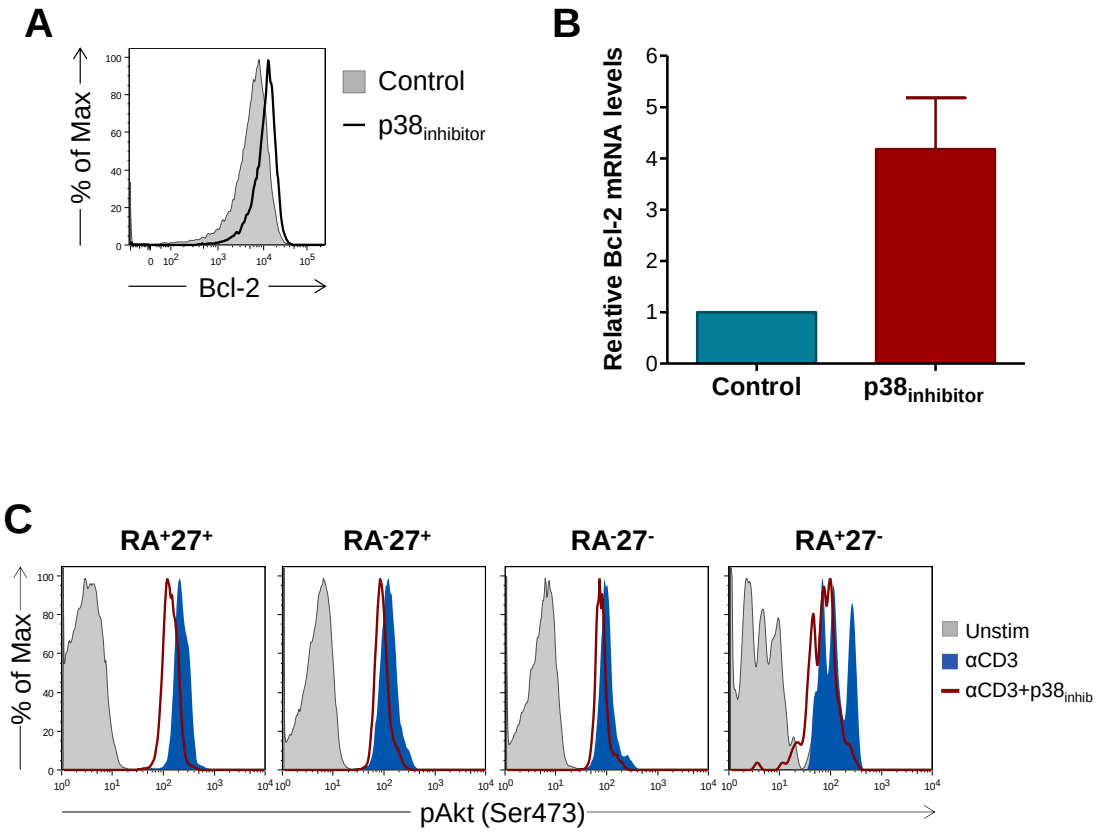

Figure 16: p38 inhibition increases Bcl-2 expression but not pAkt(Ser473) phosphorylation.

A, B) $CD4^+$ T cells were activated with anti-CD3 and IL-2 in the presence or absence of the p38 inhibitor BIRB796. A) On day 4, Bcl-2 expression was assessed at the protein level by flow cytometry. B) Bar graph shows the levels of Bcl-2 mRNA in the presence of the p38 inhibitor BIRB796 normalized for the levels observed in its absence (control). Error bars represent the SE from the mean of three separate experiments. C) pAkt(Ser473) expression was assessed within CD45RA/ CD27 $CD4^+$ subsets activated with anti-CD3 in the presence (red line) or absence (blue histogram) of the p38 inhibitor BIRB796. As a negative control, cells were left unstimulated (grey histogram). Overlays from a representative experiment out of two performed are shown.

The lack of telomerase activity upon TCR stimulation is a hallmark of pre-disposition to replicative senescence [67]. As we observed that CD45RA$^+$CD27$^-$ cells have impaired telomerase activity following *in vitro* activation (Figure 10B), we were interested in assessing if p38 inhibition could restore the ability to induce this enzyme. For that purpose, telomerase activity was measured in purified CD45RA/CD27 CD4$^+$ subsets activated with anti-CD3 and irradiated APCs in the presence or absence of the p38 inhibitor BIRB796 (Figure 17). The inhibition of p38 had a negligible impact on the telomerase activity observed in the CD45RA$^+$CD27$^+$ and CD45RA$^-$CD27$^+$ subsets (Figure 17). Although we could observe a considerable increase in the telomerase activity in the CD45RA$^-$CD27$^-$ subset in the presence of the p38 inhibitor BIRB796, it did not reach statistical significance (Figure 17B). The inhibition of p38 had a major impact on the CD45RA$^+$CD27$^-$ subset, significantly increasing telomerase activity to levels similar to those observed in CD45RA$^+$CD27$^+$ and CD45RA$^-$CD27$^+$ cells (Figure 17B). These data suggest that the p38 pathway is at least partially responsible for the impaired survival and telomerase activity following activation observed in the CD4$^+$ CD45RA$^+$CD27$^-$ subset. Moreover, these senescence traits were at least partly reversible through specific inhibition of the p38 pathway.

Subsequently, we investigated which up-stream stimulus might be triggering p38 in CD4$^+$ CD45RA$^+$CD27$^-$ cells. The frequency of this subset significantly correlates with CMV infection [2], which in turn is associated with high levels of pro-inflammatory cytokines such as TNF-α [68]. This cytokine has been proposed to be linked to the extreme T cell differentiation observed during CMV infection [69-72]. As previously described in HeLa cells [73], we observed that TNF-α induces p38 phosphorylation in CD4$^+$ T cells (Figure 18A). Interestingly, CD4$^+$ T cells activated in the presence of TNF-α showed lower levels of telomerase activity (Figure 18B). Moreover, TNF-α inhibition has been shown to increase telomerase activity and delay the onset of senescence on CD8$^+$ T cells *in vitro* [74]. Hence we investigated whether p38 inhibition would abrogate the impact of TNF-α upon telomerase activity. As illustrated in Figure 18C, the telomerase activity levels in CD4$^+$ T cells activated in the presence of both TNF-α and the p38 inhibitor BIRB796 were similar to those observed in cells activated with anti-CD3 and irradiated APCs alone (control). Thus p38 inhibition does seem to subvert the telomerase down-modulation induced by TNF-α, suggesting that the p38 pathway may act down-stream of TNF-α to hinder telomerase activity.

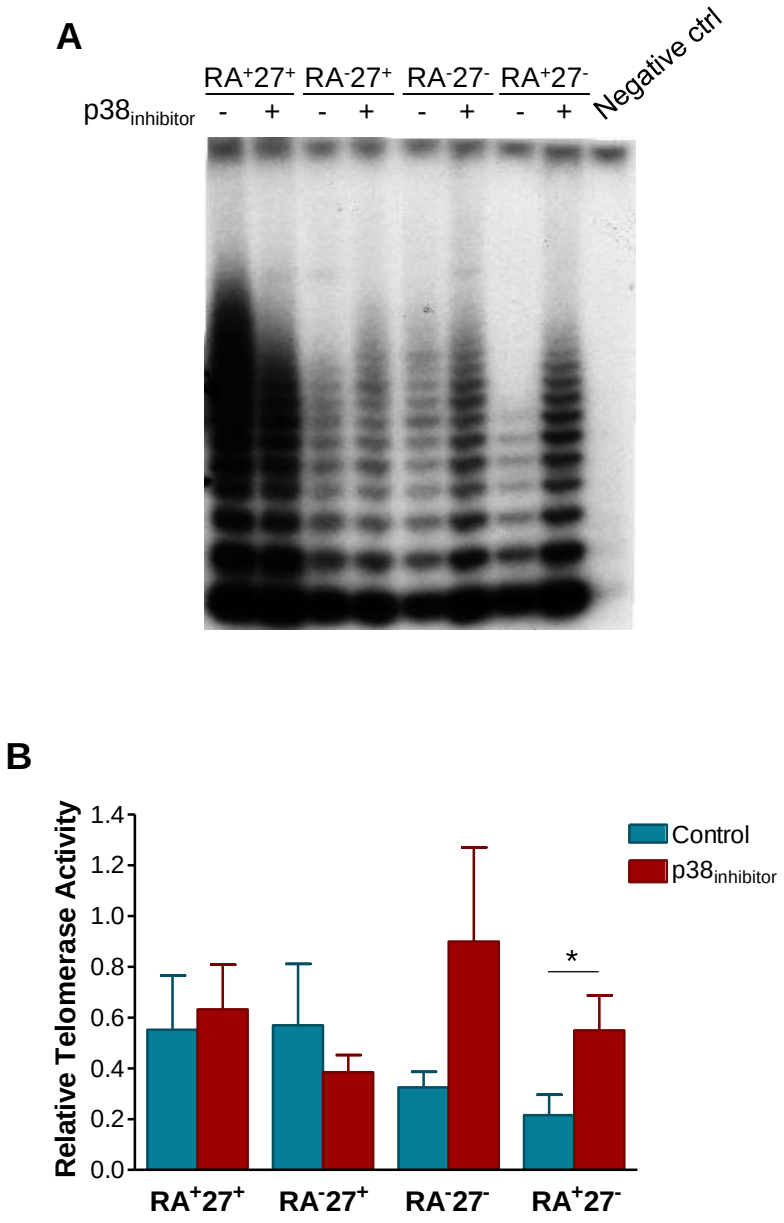

Figure 17: p38 inhibition significantly increases telomerase activity in CD4$^+$ CD45RA$^+$CD27$^-$ cells.

Telomerase activity was determined by telomeric repeat amplification protocol assay as described in Figure 10. Purified subsets were activated with anti-CD3 and irradiated APCs for 4 days in the presence or absence of the p38 inhibitor BIRB796. A) Autoradiography of a TRAP assay acrylamide gel from a representative experiment is shown. As a negative control, lysis buffer was used instead of cell extract. B) Bar graph represents telomerase activity for each subset with or without (control) the p38 inhibitor BIRB796. Error bars represent the SE from the mean of four separate experiments. Statistical analysis was performed using the Wilcoxon matched pairs test (GraphPad Prism).

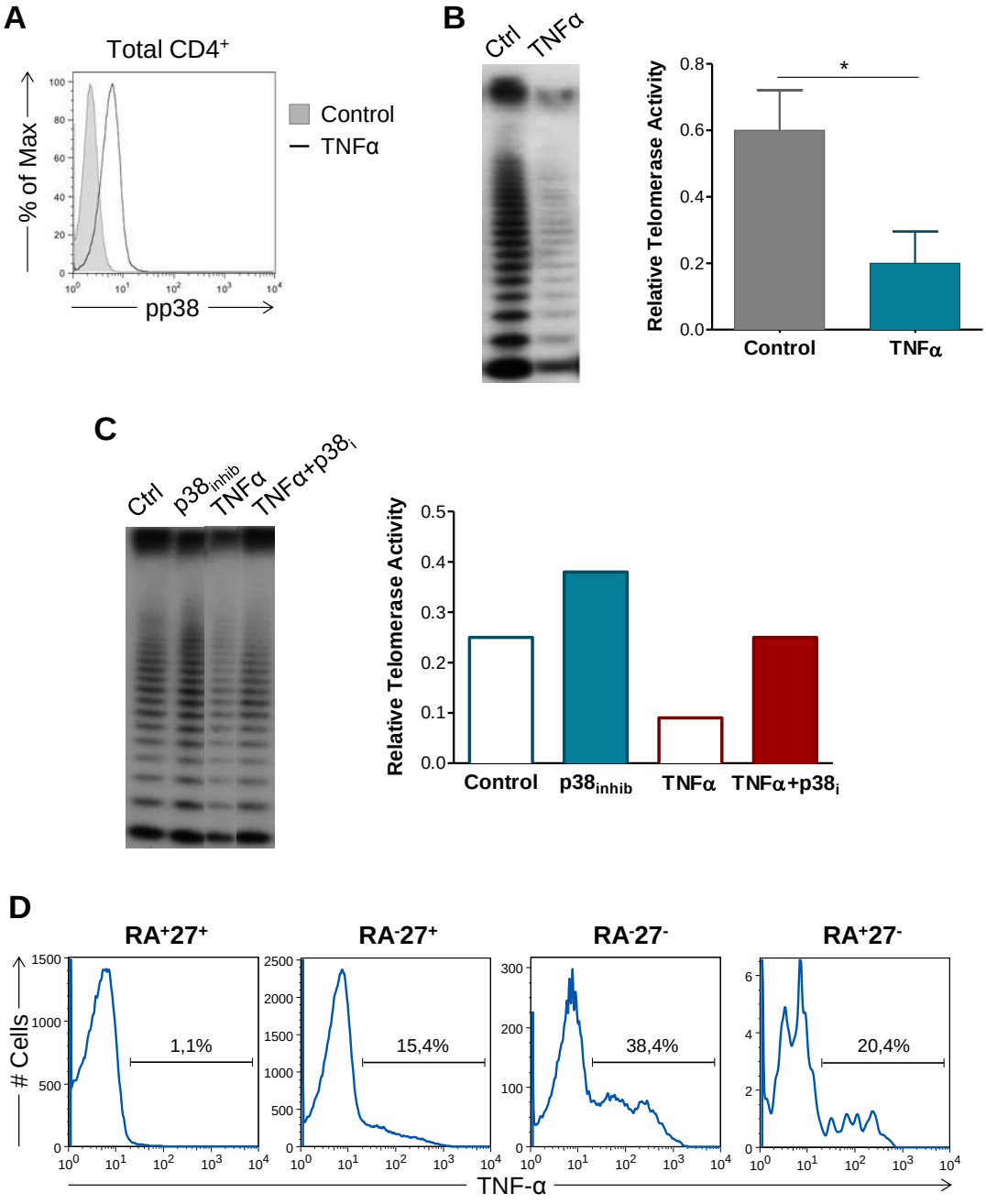

Figure 18: Inhibition of p38 abrogates the TNF-α-induced down-modulation of telomerase activity.

A) Phospho-p38 expression was assessed on $CD4^+$ T cells incubated with TNF-α or left unstimulated (control). A representative overlay is shown. B) $CD4^+$ T cells were cultured with anti-CD3 and APCs in the presence or absence (control) of TNF-α for 4 days. Telomerase activity was determined by telomeric repeat amplification protocol assay as described in Figure 10. Autoradiography of a TRAP assay acrylamide gel from a representative experiment is shown. Bar graph represents telomerase activity in activated $CD4^+$ T cells with or without (control) TNF-α. Error bars represent the SE from the mean of three separate experiments. Statistical analysis was performed using the Wilcoxon matched pairs test (GraphPad Prism). C) $CD4^+$ T cells were cultured for 4 days with anti-CD3 and APCs alone or in combination with the p38 inhibitor BIRB796, TNF-α or both. Autoradiography and bar graph show the telomerase activity observed under these culture conditions. D) PBMCs were activated with anti-CD3 and IL-2 overnight. The production of TNF-α within the CD45RA/CD27 $CD4^+$ subsets was assessed by flow cytometry. Histograms illustrate the profile of TNF-α expression observed in each CD45RA/CD27 subset upon activation.

We then sought to elucidate which subsets were the potential sources of TNF-α. Highly differentiated $CD4^{+}$ [75-77] and $CD8^{+}$ [3] T cells have been described to produce TNF-α, particularly those re-expressing CD45RA. To verify if this was also the case for $CD45RA^{+}CD27^{-}$ $CD4^{+}$ T cells, we assessed the production of TNF-α by CD45RA/CD27 subsets activated with anti-CD3 and IL-2 (Figure 18D). We observed substantial levels of TNF-α-producing cells within all the $CD4^{+}$ memory T cell subsets, particularly within $CD45RA^{-}CD27^{-}$ and $CD45RA^{+}CD27^{-}$ cells (Figure 18D).

These data indicate that $CD45RA^{+}CD27^{-}$ $CD4^{+}$ T cells are prone to cellular senescence, displaying senescence-associated markers such as γ-H2AX and lack of telomerase activity following activation. The observation that $CD45RA^{+}CD27^{-}$ $CD4^{+}$ T cells have relatively long telomeres suggests that the mechanism driving these cells to senescence might be telomere-independent. The apparent involvement of the stress-induced p38 pathway further supports this hypothesis. Interestingly, the modulating effects of p38 on cellular senescence appear to be partly reversible, raising the possibility that therapeutic approaches targeting this pathway might improve immunity during ageing and chronic viral infection.

Discussion

We reported for the first time a detailed study characterising the $CD45RA^+CD27^-$ $CD4^+$ T cell subset. The CD45RA re-expressing $CD4^+$ T cell population has only previously been described in a few reports, which portray it as a terminally differentiated population with low replicative potential [78], albeit capable of producing pro-inflammatory cytokines [77,79].

Our work has revealed that $CD45RA^+CD27^-$ $CD4^+$ T cells are multi-functional, with respect to their ability to secrete cytokines following activation [2], and are potentially capable of exerting cytotoxic functions (Figure 1). We further report that $CD45RA^+CD27^-$ $CD4^+$ T cells appear to exist in a quiescent state *in vivo*, as has been described for their $CD8^+$ counterparts [8,12,14], but can be induced to proliferate upon activation. Our results show that these cells are not exhausted, although they appear to be prone to cell death following activation. Given that $CD45RA^+CD27^-$ $CD4^+$ T cells express only intermediate levels of CD95, other cell death pathways might be involved in the high susceptibility to apoptosis observed in these cells following activation. In agreement with this hypothesis, CD45RA re-expressing $CD4^+$ T cells have been previously shown to be relatively resistant to CD95-induced apoptosis [80]. Furthermore, CD95 expression does not strictly correlate with susceptibility to apoptosis, given that this molecule can also exert co-stimulatory functions [33]. In lymphopenic settings, increased IL-7 levels favour the co-stimulatory activity of CD95, which is able to induce the proliferation of T cells activated by low-affinity antigens [81]. In addition, the CD95 levels were assessed directly *ex vivo* and resting T cells may not be susceptible to CD95 triggering, a process which probably requires previous T cell activation [82-84].

We have shown that $CD45RA^+CD27^-$ $CD4^+$ T cells accumulate in the elderly, showing a strong positive correlation with CMV infection [2], which is unexpected from a population that is prone to apoptosis following activation. This observation suggests that $CD45RA^+CD27^-$ $CD4^+$ T cells have to be constantly generated to compensate for their high susceptibility to activation-induced apoptosis. In agreement with this hypothesis, the overall CMV-specific population has been proposed to be maintained by a continuous replacement of short-lived, functional cells during chronic CMV infection in mice [85]. We report for the first time a mechanism able to induce CD45RA re-expression on memory

$CD4^+$ T cells by showing that IL-7 stimulation triggers CD45RA re-expression whilst concomitantly driving homeostatic proliferation of $CD45RA^-CD27^+$ $CD4^+$ T cells. In order to determine as to where CD45RA re-expression might occur *in vivo*, we investigated which immune compartments were enriched in both IL-7 and $CD45RA^+CD27^-$ $CD4^+$ T cells. We observed that the frequency of $CD45RA^+CD27^-$ cells within the $CD4^+$ T cell population is significantly higher in the bone marrow than in the peripheral blood of the same individuals [2]. Moreover, IL-7-producing bone-marrow stromal cells have been shown to constitute survival niches for memory $CD4^+$ T cells [86]. Taken together these results point to the bone-marrow as a potential site where IL-7-driven CD45RA re-expression might occur *in vivo*. We further hypothesise that the $CD45RA^+CD27^-$ $CD4^+$ T cell subset might be replenished *in vivo* by CD45RA re-expressing cells derived from the $CD4^+$ $CD45RA^-CD27^+$ pool through a homeostatic process driven by IL-7. Culture of the $CD45RA^-CD27^+$ $CD4^+$ T cells in the presence of IL-7 efficiently gave rise to CD45RA re-expressing cells, although it only modestly induced CD27down-modulation in this sub-population (data not shown). The fact that IL-7 stimulation alone could not lead to to a $CD45RA^+CD27^-$ phenotype upon $CD45RA^-CD27^+$ T cells might suggest that loss of CD27 expression requires TCR stimulation or other factors that can induce down-modulation of co-stimulatory molecules. Upon several rounds of stimulation, $CD4^+$ T cells successively lose CCR7, CD27 and CD28 expression [87,88]. CD27 expression has been shown to be transiently up-regulated upon TCR engagement, followed by a progressive and irreversible down-regulation following repeated antigenic stimulation *in vivo* [89,90]. On the other hand, TNF-α has been shown to promote the loss of CD28 expression on $CD8^+$ T cells [91-93], raising the possibility that this pro-inflammatory cytokine might also accelerate the down-modulation of co-stimulatory molecules on $CD4^+$ T cells.

As illustrated in Figure 10A, $CD45RA^-CD27^+$ $CD4^+$ T cells have significantly shorter telomeres than those observed on the $CD45RA^+CD27^-$ subset. Although this result apparently contradicts our hypothesis that the former subset comprises precursors of the $CD45RA^+CD27^-$ $CD4^+$ T cell population, it might be explained by the induction of telomerase activity alongside with CD45RA re-expression by IL-7, resulting in longer telomeres on the $CD45RA^+$ daughter population than on the $CD45RA^-$ precursors. In agreement, IL-7 has been shown to induce telomerase activity on cord blood naive T cells [34], as well as on naive and memory $CD4^+$ T cells [94] and $CD8^+$ T cells [33,95]. The assessment

of telomerase activity on CD45RA$^-$CD27$^+$ CD4$^+$ T cells stimulated with IL-7, in particular within the sub-population re-expressing CD45RA, would help clarify this issue.

Interestingly, CD45RA re-expressing cells generated in the presence of IL-7 alone resemble the *ex vivo* CD45RA$^+$CD27$^-$ CD4$^+$ T cell subset in that they express low levels of IL-7Rα (Figure 7) and Bcl-2 [2]. IL-7Rα expression on mouse CD8$^+$ T cells has been shown to display different kinetics when stimulated with LCMV strains associated either with viral clearance or persistence: the former only transiently down-modulated IL-7Rα expression, the restoration of which was associated with improved survival and induction of a quiescent state, whereas the latter suppressed IL-7Rα expression and this correlated with reduced Bcl-2 expression [96]. In a recent study of human naive CD4$^+$ T cells, IL-7Rα was shown to be chronically down-modulated following TCR activation [37]. Interestingly, neither CD45RA$^+$CD27$^-$ CD4$^+$ and CD8$^+$ T cells nor CMV-specific CD8$^+$ T cells were able to re-express IL-7Rα after resting over-night in medium alone, possibly due to epigenetic modifications to the IL-7Rα promoter [37,97]. Our data supports the correlation between IL-7Rα and Bcl-2 expression and shows that IL-7 stimulation, so far solely associated with a transient down-modulation of IL-7Rα and up-regulation of Bcl-2 expression, can also induce a persistent down-modulation of both these markers on a sub-population of CD45RA$^-$CD27$^+$ T cells. Although IL-15 has been shown to induce CD45RA re-expression on CD8$^+$ T cells [32], only a negligible CD45RA-expressing population was observed when CD45RA$^-$CD27$^+$ T cells were cultured in the presence of IL-15 or IL-2. The differential effect of IL-15 on CD4$^+$ and CD8$^+$ T cells mirrors the distinct dependence on IL-15 for T cell homeostasis, with CD4$^+$ T cells apparently not requiring IL-15 [98-102]. CD45RA$^-$CD27$^-$ T cells could not be induced to re-express CD45RA by any of the γ_C cytokines tested, as previously reported for CD8$^+$ T cells, where γ_C cytokines could trigger CD45RA re-expression on CD45RA$^-$CCR7$^+$ but not on CD45RA$^-$CCR7$^-$ cells [5]. The induction of CD45RA re-expression is probably linked to IL-7-driven modifications to the transcriptional program on a subset of CD45RA$^-$CD27$^+$ CD4$^+$ T cells. The T-box transcription factor T-bet emerged as the most likely candidate to be associated with CD45RA re-expression since the CD45RA$^+$CD27$^-$ subset expressed the highest levels of this transcription factor *ex vivo* and its expression could be up-regulated on CD45RA$^-$CD27$^+$ cells by IL-7. This hypothesis is supported by a recent study reporting significantly higher levels of T-bet expression on CD45RA$^+$CD27$^-$ cells than on CD45RA$^-$CD27$^+$ CD8$^+$ T cells during latent human CMV infection [38].

As mentioned above, the *ex vivo* frequency of Ki67-expressing cells within the $CD4^+$ $CD45RA^+CD27^-$ subset is very small, suggesting that this population might have a low turnover rate *in vivo* and thus represent a quiescent subset, similarly to what has been proposed for CD45RA re-expressing $CD8^+$ T cells [8,12,14]. However the susceptibility to activation-induced apoptosis, associated with low Bcl-2 levels and defective Akt(Ser473) phosphorylation, in addition to high level expression of the senescence-associated markers CD57 and KLRG1 (Di Mitri *et. al.*, manuscript under submission), raised the possibility that $CD45RA^+CD27^-$ $CD4^+$ T cells might be approaching senescence rather than being quiescent. Unlike the transient cell cycle arrest observed in quiescent cells, the growth arrest induced by cellular senescence is thought to be irreversible [15]. The relatively long telomeres observed in $CD45RA^+CD27^-$ $CD4^+$ T cells argued in favour of a quiescent rather than a senescent state. However telomere length cannot be interpreted as an absolute marker of replicative potential as it only gives a snap-shot of the relative levels of telomere erosion in the different subsets. Assessing the ability of each subset to induce telomerase activity is critical for the interpretation of the telomere data, since it reveals the potential to add back telomere repeats upon subsequent activation and thus provides a more dynamic depiction of the residual replicative potential. In contrast to the telomere length data, the inability to induce telomerase activity following TCR stimulation observed on $CD45RA^+CD27^-$ $CD4^+$ T cells indicated that this subset had a limited capacity to be maintained *in vivo* by continuous proliferation and is therefore prone to senescence. The susceptibility to cellular senescence of $CD45RA^+CD27^-$ $CD4^+$ T cells was further supported by the assessment of the senescence marker γ-H2AX, which reached the highest levels on $CD45RA^-CD27^-$ and $CD45RA^+CD27^-$ $CD4^+$ T cells. The p38 MAPK pathway appears to be at least partly responsible for the senescence traits displayed by these subsets, most strikingly so in the case of the $CD45RA^+CD27^-$ $CD4^+$ T cells. The expression of total and phosphorylated p38 was highest within the $CD45RA^+CD27^-$ $CD4^+$ T cell subset, although $CD45RA^-CD27^-$ cells also expressed considerable levels of these proteins. Of note, the expression of total p38 was exclusive to the CD45RA re-expressing population that emerged during the culture of $CD45RA^-$ $CD27^+$ cells in the presence of IL-7. It remains to be elucidated whether p38 expression is a cause or consequence of CD45RA re-expression in these cells, but this observation further implies that p38 expression is characteristic of highly differentiated cells, particularly CD45RA re-expressing cells.

Inhibition of the p38 MAPK pathway has been shown to delay the onset of senescence in human fibroblasts [62]. Thus we ascertained the involvement of the p38 MAPK pathway in the senescence-associated features of CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ CD4$^+$ T cells by re-assessing these traits in the presence of the p38 inhibitor BIRB796. We found that p38 inhibition had a greater impact on the CD45RA$^+$CD27$^-$ subset, inducing a slight decrease on γ-H2AX expression and boosting cell recovery during TCR stimulation. We further determined that the increase in CD45RA$^+$CD27$^-$ cell numbers following activation was due to improved cell survival rather than elevated proliferation levels induced by p38 inhibition. Our data suggest that the p38 pathway might hamper cell survival by down-modulating Bcl-2 levels in an Akt-independent fashion. The persistent activation of p38 MAP kinase in transgenic mice expressing a constitutively activated form of MKK6 led to a decrease in the CD8$^+$ but not CD4$^+$ T cell numbers [66]. The selective loss of the CD8$^+$ T cell population was associated with increased apoptosis and lower Bcl-2 levels, although the rate of *in vivo* proliferation was not altered by p38 activation [66]. The MKK6-driven *in vivo* activation of p38 had a negligible effect on *in vivo* and spontaneous apoptosis of CD4$^+$ T cells [66]. Although *in vitro* p38 inhibition was shown to increase cell recovery of CD8$^+$ T cells following polyclonal T cell activation with concanavalin A (Con A), the same experiment was not performed on CD4$^+$ T cells [66]. As mentioned above, p38 can be activated by two specific kinases: MKK3 and MKK6. Interestingly, CD4$^+$ T cells from MKK3- but not from MKK6-deficient mice have been shown to be more resistant to cell death induced by TCR activation or by cytokine withdrawal [103]. Proliferation of CD4$^+$ T cells was not greatly affected by either MKK3 or MKK6 knock-down [103]. In addition, CD4$^+$ T cells from MKK3-deficient mice had a greater reduction in p38 activation compared to those from MKK6-deficient mice [103]. These data suggest that the p38 pathway negatively impacts the survival of both CD4$^+$ and CD8$^+$ T cells, although p38 might be activated by different MAPK kinases in these populations. A major effect of p38 inhibition was to endow CD45RA$^+$CD27$^-$ cells with the ability to induce telomerase activity upon TCR stimulation. The telomerase activity on CD45RA$^-$CD27$^-$ cells was also improved by p38 inhibition. Interestingly, the telomerase activity boost induced by p38 inhibition was not associated with improved Akt(Ser473) phosphorylation, further supporting the view that pAkt(Ser473) is not as crucial as initially thought for the triggering of telomerase activity [26]. Although CD45RA$^+$CD27$^-$ cells had significantly longer telomeres than CD45RA$^-$CD27$^-$ cells, p38 inhibition appears to revert some of the

senescence-associated traits of both these subsets. Our data suggests that the p38 pathway might be involved in both telomere-dependent and –independent senescence of $CD4^+$ T cells, as previously demonstrated for other cell types [62]. The potential role of the p38 pathway in the generation and/or maintenance of senescence-prone $CD45RA^+CD27^-$ $CD4^+$ T cells which accumulate in the elderly, mainly in CMV-infected individuals [2], compels the investigation of putative triggers that may be inducing p38 signalling in these cells. A likely candidate is TNF-α, a pro-inflammatory cytokine reportedly associated with the pronounced T cell differentiation observed during CMV infection [69-72]. We confirmed that TNF-α is able to activate the p38 pathway and to inhibit telomerase activity, two outcomes that are probably interlinked. In addition, the production of TNF-α has been shown to increase not only during CMV infection [68] but also upon the establishment of cellular senescence [104]. We observed that the $CD45RA^-CD27^-$ and $CD45RA^+CD27^-$ subsets display the highest frequency of TNF-α-producing cells. These data allude to a hypothetical scenario where the high levels of TNF-α present during CMV infection might lead to the generation of highly differentiated $CD4^+$ T cells, which would in turn produce more TNF-α, further contributing to the pro-inflammatory environment and the accumulation of these cells. TNF-α-induced signalling would activate the p38 pathway, potentially impairing survival and telomerase activity following TCR stimulation of these highly differentiated $CD4^+$ T cells. The apparently incongruous accumulation of apoptosis-susceptible cells might be due to a continuous replenishment of the subset by precursors driven to differentiate, as has been proposed for CD45RA re-expressing $CD8^+$ T cells [5]. Interestingly, TNF-α has been shown to increase the production of IL-7 by bone-marrow stromal cells [105] as well as to increase IL-7Rα expression in both $CD4^+$ and $CD8^+$ mouse T cells [36]. Furthermore, IL-7 has been shown to stimulate TNF-α production by intra-articular $CD4^+$ T lymphocytes and accessory cells in patients with rheumatoid arthritis, exacerbating the pro-inflammatory responses in these patients [106]. These data suggest a potential positive feedback-loop between TNF-α and IL-7 production which might contribute to the accumulation of CD45RA re-expressing memory $CD4^+$ T cells.

Taken together, our data indicate that $CD45RA^+CD27^-$ $CD4^+$ T cells do not constitute an exhausted subset, displaying cytotoxic potential and proliferative capacity upon TCR activation. Given that $CD45RA^+CD27^-$ $CD4^+$ T cells are highly susceptible to cell death following activation, we hypothesize that this subset is comprised by short-lived cells and

thus has to be constantly replenished. We demonstrated that CD45RA re-expression can be induced by IL-7 in $CD45RA^-CD27^+$ $CD4^+$ T cells, suggesting a role for this cytokine in the generation of the $CD4^+$ $CD45RA^+CD27^-$ subset, although this process appears to require other factors. Finally, we found that $CD45RA^+CD27^-$ $CD4^+$ T cells are prone to telomere-independent senescence through a process partly driven by the p38 MAPK pathway.

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CONCLUSIONS

Homeostasis of the T cell pool allows the preservation of relatively constant cell numbers and adequate diversity in face of declining thymic output and constant antigenic challenge [1]. IL-7 is a key mediator in both naive and memory T cell homeostasis through its ability to induce signalling pathways that promote cell survival and proliferation [1-6]. We assessed the potentially distinct effects of IL-7 in the homeostasis of naive CD4$^+$ T cell subsets defined by CD31 expression. Furthermore, we investigated the mechanisms involved in the restoration of T cell homeostasis following haploidentical HSCT, particularly in the maintenance of the CD31$^+$ naive CD4$^+$ T cell pool. As pertaining to memory CD4$^+$ T cell homeostasis, we sought to determine the replicative and functional potential of highly differentiated CD45RA$^+$CD27$^-$ cells, in order to clarify the impact of the increasing representation of this subset observed during CMV infection, as well as the putative involvement of IL-7 in CD45RA re-expression on memory CD4$^+$ T cells.

During ageing, the replenishment of the naive T cell pool with recent thymic emigrants progressively declines due to thymic involution [7]. Hence homeostatic mechanisms in the periphery are required to maintain the size and diversity of the naive CD4$^+$ T cell pool. IL-7 has been shown to induce homeostatic proliferation of umbilical cord blood naive CD4$^+$ T cells, a population that can be used as a model for RTEs [8-10]. Hence we sought to investigate if IL-7 was also able to expand the RTE-enriched subset in adult peripheral blood identifiable by CD31 expression within naive CD4$^+$ T cells. We described for the first time a selective proliferation of the CD31$^+$ subset within adult naive CD4$^+$ T cells in response to IL-7 stimulation. Furthermore, we showed that IL-7-induced proliferation did not lead to down-modulation of CD31 and consequent generation of a CD31$^-$ sub-population. On the contrary, IL-7 sustained or even increased the level of CD31 expression in CD31$^+$ naive CD4$^+$ T cells, although it did not induce CD31 re-expression in the CD31$^-$ subset. We also demonstrated that both IL-7-induced proliferation and CD31 maintenance required the activation of the PI3K pathway. Conversely, other characteristic IL-7 read-outs, namely up-regulation of the anti-apoptotic protein Bcl-2 and promotion of *in vitro* cell survival, were observed both in CD31$^+$ and CD31$^-$ naive CD4$^+$ T cells and were not dependent on PI3K activation. These data allow us to hypothesize

that IL-7-induced PI3K signalling might be defective in adult $CD31^-$ naive $CD4^+$ T cells. In order to clarify this issue, Akt phosphorylation levels should be assessed in purified $CD31^+$ and $CD31^-$ naive $CD4^+$ T cells stimulated with IL-7. This assessement was precluded by the low levels of pAkt induced by IL-7 alone in primary naive $CD4^+$ T cells, which were undetectable both by Western blot and flow cytometry.

The role of CD31 expression on naive $CD4^+$ T cells is yet to be clarified, although it has been proposed to hamper the proliferation of $CD31^+$ naive $CD4^+$ T cells in response to TCR triggering through its cytoplasmic ITIMs [11]. Alternatively, CD31 might be required for transendothelial migration, as described for other cell types [12], potentially driving the migration of $CD31^+$ naive $CD4^+$ T cells into IL-7-rich microenvironments where they might undergo homeostatic proliferation. A transendothelial migration assay in the presence or absence of CD31-blocking antibodies might elucidate whether CD31 plays a role in naive $CD4^+$ T cell migration.

Our data suggest the $CD31^+$ naive $CD4^+$ T cell pool is maintained throughout adulthood, at least partly, by IL-7 signalling and hence IL-7-based therapies might exhert a preferential effect on this population. For instance, we hypothesise that the increased $CD31^+$ naive $CD4^+$ T cell numbers observed after IL-7 administration during a phase I clinical trial in cancer patients [13] was likely driven by IL-7-induced expansion of this naive $CD4^+$ subset. The expansion of RTE-enriched $CD31^+$ naive $CD4^+$ T cells has been associated with an age-independent broadening of the T cell repertoire diversity [13]. Therefore IL-7 administration has a promising therapeutic potential in a variety of clinical settings, namely those associated with limited naive T cell numbers and skewed T cell repertoire. IL-7 therapy might thus be relevant as an aid for immune reconstitution following stem cell transplantation, potentially accelerating the restoration of T cell numbers and diversity by promoting thymic output and peripheral expansion of naive T cells, particularly the $CD31^+$ naive $CD4^+$ T cell subset.

Haploidentical HSCT constitutes a particularly challenging clinical setting because the number of mature T cells in the graft needs to be minimal in order to prevent GVHD, delaying early immune reconstitution through peripheral expansion and limiting the GVL effect, whereas the conditioning regimen might damage thymic or peripheral lymphoid tissues, hindering naive T cell output as well as memory T cell maintenance [14]. We performed a cross-sectional evaluation of long term immune reconstitution following haploidentical HSCT in a group of five patients who had received a $CD34^+$ purified stem

cell graft from full-haplotype mismatched related donors. The study took place four to six years post-transplant and allowed us to assess whether T cell homeostasis was successfully restored in these patients, not only in terms of cell numbers but also of T cell diversity. We were also interested in elucidating the mechanisms involved in T cell reconstitution, particularly in the replenishment of the naive $CD4^+$ T cell population. We observed that these patients had comparable absolute numbers of $CD4^+$ and $CD8^+$ T cells, as well as of B and NK cells, to healthy age-matched controls. We next assessed the proportion of naive and memory subsets as defined by the expression of CD45RA and CD27 within $CD4^+$ and $CD8^+$ T cells. We found that transplant recipients tended to have slightly increased frequencies of naive $CD45RA^+CD27^+$ cells, equivalent frequencies of early stage differentiation $CD45RA^-CD27^+$ cells and lower frequencies of highly differentiated $CD45RA^-CD27^-$ and $CD45RA^+CD27^-$ cells when compared to the other two cohorts. We demonstrated that $CD45RA^+CD27^-$ $CD4^+$ T cells are prone to cellular senescence and that these cells are accumulated during chronic CMV infection, a setting associated with persistent antigen stimulation. Hence, the absence of inflated $CD45RA^+CD27^-$ T cell populations in these transplant recipients suggests that restoration of T cell numbers was not mainly driven by clonal expansions but rather relied largely on homeostatic mechanisms. Furthermore, the proportion of $CD31^+$ cells within the naive $CD4^+$ T cell subset in patients was similar to the one observed in age-matched controls and it was significantly higher than in donors, which was likely due to the age gap between the two cohorts, given that the donors were always one of the parents. The $CD31^+$ naive $CD4^+$ T cell subset is highly enriched in RTEs and hence this result might lead us to speculate that thymic output played a major role in the maintenance of this population. However, as mentioned above, IL-7-driven peripheral expansion might also have contributed to the expansion of $CD31^+$ naive $CD4^+$ T cells, particularly since IL-7 levels have been described to be increased during lymphopenia, such as the one established immediately following stem cell transplantation. At the time of the study, transplant recipients did not have higher IL-7 serum levels than the other two cohorts. As previously reported, IL-7 levels return to steady-state levels once $CD4^+$, particularly naive $CD4^+$, T cell numbers are restored [15-17]. Hence, the absence of elevated IL-7 levels is in agreement with the observation of comparable $CD4^+$ T cell counts as well as $CD31^+$ naive $CD4^+$ T cell frequencies between recipients and age-matched controls. In order to elucidate the relative contribution of thymic output and peripheral expansion to immune

reconstitution, we quantified TREC content within PBMCs and measured telomere length within $CD4^+$ and $CD8^+$ T cell subsets. Both these assays revealed similar results when the three cohorts were compared, further suggesting that thymic output contributed, at least partly, to immune reconstitution. In agreement with these observations, we found comparably diverse TCRVB repertoires in transplant recipients and age-matched controls. It would be interesting to analyse the TREC content and telomere length within $CD4^+$ and $CD8^+$ T cell subsets in order to get a clearer picture of the replicative histories of each population. These data would also inform us of the relative contribution of thymic output and IL-7-driven expansion to the maintenance of the $CD31^+$ naive $CD4^+$ T cell subset. Overall, our data suggest that long term immune reconstitution was successfully achieved in this cohort of haploidentical HSCT recipients, likely through a combination of thymus-dependent and -independent mechanisms which gave rise to balanced $CD4^+$ and $CD8^+$ T cell subsets and to a diverse T cell repertoire. In order to assess the functional properties of these T cells, we are currently assessing proliferative responses and cytokine production profiles after polyclonal as well as antigen-specific stimulation.

As mentioned above, $CD45RA^+CD27^-$ $CD4^+$ T cells are accumulated during CMV infection [18], constituting large clonal expansions of CMV-specific cells [19,20]. Given the high prevalence of CMV infection, particularly in elderly individuals, we sought to investigate if the accumulation of $CD45RA^+CD27^-$ $CD4^+$ T cells might constitute a hindrance for immune competence. For that purpose, we compared the phenotypic and functional characteristics of these cells with the remaining CD45RA/CD27 $CD4^+$ T cell subsets. Our data indicate that $CD45RA^+CD27^-$ $CD4^+$ T cells are not exhausted, being able to produce multiple cytokines [18] and to proliferate in response to TCR activation. However, these cells are highly susceptible to cell death following activation, which is associated with low Bcl-2 and IL-7Rα basal levels [18], as well as with defective Akt(Ser473) phosphorylation. Nevertheless, $CD45RA^+CD27^-$ $CD4^+$ T cells do accumulate in CMV-infected individuals during ageing [18], which suggests a continual replenishment of this subset from $CD45RA^-$ precursors. The highly differentiated phenotype of $CD45RA^+CD27^-$ $CD4^+$ T cells indicates that they are not directly derived from $CD45RA^+CD27^+$ naive cells induced to lose CD27 expression. This scenario can be ruled out by comparing the TREC content within these subsets, which will reveal their replicative histories and thus clarify if $CD45RA^+CD27^-$ cells display TREC levels consistent with them being a potential direct differentiation product of $CD45RA^+CD27^+$

precursors. We demonstrated that $CD45RA^-CD27^+$ $CD4^+$ T cells can be induced to re-express CD45RA as a result of IL-7 stimulation. In contrast, IL-2 or IL-15 stimulation only induced modest levels of CD45RA-expression in these cells, whereas $CD45RA^-$ $CD27^-$ $CD4^+$ T cells failed to re-express CD45RA in the presence of IL-2, IL-7 or IL-15. Similarly to what we observed in *ex vivo* $CD45RA^+CD27^-$ $CD4^+$ T cells [18], the IL-7-induced CD45RA re-expressing population displayed low levels of Bcl-2 and IL-7Rα. Interestingly, we found that a larger proportion of CD45RA re-expressing cells proliferated during IL-7 stimulation when compared to the population that remained $CD45RA^-$, which appears incongruous with the persistently low IL-7Rα levels observed in the former population. However, IL-7Rα expression levels have been shown not to correlate with IL-7-induced signalling in human $CD4^+$ T cells [21]. Our results point to a role for IL-7 in the induction of CD45RA re-expression on memory $CD4^+$ T cells. A potential site where IL-7-driven CD45RA re-expression might occur *in vivo* is the bone marrow, an IL-7-rich immune compartment which also has increased frequencies of $CD45RA^+CD27^-$ $CD4^+$ T cells in comparison to peripheral blood [18]. CD45RA re-expression is likely to entail changes in the transcriptional program, namely IL-7-driven up-regulation of certain transcription factors in a sub-population of $CD45RA^-CD27^+$ $CD4^+$ T cells. Our results point to T-bet as a potential transcription factor involved in this process, given that *ex vivo* $CD45RA^+CD27^-$ $CD4^+$ T cells expressed the highest levels of T-bet in relation to the other subsets and that IL-7 up-regulated its expression in $CD45RA^-CD27^+$ $CD4^+$ T cells. The specific knock-down of T-bet expression in $CD45RA^-CD27^+$ $CD4^+$ T cells stimulated with IL-7 would help to clarify if this transcription factor is required for IL-7-induced CD45RA re-expression in these cells. Nevertheless, we found that IL-7-induced CD45RA re-expressing cells only modestly decreased CD27 expression, suggesting that other factors might co-operate with IL-7 in the generation of $CD45RA^+CD27^-$ $CD4^+$ T cells from $CD45RA^-CD27^+$ precursors. A likely candidate is TNF-α, a pro-inflammatory cytokine which has been shown to down-modulate the expression of co-stimulatory molecules on $CD8^+$ T cells [22-24]. The effects on the CD45RA/CD27 profile of $CD45RA^-CD27^+$ $CD4^+$ T cells cultured in the presence of IL-7 together with TNF-α are currently being assessed.

Although we found that $CD45RA^+CD27^-$ $CD4^+$ T cells retain functional and proliferative potential, these cells also displayed senescence-associated traits, such as high levels of CD57 and KLRG1 (Di Mitri *et. al.*, manuscript under submission) expression.

Surprisingly, CD45RA$^+$CD27$^-$ CD4$^+$ T cells showed relatively long telomeres but were defective in the induction of telomerase activity following TCR stimulation, giving contradictory indications about the replicative capacity and susceptibility to senescence of these cells. In order to clarify this issue, we assessed the expression of the senescence marker γ-H2AX after activation and found that CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ CD4$^+$ T cells expressed the highest levels, suggesting that the latter subset is prone to telomere-independent senescence. Given that the p38 MAPK pathway has been shown to mediate both telomere-dependent and -independent senescence in human fibroblasts [25], we investigated if blocking this pathway might reverse the senescence characteristics observed in CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ CD4$^+$ T cells. We found that p38 inhibition led to a modest decrease in γ-H2AX expression in the latter subset and substantially improved cell survival in both subsets, which was probably associated with increased Bcl-2 levels, following TCR activation. Moreover, blocking the p38 pathway enhanced telomerase activity upon TCR stimulation in CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ CD4$^+$ T cells, although its impact was more striking in the latter subset. These data, together with our observation that p38 expression was only detectable on CD45RA-expressing cells when CD45RA$^-$CD27$^+$ CD4$^+$ T cells were cultured with IL-7, suggest that the p38 pathway might mediate the generation and/or maintenance of CD45RA re-expressing memory CD4$^+$ T cells. We sought to investigate which factors might be responsible for the activation of the p38 pathway, potentially prompting the replenishment and accumulation of senescence-prone CD45RA$^+$CD27$^-$ CD4$^+$ T cells during CMV infection. Again, we found evidence to suggest that TNF-α may be involved in this process, given that p38 inhibition reversed the TNF-α-induced down-modulation of telomerase activity in CD4$^+$ T cells. Interestingly, this pro-inflammatory cytokine has been shown to be elevated during ageing [26], CMV infection [27] and in CD8$^+$ T cells that have reached replicative senescence *in vitro* [28], as well as to increase IL-7 production by bone marrow stromal cells [29]. Hence we can speculate that the high levels of TNF-α present during CMV infection would increase the levels of IL-7 in the bone-marrow, which in turn would be conducive to the generation of CD45RA$^+$CD27$^-$ CD4$^+$ T cells from CD45RA$^-$CD27$^+$ precursors through a mechanism involving IL-7-induced CD45RA re-expression and TNF-α-driven CD27 down-modulation. These CD45RA$^+$CD27$^-$ CD4$^+$ T cells would in turn produce more TNF-α, thus exhacerbating the pro-inflammatory environment and perpetuating the accumulation of these cells. Moreover, TNF-α would

also contribute to the onset of telomere-independent senescence in these cells through the activation of the p38 pathway. Although this is an hypothetical scenario, TNF-α inhibition during long-term cultures of human CD8$^+$ T cells has been shown to delay the onset of senescence [30], whereas anti-TNF-α therapy in patients with rheumatoid arthritis has been associated with the restoration of the CD28$^+$ T cell population within diseased joints [23,31], further suggesting a role for TNF-α in the accumulation of senescent T cells during chronic inflammation. Overall, our data show that the telomere-independent senescence traits found in CD45RA$^+$CD27$^-$ CD4$^+$ T cells were at least partly mediated by the p38 MAPK pathway and could be reversed to an extent by p38 inhibition. This link between p38 activation and senescence in lymphocytes identifies a potential target for therapeutic interventions.

Taken together, our results further emphasize the contribution of IL-7 signalling to naive and memory CD4$^+$ T cell homeostasis, ensuring the maintenance of the CD31$^+$ naive T cell pool and potentially contributing the generation of a CD45RA re-expressing reservoir of memory T cells which can be re-activated to perform effector functions.

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LIST OF PUBLICATIONS

Peer-reviewed articles

Cytomegalovirus infection induces the accumulation of short-lived, multifunctional CD4$^+$ CD45RA$^+$CD27$^-$ T cells: the potential involvement of interleukin-7 in this process.

Valentina Libri[1*], Rita I Azevedo[1,2*], Sarah E. Jackson[1*], Diletta Di Mitri[1,3], Raskit Lachmann[4], Stephan Fuhrmann[4,5], Milica Vukmanovic-Stejic[1], Kwee Yong[6], Luca Battistini[3], Florian Kern[4], Maria V.D. Soares[7] and Arne N. Akbar[1]

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Article first published online in Immunology on 7 January 2011.
DOI: 10.1111/j.1365-2567.2010.03386.x

KLRG1 signaling inhibits Akt (Ser473) phosphorylation and proliferation of highly differentiated CD8$^+$ T cells.

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Published in Blood Journal, June 25th 2009, Volume 113, Number 26, 6619-6628.

IL-7 sustains CD31 expression in human naive CD4$^+$ T cells and preferentially expands the CD31$^+$ subset in a PI3K-dependent manner.

Rita I. Azevedo[1*], Maria Vieira D. Soares[1*], João T. Barata[2], Rita Tendeiro[1], Ana Serra-Caetano[3], Rui M.M. Victorino[1] and Ana E. Sousa[1]

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Published in Blood Journal, March 26th 2009, Volume 113, Number 13, 2999-3007.

Manuscripts under submission

CD45RA Re-Expressing CD4$^+$ Memory T Cells Exhibit p38 MAP kinase Regulated Telomere Independent Senescence

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Submitted to the Journal of Experimental Medicine on December 2010.

Manuscripts in preparation

Long term immune reconstitution following haplotype-mismatched hematopoietic stem cell transplantation.

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Communications

Oral presentations

IL-7 sustains CD31 expression in human naive CD4$^+$ T cells and preferentially expands the CD31$^+$ subset in a PI3K-dependent manner

Rita I. Azevedo[1] and Maria Vieira D. Soares[1], João T. Barata[2], Rita Tendeiro[1], Ana Serra-Caetano[3], Rui M. M. Victorino[1], Ana E. Sousa[1]

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7th EAACI-GA^{2}LEN-Immunology-winter School, Davos, Switzerland, February 5th-8th 2009.

Long term immune reconstitution following haplotype-mismatched hematopoietic stem cell transplantation.

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13th International Congress of Immunology, Rio de Janeiro, Brazil, August 21st-25th 2007.

Poster presentations

IL-7 sustains CD31 expression in human naive CD4$^+$ T cells and preferentially expands the CD31$^+$ subset in a PI3K-dependent manner

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2nd European Congress of Immunology, Berlin, Germany, September 13th-16th 2009.

IL-7-driven homeostatic proliferation induces the generation of short-lived functionally distinct memory CD4$^+$ T cells re-expressing CD45RA

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2nd European Congress of Immunology, Berlin, Germany, September 13th-16th 2009.

Human naive CD4$^+$CD45RA$^+$CD31$^+$ recent thymic emigrants can be maintained by IL-7.

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13th International Congress of Immunology, Rio de Janeiro, Brazil, August 21st-25th 2007.

IL-7 and maintenance of naive CD4$^+$ T cell pools: insights from HIV-1 and HIV-2 infections.

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16th European Congress of Immunology – ECI, Paris, 2006.

Major Conferences Attended

7th European Congress of Biogerontology, "Centenarian lesson: a life beyond time" Palermo, Italy, October 14th-17th 2010.

2nd European Congress of Immunology, "Immunity for Life – Immunology for Health", Berlin, Germany, September 13th-16th 2009.

7th EAACI-GA^{2}LEN-Immunology-winter School, "Immune Responses in Allergy and Asthma", Davos, Switzerland, February 5th-8th 2009.

BSI Joint London Immunology Group / Differentiation and Immunosenescence Meeting, "Leukocyte differentiation and regulation in disease**"**, Institute of Child Health, London, September 12th 2008.

14th FEBS International Summer School on Immunology, "Immune System: Genes, Receptors and Regulation", Hvar, Croatia, September 10th -17th 2007.

13th International Congress of Immunology, "ImmunoRio 2007", Rio de Janeiro, Brazil, August 21st-25th 2007.

APPENDIX

Related Publications

In agreement with the Decreto-Lei 388/70, art. 8º, parágrafo 2, the results presented here were published or are currently being prepared for publication in the following scientific journals:

Chapter 1

Chapter 1.1

IL-7 sustains CD31 expression in human naive CD4$^+$ T cells and preferentially expands the CD31$^+$ subset in a PI3K-dependent manner.

Rita I. Azevedo*, Maria Vieira D. Soares*, João T. Barata, Rita Tendeiro, Ana Serra-Caetano, Rui M.M. Victorino, and Ana E. Sousa.

Blood, 26 March 2009, Vol. 113, No. 13, pp. 2999-3007.

Chapter 2

Chapter 2.1

Cytomegalovirus infection induces the accumulation of short-lived, multifunctional CD4$^+$ CD45RA$^+$CD27$^-$ T cells: the potential involvement of interleukin-7 in this process.

Valentina Libri*, Rita I. Azevedo*, Sarah E. Jackson*, Diletta Di Mitri, Raskit Lachmann, Stephan Fuhrmann, Milica Vukmanovic-Stejic, Kwee Yong, Luca Battistini, Florian Kern, Maria V.D. Soares and Arne N. Akbar.

Article first published online in Immunology on 7 January 2011.

Chapter 2.2

CD45RA Re-Expressing CD4$^+$ Memory T Cells Exhibit p38 MAP kinase Regulated Telomere Independent Senescence.

Diletta Di Mitri, Rita Azevedo, Valentina Libri, Sian M. Henson, Luca Battistini, David Bagley, David Kipling, Arne N. Akbar.

Submitted to the Journal of Experimental Medicine in December 2010.

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blood

2009 113: 2999-3007
Prepublished online Nov 13, 2008;
doi:10.1182/blood-2008-07-166223

IL-7 sustains CD31 expression in human naive CD4+ T cells and preferentially expands the CD31 + subset in a PI3K-dependent manner

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published semimonthly by the American Society of Hematology, 1900 M St, NW, Suite 200, Washington DC 20036.

IL-7 sustains CD31 expression in human naive CD4$^+$ T cells and preferentially expands the CD31$^+$ subset in a PI3K-dependent manner

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The CD31$^+$ subset of human naive CD4$^+$ T cells is thought to contain the population of cells that have recently emigrated from the thymus, while their CD31$^-$ counterparts have been proposed to originate from CD31$^+$ cells after homeostatic cell division. Naive T-cell maintenance is known to involve homeostatic cytokines such as interleukin-7 (IL-7). It remains to be investigated what role this cytokine has in the homeostasis of naive CD4$^+$ T-cell subsets defined by CD31 expression. We provide evidence that IL-7 exerts a preferential proliferative effect on CD31$^+$ naive CD4$^+$ T cells from adult peripheral blood compared with the CD31$^-$ subset. IL-7–driven proliferation did not result in loss of CD31 expression, suggesting that CD31$^+$ naive CD4$^+$ T cells can undergo cytokine-driven homeostatic proliferation while preserving CD31. Furthermore, IL-7 sustained or increased CD31 expression even in nonproliferating cells. Both proliferation and CD31 maintenance were dependent on the activation of phosphoinositide 3-kinase (PI3K) signaling. Taken together, our data suggest that during adulthood CD31$^+$ naive CD4$^+$ T cells are maintained by IL-7 and that IL-7–based therapies may exert a preferential effect on this population. (Blood. 2009;113: 2999-3007)

Introduction

Human naive CD4$^+$ T cells have recently been shown to contain 2 subpopulations distinguished by the expression of CD31 (platelet endothelial cell adhesion molecule-1, PECAM-1). The CD31$^+$ subset is thought to incorporate the population of cells recently emigrated from the thymus, whereas the CD31$^-$ subset has been proposed to derive from CD31$^+$ after homeostatic cell division.[1] During T-cell development in the thymus, rearrangement of the T-cell receptor (TCR) genes generates stable episomal DNA excision circles (TRECs) that are progressively diluted with cell division.[2-4] Accordingly, CD31$^+$ naive CD4$^+$ T cells have higher TREC content compared with the CD31$^-$ naive subset.[1] Moreover, the progressive age-associated decline in naive CD4$^+$ T cells is mainly due to a reduction in the CD31$^+$ naive subset while the CD31$^-$ subset persists,[5,6] further supporting the contribution of thymic output to the maintenance of CD31$^+$ cells. However, the decrease in TREC levels observed during aging is disproportionally greater compared with the decline in CD31$^+$ naive T cells, implicating other mechanisms, in addition to thymic output, in the persistence of these cells into old age.[4]

Cytokine-driven expansion has been proposed to significantly contribute to a low level of homeostatic proliferation that maintains naive T-cell numbers.[7] Besides its established importance in thymopoiesis, interleukin-7 (IL-7) is considered to play a key role in naive T-cell survival and proliferation in the periphery.[2,7] In vitro studies of human naive CD4$^+$ T cells cultured in the presence of IL-7 revealed, alongside with its antiapoptotic properties, an ability to induce proliferative responses without a switch to a memory phenotype.[8] IL-7 seems to exert a preferential effect on umbilical cord blood (CB) naive T cells that proliferate significantly more than adult peripheral blood naive T cells in response to IL-7.[8,9] Despite this, a considerable reliance upon IL-7 in naive T-cell homeostasis after T-cell depletion has been established.[7,10] IL-7 was able to promote T-cell reconstitution after bone marrow transplantation in mice acting not only at the thymic but also at the peripheral level,[11-13] and to expand naive and memory T cells in uninfected[14] and simian immunodeficiency virus (SIV)–infected nonhuman primates.[15] Furthermore, IL-7 serum levels were shown to increase in different lymphopenic settings in humans in strong inverse correlation with naive CD4$^+$ T-cell counts, suggesting a feedback mechanism to counteract T-cell depletion.[16-19] IL-7 administration to patients with metastatic melanoma led to CD4$^+$ and CD8$^+$ T-cell expansion, particularly of CD45RA$^+$ naive T cells,[20] and further clinical trials are currently exploring its therapeutic potential.

The possibility of IL-7 having distinct effects on human CD31$^+$ and CD31$^-$ naive subsets has not yet been investigated. These data are relevant not only to further clarify the mechanisms involved in the maintenance of these 2 naive populations during aging, but also to better characterize the potential cellular targets of therapeutic interventions involving IL-7 administration. In this respect, a recently published phase 1 clinical trial with IL-7 in refractory cancer shows a preferential expansion of the CD31$^+$ naive CD4$^+$ subset.[21] This was associated with a decrease in TREC content in this population consistent with the induction of proliferation by IL-7 in this subset.[21]

Here, we report that IL-7 exerted a selective proliferative effect on CD31$^+$ naive CD4$^+$ T cells from adult peripheral blood compared with their CD31$^-$ counterparts. We further observed that proliferation of adult CD31$^+$ naive CD4$^+$ T cells was dependent on

Submitted July 2, 2008; accepted November 6, 2008. Prepublished online as *Blood* First Edition paper, November 13, 2008; DOI 10.1182/blood-2008-07-166223.

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The online version of this article contains a data supplement.

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the activation of phosphoinositide 3-kinase (PI3K) signaling pathway and was not associated with loss of CD31 expression. IL-7 also promoted the preservation of CD31 levels in nonproliferating naive T cells through PI3K activation. Taken together, our data suggest that IL-7 may play a preferential role in the maintenance of $CD31^+$ naive $CD4^+$ T cells during adult life.

Methods

Cell isolation

This study was approved by the Ethics Board of the Faculty of Medicine of Lisbon. Mononuclear cells were isolated from heparinized adult peripheral blood of healthy volunteers, and from umbilical cord blood (CB) obtained immediately after delivery of full-term infants, with informed consent obtained in accordance with the Declaration of Helsinki, by Ficoll-Hypaque density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). $CD4^+$ T cells were negatively selected using the EasySep Human $CD4^+$ T-Cell Enrichment Kit (StemCell Technologies, Vancouver, BC) and subsequently sorted into $CD31^+$ and $CD31^-$ naive subsets using a FACSAria flow cytometer (BD Biosciences, San Jose, CA) after staining for CD45RA, CD45RO, CD4, and CD31 as described below.

Cell culture

Cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated human AB serum (Sigma-Aldrich, St Louis, MO), 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine (Invitrogen), in the presence or absence of recombinant human IL-7 (10 ng/mL; R&D Systems, Minneapolis, MN) or recombinant human IL-2 (10 U/mL; obtained through the National Institutes of Health (NIH)/AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH [IL-2] from Hoffman-La Roche). PI3K and mitogen-activated protein kinase (MEK)–extracellular signal-regulated kinase (ERK) activity were respectively blocked by incubation of cells for 1 hour at 37°C before IL-7 stimulation with either 10 μM LY294002 or 10 μM PD98059 (both from Calbiochem, Merck Biosciences, Nottingham, United Kingdom) or the equivalent volume of the vehicle control dimethyl sulfoxide (DMSO; Sigma-Aldrich) alone. LY294002, PD98059, and DMSO were readded to the culture at day 4.

Phenotypic analysis

Cells resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich) were stained for 20 minutes at room temperature with the following anti–human monoclonal antibodies: CD4-phycoerythrin (PE)–cyanin 7 (PE-CY7; clone, L3T4), CD45RA–fluorescein isothiocyanate (FITC) or allophycocyanin (APC; clone, HL100), CD45RO-PE (clone; UCHL1), CD62L-APC-cyanin 7 (APC-Cy7; clone, DREG 56) and CD31 PE or APC (clone, WM59) from eBioscience (San Diego, CA); CD38 PE (clone, HB7) and CD3–peridinin chlorophyll protein (PerCP; clone, SK7) from BD Biosciences; and CD127 PE (IL-7Rα; clone 40131; R&D Systems). Intracellular staining for Bcl-2 FITC (clone 124; Dako, Glostrup, Denmark) and Ki67 FITC (clone B56; BD Biosciences) was performed using fixation and permeabilization reagents from eBioscience. Cells were labeled with 0.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes-Invitrogen, Carlsbad, CA) at 37°C for 15 minutes in the dark, quenched with ice-cold culture medium at 4°C for 5 minutes, and washed 3 times before culture. Apoptosis was assessed using 7-amino-actinomycin D (7-AAD) viability Staining Solution (eBioscience) or by annexin V/propidium iodide (PI) detection kit (BD Biosciences). Samples were acquired on a BD FACSCanto flow cytometer (BD Biosciences) after fixation with 1% formaldehyde (Sigma-Aldrich). Data were analyzed using FlowJo software version 8.1.1 (TreeStar, Ashland, OR).

STAT5 tyrosine phosphorylation analysis

Cells were surface stained and stimulated with 50 ng/mL IL-7 for 15 minutes, fixed with 2% formaldehyde at 37°C for 10 minutes, and placed on ice. Cells were then permeabilized with ice-cold 90% methanol (Sigma-Aldrich) at 4°C for 30 minutes and incubated with anti–phospho-STAT5 (pY694) antibody coupled to Alexa Fluor 488 (BD Biosciences) at room temperature for 1 hour. Samples were immediately acquired on FACSCanto.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). Data are presented as mean plus or minus standard error of mean (SEM). *P* less than .05 was considered significant.

Results

IL-7–induced proliferation of adult naive $CD4^+$ T cells is restricted to the $CD31^+$ subset

IL-7 is known to induce proliferation of naive $CD4^+$ T cells,[8,22,23] but the possibility of distinct effects on naive subsets defined by CD31 expression has not been determined. Our preliminary data from the culture of adult total naive $CD4^+$ T cells ($CD4^+CD45RA^+CD45RO^-$) with recombinant human IL-7 for 7 days suggested that the proliferative response was confined to $CD31^+$ cells (data not shown). Of note, in agreement with previous reports,[23,24] similar results were obtained when the concentration of IL-7 was increased from 10 to 50 ng/mL. Proliferation was assessed using the cell-cycle entry marker Ki67, because we found it to be the most reliable method to quantify low levels of proliferation. Although we cannot guarantee that all $Ki67^+$ cells complete the proliferative cycle, we were able to confirm the proliferation using CFSE staining in adult cells upon IL-7 stimulation (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Moreover, IL-7 has previously been shown to induce similar levels of cell division in adult naive $CD4^+$ T cells.[9,25]

To exclude a gain of CD31 upon proliferation, we proceeded by investigating the ability of purified $CD31^+$ and $CD31^-$ naive ($CD45RA^+CD45RO^-$) $CD4^+$ T cells from adult peripheral blood as well as umbilical CB to proliferate in response to IL-7 after 7 days of in vitro culture. Figure 1 illustrates representative flow cytometry dot plots of CD31/CD45RA profiles of freshly isolated $CD4^+$ T cells from adult and CB as well as the gating strategy used to purify the subsets. We confirmed that proliferative responses from adult naive $CD4^+$ T cells were only observed within the $CD31^+$ subset (Figure 2). In agreement with previous reports,[8] CB naive T cells showed consistently stronger proliferative responses to IL-7 stimulation than adult naive T cells. Only 12 of the 22 studied adult samples proliferated in response to IL-7, whereas all 12 CB samples proliferated. Purified $CD31^+$ naive $CD4^+$ T cells from adults also proliferated significantly less than $CD31^+$ from CB (2.82% ± 1.11% vs 26.7% ± 3.22% $Ki67^+$ cells, respectively; $P = .001$). Of note, both $CD31^+$ and $CD31^-$ naive $CD4^+$ T-cell subsets isolated from CB were found to proliferate in response to IL-7, while in all analyzed adults proliferation was restricted to the $CD31^+$ subset, as illustrated in Figure 2A. Adult cells able to proliferate in response to IL-7 did not significantly differ from nonresponders with respect to the proportion of males and females, the percentage of naive ($CD45RA^+$) or $CD31^+$ naive within $CD4^+$ cells, or the percentage of $CD31^+$ within the naive $CD4^+$ subset (data not shown). We also did not find any differences comparing

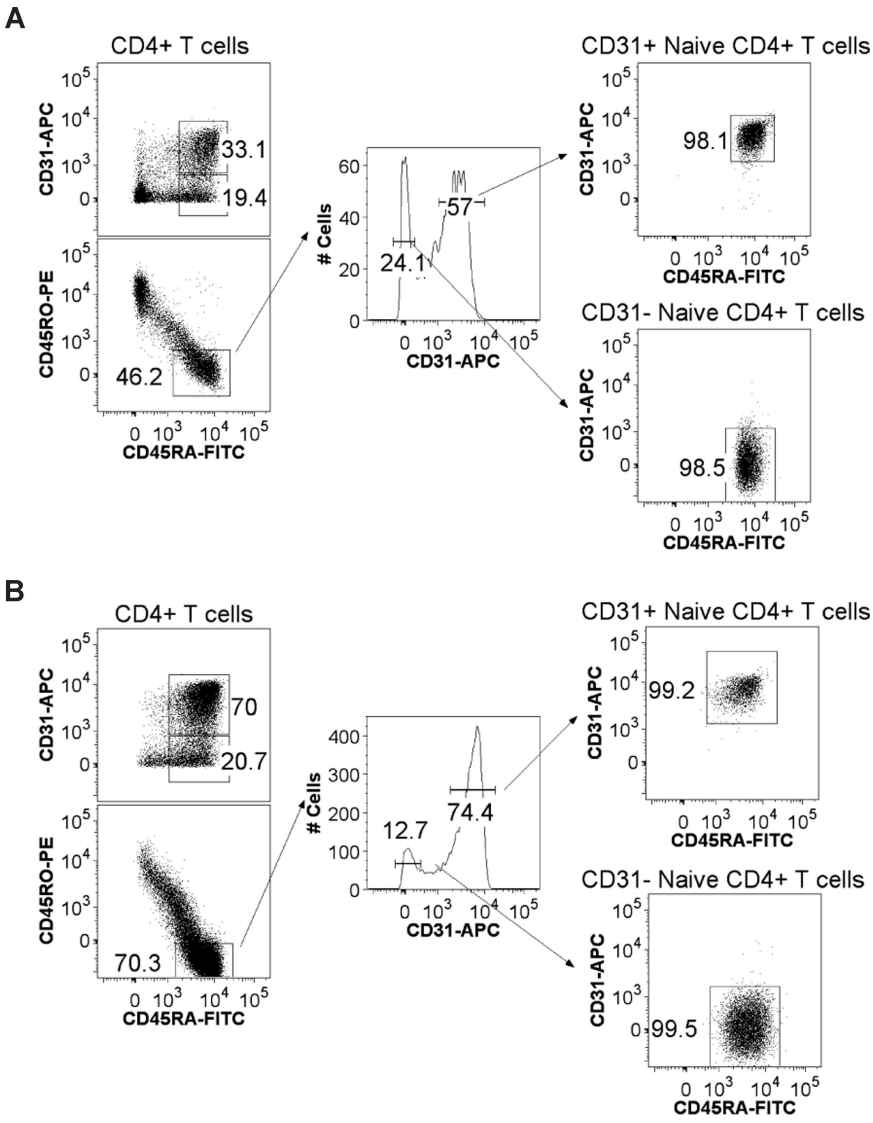

Figure 1. CD31 expression profiles and gating strategy used to purify CD31+ and CD31− naive CD4+ T-cell subsets from adult and cord blood. CD4+ T cells were negatively selected using the EasySep Human CD4+ T-cell Enrichment Kit and stained using monoclonal antibodies for CD45RA, CD45RO, CD4, and CD31. Flow cytometry profiles of CD4+ T cells stained for CD45RA and CD31 are shown for representative adult (A) and cord blood (B) samples. Also shown is the gating strategy used for FACS sorting. After gating on viable lymphocytes and CD4+ T cells, cells were gated on CD45RA+ and CD45RO− expression followed by tight gates on CD31+ and CD31− cells as illustrated by the resulting postsorting profiles.

the expression of the alpha chain of the IL-7 receptor (IL-7Rα) within the total naive CD4+ gate, or within the CD4+CD45RA+CD31+ and CD4+CD45RA+CD31− gates (data not shown). Interestingly, responders tended to be younger than nonresponders, although this did not reach statistical significance (28.9 ± 2.42 years and 36.4 ± 3.41 years, respectively; $P = .109$).

CD31− naive CD4+ T cells are thought to represent a subpopulation that has undergone peripheral expansion.[1] Thus, we next addressed whether in vitro IL-7–induced proliferation resulted in loss of CD31 expression. We found that proliferating CD31+ naive CD4+ T cells did not lose CD31, and that the CD31 median fluorescence intensity (MFI) was significantly higher in Ki67-expressing than in noncycling CD31+ cells (Figure 2B). Furthermore, we were able to monitor cell divisions using CFSE labeling in CB naive CD4+ T cells given their strong proliferative responses to IL-7, and observed that cells that divided up to 4 times during the culture period maintained CD31 expression. Statistical analysis using paired *t* test showed no statistically significant differences in CD31 expression levels between undivided populations and those that had undergone proliferation (Figure 2C).

We next evaluated whether the different levels of proliferative responses could be attributed to a distinct basal expression of IL-7Rα. We measured ex vivo IL-7Rα expression levels by flow cytometry in freshly isolated lymphocytes, and found that adult CD31− naive CD4+ T cells expressed lower levels than their CD31+ counterparts (Figure 2D). The opposite was found in CB subsets where CD31− cells showed higher IL-7Rα expression than CD31+. Although the levels of IL-7Rα expression were significantly higher in the CD31− subset of CB compared with adults, they were similar in adult CD31− and CB CD31+ subsets (Figure 2D). Thus, the proliferative outcome of IL-7 stimulation is unlikely to rely solely on IL-7Rα expression levels.

These data suggest that in adulthood, the ability of naive CD4+ T cells to proliferate in response to IL-7 is restricted to the CD31+ subset and show that CD31 is not lost after IL-7–induced proliferation.

IL-7–induced proliferation of adult CD31+ naive CD4 T cells is dependent on the PI3K pathway

We next investigated whether the decreased proliferation of the CD31− naive CD4+ subset was associated with a general inability to respond to IL-7. A consequence of IL-7 binding is the down-regulation of its own receptor which has been shown to be controlled at the transcriptional level.[9,26] We found a clear down-regulation of the IL-7Rα in all populations compared with freshly

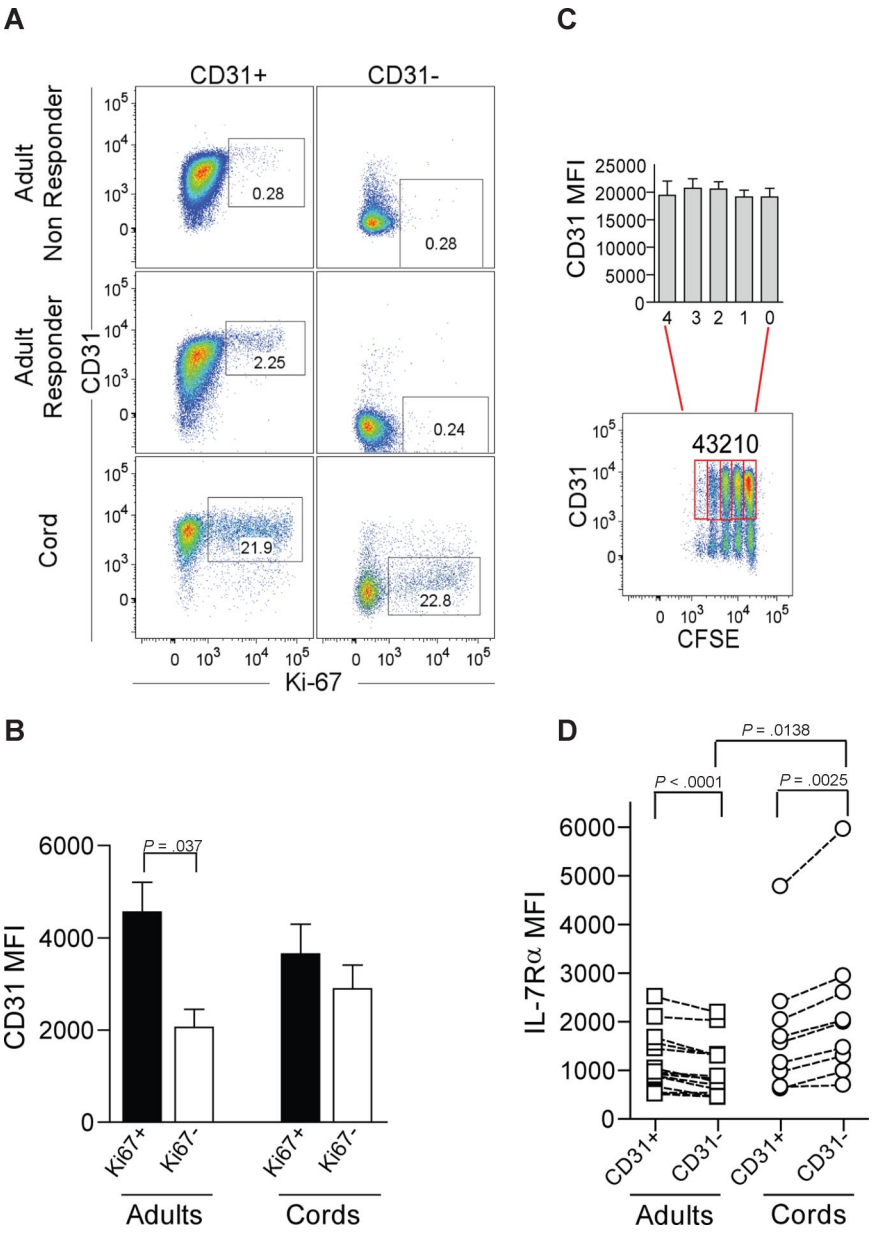

Figure 2. IL-7–induced proliferation of adult naive CD4$^+$ T cells is restricted to the CD31$^+$ subset. (A) Representative dot-plots of CD31 and Ki67 flow cytometry analysis after 7-day culture in the presence of IL-7 of purified CD31$^+$ and CD31$^-$ naive CD4$^+$ T-cell subsets from adult peripheral blood, for an IL-7 "nonresponder" (top panel), an IL-7 "responder" (middle panel), and CB (bottom panel). Cells were successively gated on a viable lymphogate, CD3$^+$, CD4$^+$, and CD45RA$^+$. (B) CD31 MFI was assessed within the purified CD31$^+$ naive subset further gated on Ki67$^+$ or Ki67$^-$ cells after 7-day culture with IL-7. Three adults and 4 CB samples were studied. (C) Representative dot-plot illustrating CD31 expression plotted against CFSE labeling of CB CD4$^+$CD45RA$^+$ T cells cultured with IL-7 for 7 days. CD31$^+$ cells were further gated according to the number of cell divisions, and bars show CD31 MFI from 4 experiments. (D) Ex vivo analysis of IL-7Rα MFI on freshly isolated mononuclear cells from adult and CB samples sequentially gated on CD3$^+$, CD4$^+$, CD45RA$^+$, and CD31$^+$ or CD31$^-$ lymphocytes. Each symbol represents one individual. Bars represent mean plus or minus SEM. Data were compared using paired or unpaired *t* test as appropriate and significant *P* values are shown.

isolated cells (Figure 3A). IL-7–mediated signaling is known to induce signal transducer and activator of transcription-5 (STAT5) phosphorylation that promotes not only cell cycling but also cell survival through Bcl-2 up-regulation.[8,27,28] We found induction of STAT5 phosphorylation (Figure 3B) and Bcl-2 up-regulation (Figure 3C) in both adult and CB CD31$^+$ and CD31$^-$ naive CD4 subsets after IL-7 stimulation in comparison with freshly isolated cells. In agreement, similar levels of inhibition of apoptosis (ranging from 60%-70%) were observed in all subsets, using 7-AAD incorporation to compare unstimulated with IL-7 stimulated cells after 7 days of culture (Figure 3D). These data show that despite exerting distinct proliferative effects, IL-7 is able to induce STAT5 phosphorylation, to up-regulate Bcl-2 expression, to prevent apoptosis, and to down-regulate IL-7Rα in both CD31$^+$ and CD31$^-$ naive CD4$^+$ subsets.

IL-7–mediated signaling leads to PI3K activation, a pathway that regulates cell proliferation and metabolism.[23,27] In particular, IL-7–induced proliferation and glucose uptake of naive CD4$^+$ T cells from CB was shown to be dependent upon the PI3K pathway.[23] Through the use of the cell-permeable PI3K-specific inhibitor LY294002, we investigated whether the PI3K pathway was required for IL-7–mediated proliferation of adult and CB CD31$^+$ and CD31$^-$ subsets. As shown in Figure 4A, LY294002 was very effective at blocking proliferation of adult CD31$^+$ naive CD4$^+$ T cells cultured in IL-7 for 7 days. IL-7Rα down-modulation was found to be PI3K independent (Figure 4B). Despite blocking proliferation, LY294002 did not affect Bcl-2 levels, showing a dissociation of these pathways in these cells (Figure 4C). As previously reported,[23,29] we observed a minor increase in apoptosis in the presence of LY294002 in adult naive CD4$^+$ T-cell subsets that was not observed in CB cultures (Figure 4D). Although the possibility of a contribution of apoptosis to the observed block in proliferation induced by PI3K inhibition in the adult CD31$^+$ subset cannot be excluded, this is unlikely to be the case because LY294002 completely blocked proliferation in CB cultures (Figure 4A) without an increase in apoptosis (Figure 4D).

The ability of IL-7 to activate the MEK-ERK pathway in T cells remains controversial. Although IL-7 is able to induce ERK1/2

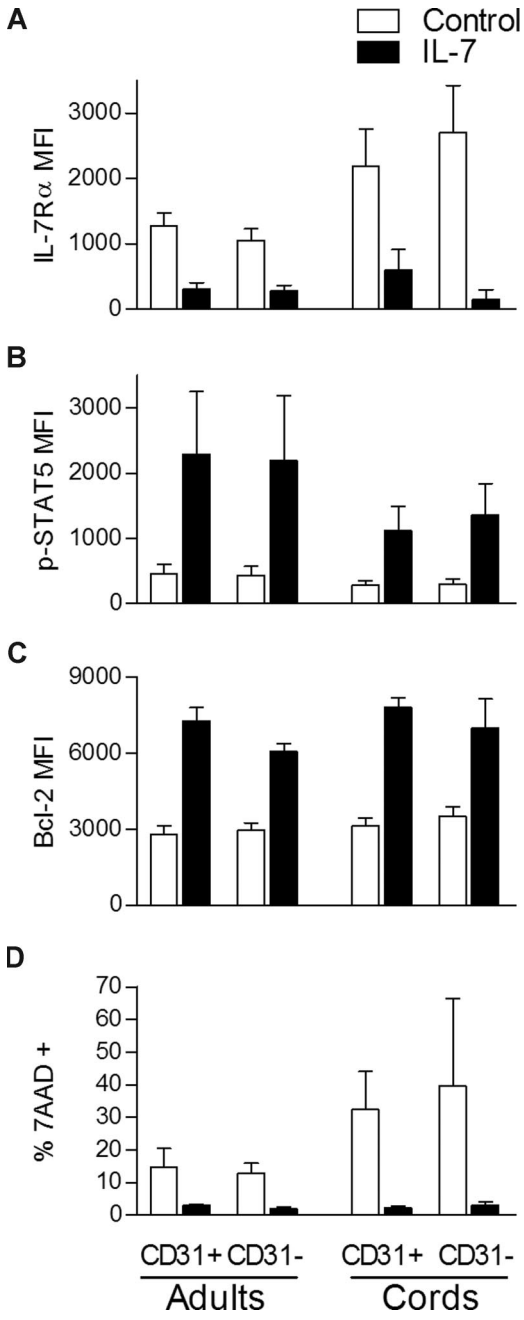

Figure 3. IL-7 stimulation leads to STAT5 phosphorylation, Bcl-2 up-regulation, and IL-7Rα down-modulation in both CD31+ and CD31− naive CD4+ subsets. IL-7Rα expression (A), STAT5 phosphorylation (B), Bcl-2 expression (C), and 7-AAD incorporation (D) were evaluated by flow cytometry within gated CD31+ and CD31− naive CD4 subsets. p-STAT5 was assessed on freshly isolated mononuclear cells from adult (n = 5) and CB (n = 3) samples either unstimulated or stimulated with IL-7 for 15 minutes. Bcl-2 and IL-7Rα MFI were evaluated ex vivo in adult PBMC (n = 6 and n = 9, respectively) and CB cells (n = 4 and n = 6, respectively) and in the corresponding purified CD31+ and CD31− naive subsets cultured in the presence of IL-7 for 7 days. 7-AAD incorporation was measured in purified CD31+ and CD31− subsets after 7 days of culture in the presence of IL-7 and in its absence (control). Bars represent mean MFI values plus or minus SEM.

phosphorylation in human leukemia T-cell precursors,[27] it does not appear to do so in some mouse T-cell lines,[30] in normal human thymocytes,[31] or in human peripheral blood T cells.[32] We used the MEK-specific inhibitor PD98059 to test the involvement of this pathway in IL-7–mediated effects on human adult CD31+ naive CD4+ subset. As illustrated in Figure 4, PD98059 did not impair any of the IL-7–dependent effects assessed, indicating that the MEK-ERK pathway does not play a critical role in the overall effects of IL-7 in human naive CD4+ T cells.

The same findings were observed for CB CD31+ and CD31− naive CD4+ T cells. Namely, proliferation was blocked by LY294002 but not PD98059, while all the other IL-7 readouts assessed were unaffected by PI3K or MEK-ERK inhibition (Figure 4).

Overall, we show that despite their inability to proliferate in response to IL-7, adult CD31− naive CD4+ T cells are not refractory to IL-7–mediated signaling as measured by STAT5 phosphorylation, Bcl-2 up-regulation or IL-7Rα down-modulation. These data suggest a selective inability of IL-7 to activate the signaling pathways that lead to proliferation in these cells. Moreover, we show for the first time that adult CD31+ naive CD4+ T-cell proliferation is dependent on PI3K activation.

IL-7 promotes the maintenance of CD31 expression in both adult and CB naive CD4+ T cells in a PI3K-dependent manner

As shown in Figure 2B, cells actively proliferating in response to IL-7 do not lose CD31 expression. We further assessed whether CD31− cells could reexpress CD31 after culture in the presence of IL-7. As shown in Figure 5A, purified CD31− cells from either adult or CB did not acquire CD31 during the culture period. In addition, Figure 5A clearly shows that the levels of CD31 expression were maintained or even increased in CD31+ naive CD4+ cells after in vitro culture with IL-7, whereas cells cultured in medium alone showed reduced CD31 expression ($P = .008$, paired t test comparison of adult CD31+ cells cultured in the presence of IL-7 and in its absence). This was also the case when cells were cultured for up to 13 days, where CD31 levels were maintained in the presence of IL-7, while cells cultured in medium alone or in the presence of IL-2 exhibited decreased CD31 expression (Figure 5B).

We next asked whether the preservation of CD31 expression in cells cultured with IL-7 was dependent on the PI3K pathway. For this purpose, we monitored CD31 levels in the presence of IL-7 alone or with the PI3K inhibitor (Figure 5C), and found that blocking the PI3K pathway led to a statistically significant decrease in CD31 expression levels in both adult and CB naive cells ($P = .002$ and $P = .009$, for adults and CB, respectively, paired t test comparison of IL-7 culture with and without LY294002). DMSO, used as a vehicle control, and PD98059 had no effect on CD31 MFI compared with IL-7 alone (Figure 5C).

As mentioned above, CB samples always proliferated in response to IL-7, while approximately one-half of the adults studied exhibited proliferative responses in vitro. Importantly, blocking the PI3K pathway prevented CD31 maintenance in all adults tested regardless of their ability to proliferate in response to IL-7. This is shown in Figure 5C, where individuals who proliferated in response to IL-7 and those who did not are represented. These data suggest that the preservation or increase of CD31 expression is independent of proliferation.

We also assessed the possible effects of blocking PI3K signaling on the expression of the CD31 ligand, CD38.[33] This molecule has been shown to decrease on naive CB T cells cultured in the presence of IL-7.[22] As shown in Figure 5D, we observed that adult and CB CD31+ naive CD4+ T cells exhibited a significant reduction of CD38 expression after culture with IL-7 ($P < .001$, paired t test), and this was not altered by the presence of PI3K or

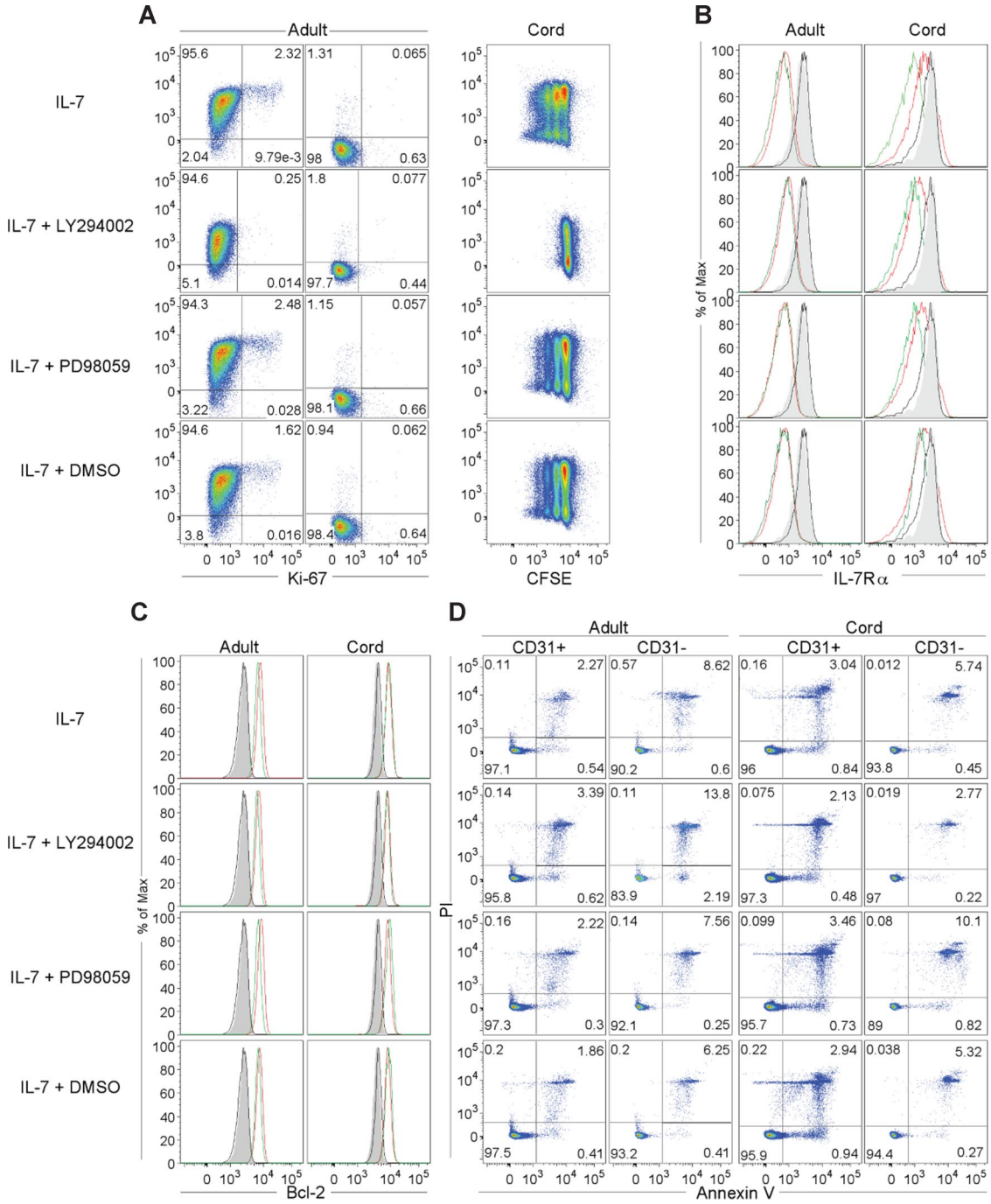

Figure 4. The IL-7–induced proliferation of adult CD31$^+$ naive CD4$^+$ T cells is dependent on the PI3K pathway. CD31$^+$ and CD31$^-$ naive CD4$^+$ T cells were purified from adult peripheral blood and CB, cultured in the presence of IL-7 with or without the PI3K inhibitor LY294002 or the MEK-ERK inhibitor PD98059 as indicated, and harvested at day 7 of culture. DMSO was used as a vehicle control. Representative examples of the 6 adults and 4 CBs studied are shown. (A) Assessment of proliferation using Ki67 in an adult sample. Representative analysis of a CB (1 of 4) is also shown illustrating the blocking effects of LY294002 on whole naive CD4$^+$ T-cell subset proliferation as assessed by CFSE labeling. CD31 staining is shown on the y-axis. (B) IL-7Rα and (C) Bcl-2 expression analyzed at day 0 within CD31$^+$ (gray filled histograms) and CD31$^-$ cells (black line). Analysis at day 7 within CD31$^+$ (red line) and CD31$^-$ (green line) purified populations cultured in the presence of IL-7 and the indicated inhibitors are also shown. (D) Evaluation of apoptosis by annexin V and PI staining after 7 days of culture of the purified CD31$^+$ and CD31$^-$ naive subsets.

MEK-ERK inhibitors. These data show that LY294002 is unable to recover the reduction of CD38 expression associated with IL-7 culture, suggesting that IL-7 may regulate CD31 expression independently of its ligand.

Overall, we report a role for IL-7 not only in the proliferation of adult CD31$^+$ naive CD4$^+$ T cells, but also in the maintenance or increase of CD31 expression levels in a PI3K-dependent manner.

Discussion

Our data indicate that IL-7 preferentially promotes proliferation of CD31$^+$CD4$^+$ naive T cells in adults, while preventing the loss of CD31 expression in both cycling and noncycling cells. The 2 mechanisms appear to depend upon the activation of the PI3K

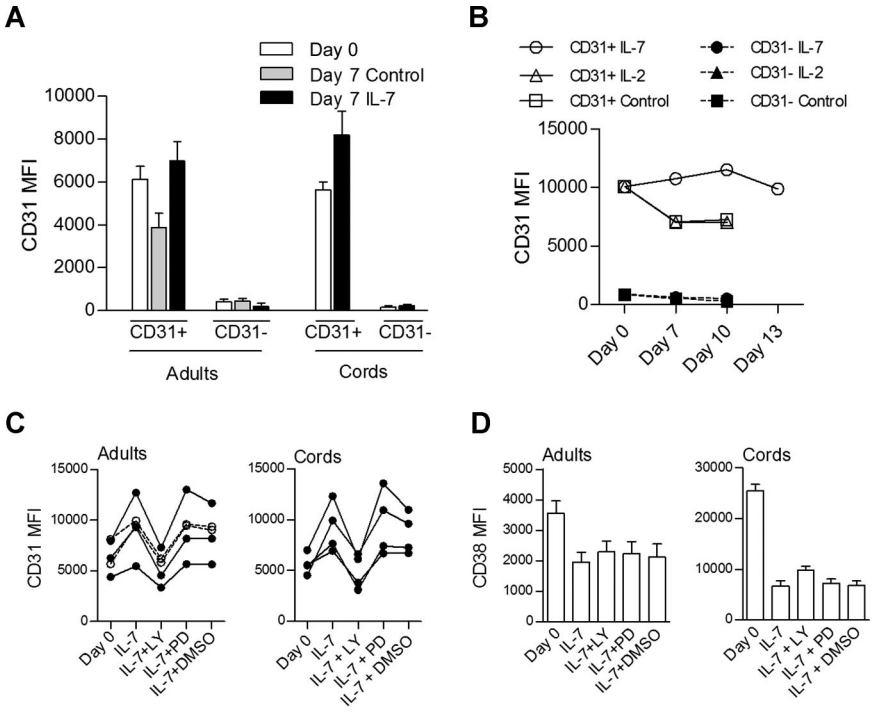

Figure 5. IL-7 promotes the maintenance of CD31 expression on both adult and CB naive CD4 T cells through the PI3K pathway. (A) CD31 MFI on CD31$^+$ and CD31$^-$ sorted subpopulations of naive CD4$^+$ T cells from adult (n = 13) and CB (n = 5) at day 0 and day 7 in the presence or absence (control) of IL-7. Analysis of CB subsets cultured in the absence of IL-7 was precluded by the high rate of cell death. (B) Longitudinal analysis of CD31 MFI of adult naive CD4$^+$ subsets cultured in the presence of IL-7, IL-2, or medium alone (control) for up to 13 days (data representative of 3 individuals). Open symbols represent CD31$^+$ purified cells while closed symbols correspond to the CD31$^-$ fraction. (C) CD31 MFI assessed on purified CD31$^+$ naive CD4$^+$ T cells at day 0 and after 7-day culture in the presence of IL-7 alone or in addition to LY294002, PD98059, or DMSO. Each symbol represents one individual. Filled symbols refer to individuals with a proliferative response to IL-7 and open symbols to those that did not proliferate. (D) CD38 MFI are shown in the same culture conditions in adult (n = 6) and CB (n = 4) samples, respectively. Bars represent mean values plus or minus SEM.

pathway and likely contribute to the maintenance of CD31$^+$ naive CD4$^+$ T cells promoted by IL-7. In contrast, the CD31$^-$ subset appears to rely on other homeostatic cues.

The selective ability of IL-7 to induce proliferation of the CD31$^+$ subset during adulthood, and in this way contribute to the maintenance of a population that is known to incorporate recent thymic emigrants,[1] is expected to have a physiologic role in the preservation of the TCR repertoire diversity within naive CD4$^+$ T cells.

Thus, as thymic output is reduced during aging, IL-7 may contribute to the persistence of the CD31$^+$ population through low-level proliferation. This is in agreement with recent data showing that both TREC content and telomere length decrease in CD31$^+$ naive CD4$^+$ T cells during aging, implying that their persistence is dependent on proliferation in the periphery.[6] The persistence of relatively high TREC content in the CD31$^+$ subset can be attributed to both residual thymic output and to a low rate of peripheral cell division.

Importantly, we have previously associated IL-7 serum levels and preservation of CD31$^+$ naive subset during aging in lymphopenic settings, and suggested that this positive correlation may contribute to the slower rate of CD4$^+$ T-cell decline in HIV-2 compared with HIV-1 infection.[16]

Our observation that the adult CD31$^-$ subset did not proliferate in response to IL-7 in vitro does not exclude the possibility of IL-7 acting as a costimulus to other homeostatic proliferation mechanisms, such as self-peptide–major histocompatibility complex (MHC) interactions.[34] However, our findings suggest that the CD31$^-$ subset may be preferentially regulated by mechanisms other than direct IL-7–driven proliferation.

The maintenance of CD31 expression upon IL-7 stimulation raises questions regarding the mechanisms underlying the loss of CD31 in naive CD4$^+$ T cells. CD31 expression has been shown to be lost after TCR stimulation of naive CD4$^+$ T cells,[35] and therefore low-affinity self-peptide–MHC interactions may be implicated in the generation as well as maintenance of the CD31$^-$ subset.[34] Our observation of a restricted IL-7 proliferative effect on adult CD31$^+$ naive CD4$^+$ T cells further support this possibility. In agreement with this, Kholer et al[5] reported that the CD31$^-$ subset expresses increased levels of BFL-1/A1 ex vivo compared with the CD31$^+$ subset. BFL-1/A1 has been described as a marker of recent TCR engagement whose expression is not induced by cytokine stimulation,[36] further implying that the CD31$^-$ subset is likely to be maintained by mechanisms that rely on TCR engagement rather than cytokine-induced proliferation. On the other hand, the presence of CD31 may impair TCR-mediated maintenance of CD31$^+$ cells, since there are data supporting an inhibitory function for CD31 in TCR activation through its cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs).[37,38] Our data further support the view that CD31 expression may impact on the homeostatic mechanisms involved in the maintenance of the adult naive CD4$^+$ T-cell pool.

We also demonstrated that the distinct responses of the CD31$^+$ and CD31$^-$ subsets to IL-7 could not be solely attributed to differences in IL-7Rα expression. Interestingly, a previous study addressing the effects of IL-7 in human B-cell progenitors comparing pro-B and pre-B cells reported that only pro-B cells proliferate in response to IL-7 despite similar levels of IL-7Rα in both subsets.[39] In addition, this study demonstrated that in contrast to the pre–B-cell subset, pro-B cells expressed CD31, further demonstrating an association between CD31 expression and the ability to proliferate in response to IL-7.[39]

In addition, we show that IL-7–induced proliferation of CD31$^+$ naive CD4$^+$ T cells from adults is dependent on PI3K activation, in agreement with what was previously reported for umbilical cord blood naive T cells.[23] Furthermore, we show for the first time that IL-7 induces maintenance or an increase of CD31 expression in a PI3K-dependent manner and that this occurs irrespectively of the induction of proliferation. The biologic significance of this finding is further emphasized by the absence of changes in the expression of the CD31 ligand (CD38) upon PI3K inhibition.

While contributing to the understanding of the role of IL-7 in the maintenance of naive CD4$^+$ subsets in humans, our data further imply that the CD31$^+$ subset is likely to be the main target of IL-7–driven proliferation during its therapeutic use. This is in agreement with data recently published of a phase 1 trial using recombinant IL-7.[21] A clear induction of T-cell proliferation was shown, whereby naive CD4$^+$ expansion was accounted by proliferation of the CD31$^+$ naive CD4$^+$ T-cell subset that was associated with a decrease in TREC content which is highly suggestive of IL-7–driven peripheral expansion.[21]

In conclusion, our data support the view that the adult naive CD4$^+$ T-cell subset identified by the CD31 marker, besides including the recent thymic emigrants,[1] represents a population with a unique ability to proliferate in response to IL-7. Moreover, we show that IL-7 sustains CD31 expression in naive CD4$^+$ T cells in a PI3K-dependent manner. This preferential effect of IL-7 on the CD31$^+$ population provides a biologic rationale for the use of IL-7 therapy in clinical settings where the expansion of the T-cell repertoire diversity is required.

Acknowledgments

We are grateful to Professor Arne N. Akbar (University College London) and Russell B. Foxall and Helena Cabaço (both from Instituto de Medicina Molecular, Lisboa) for critical review of this manuscript and for scientific discussions. We acknowledge Dr Helena Ferreira (Hospital Universitário de Santa Maria, Lisboa) for providing umbilical cord blood samples.

This work was supported by grant POCI/BIA-BCM/61079/2004 from Fundação para a Ciência e a Tecnologia (FCT) and by Programa Operacional Ciência e Inovação 2010 (POCI2010; to M.V.D.S.) R.I.A., M.V.D.S., and R.T. received scholarships from FCT cofinanced by POCI 2010 and FSE.

Authorship

Contribution: R.I.A. and M.V.D.S. designed and performed research, analyzed and interpreted data, and wrote the paper; J.T.B. designed research and discussed data; R.T. and A.S.-C. performed research; R.M.M.V. discussed data; and A.E.S. designed research, supervised the work, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Immunology
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IMMUNOLOGY ORIGINAL ARTICLE

Cytomegalovirus infection induces the accumulation of short-lived, multifunctional CD4$^+$ CD45RA$^+$ CD27$^-$ T cells: the potential involvement of interleukin-7 in this process

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doi:10.1111/j.1365-2567.2010.03386.x
Received 9 November 2010; revised 10 November 2010; accepted 10 November 2010.
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Summary

The relative roles that ageing and lifelong cytomegalovirus (CMV) infection have in shaping naive and memory CD4$^+$ T-cell repertoires in healthy older people is unclear. Using multiple linear regression analysis we found that age itself is a stronger predictor than CMV seropositivity for the decrease in CD45RA$^+$ CD27$^+$ CD4$^+$ T cells over time. In contrast, the increase in CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T cells is almost exclusively the result of CMV seropositivity, with age alone having no significant effect. Furthermore, the majority of the CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T cells in CMV-seropositive donors are specific for this virus. CD45RA$^+$ CD27$^-$ CD4$^+$ T cells have significantly reduced CD28, interleukin-7 receptor α (IL-7Rα) and Bcl-2 expression, Akt (ser473) phosphorylation and reduced ability to survive after T-cell receptor activation compared with the other T-cell subsets in the same donors. Despite this, the CD45RA$^+$ CD27$^-$ subset is as multifunctional as the CD45RA$^-$ CD27$^+$ and CD45RA$^-$ CD27$^-$ CD4$^+$ T-cell subsets, indicating that they are not an exhausted population. In addition, CD45RA$^+$ CD27$^-$ CD4$^+$ T cells have cytotoxic potential as they express high levels of granzyme B and perforin. CD4$^+$ memory T cells re-expressing CD45RA can be generated from the CD45RA$^-$ CD27$^+$ population by the addition of IL-7 and during this process these cells down-regulated expression of IL-7R and Bcl-2 and so resemble their counterparts *in vivo*. Finally we showed that the proportion of CD45RA$^+$ CD27$^-$ CD4$^+$ T cells of multiple specificities was significantly higher in the bone marrow than the blood of the same individuals, suggesting that this may be a site where these cells are generated.

Keywords: ageing; CD4 T cells; CD45RA; CMV; IL-7

Introduction

The function of the immune system declines with age leading to increased susceptibility to infectious diseases and poor responses to vaccination.[1] With the demographic shift towards an older age in many countries it is of increasing importance to understand the nature of the dysfunctional immunity in older subjects.[2] This information will provide information on possible strategies for intervention to boost immunity during ageing.

The immune dysfunction in older humans is partly the result of thymic involution, which restricts the production of naive T cells in older individuals, compromising their ability to respond to new antigens.[3] In addition, memory T cells, especially those that are specific for antigens that are encountered frequently, are driven to differentiate continuously towards an end-stage, marked by poor survival, telomere erosion, replicative senescence[3] and functional exhaustion.[4] This may result in 'holes' in the T-cell repertoire as T cells that are specific for certain antigens

are lost, which in turn may make older humans susceptible to certain infectious agents.[2] However, instead of the potential loss of specific T cells through replicative senescence, immune dysfunction during ageing may also arise from accumulation of certain T-cell populations. Longitudinal studies have defined a cluster of immune parameters in healthy older individuals, which are predictive of significantly decreased 2-year and 4-year survival of subjects over 80 years of age (reviewed in Derhovanessian *et al.*[5]). These parameters include a CD4 : CD8 ratio of < 1, which is the result of clonal expansion of highly differentiated $CD8^+$ $CD28^-$ T cells, cytomegalovirus (CMV) seropositivity and elevated levels of pro-inflammatory cytokines in the serum.[5] Furthermore, a large proportion of the expanded $CD8^+$ T cells in older subjects may be CMV-specific.[6–8] Therefore, although CMV infection is harmless to healthy young individuals, infection with this virus may have a previously unappreciated role in immune dysfunction during ageing, which is associated with the accumulation of CMV-specific T cells. This suggests that CMV infection may induce the accumulation of $CD8^+$ effector T cells that hinder the function of other memory T-cell populations.[8] This possibility is indirectly supported by data in mice indicating that expanded T-cell clones reduce T-cell diversity and inhibit the function of non-clonal cells *in vivo*[9] and that there is a negative effect of CMV infection on the size and function of Epstein–Barr virus-specific T-cell populations in humans.[10]

There have been many studies on the CMV-specific $CD8^+$ T-cell population,[6,11–13] but less is known about the characteristics of CMV-specific $CD4^+$ T cells and the impact that CMV infection has in shaping the $CD4^+$ T-cell pool in infected healthy humans.[14–16] Progressive stages in T-cell differentiation can be identified by sequential changes of expression of surface receptors such as CD45RA, CD28, CD27 and CCR7.[8,17] The most differentiated T cells in both the $CD8^+$ and $CD4^+$ populations are $CD28^-$ $CD27^-$ $CCR7^-$.[17] It has been shown that CMV-specific $CD8^+$ T cells are more differentiated phenotypically than those that are specific for other persistent viruses.[6] A proportion of these highly differentiated T cells can re-express CD45RA, a marker that was considered to identify unprimed T cells.[18–20] The $CD8^+$ $CD45RA^+$ $CD27^-$ T-cell population is expanded in CMV-infected individuals and although some reports suggest that these cells are terminally differentiated,[21–23] other studies indicate that these cells can be re-activated to exhibit potent functional responses.[24,25] Some studies have shown that $CD45RA^+$ $CD27^-$ $CD4^+$ T cells increase during ageing and in some autoimmune diseases,[26,27] but it is currently not clear whether CMV infection has an impact on their generation and whether these cells are functionally competent.

In this study we show that CMV infection significantly increases the proportion of $CD45RA^-$ $CD27^-$ and $CD45RA^+$ $CD27^-$ effector memory-like $CD4^+$ T cells in older humans. Furthermore, $CD45RA^+$ $CD27^-$ $CD4^+$ T cells were found to be multifunctional but potentially short lived after activation and may arise through interleukin-7 (IL-7) -mediated homeostatic proliferation, possibly in the bone marrow. These results suggest the possible involvement of homeostatic cytokines in the CMV infection-induced expansion of $CD45RA^+$ $CD27^-$ $CD4^+$ T cells during ageing.

Materials and methods

Volunteer sample collection and isolation

Heparinized peripheral blood was collected from young (mean age, 29 years; range, 20–39 years; $n = 67$), middle-aged (mean age, 51 years; range, 40–65 years; $n = 18$) and old (mean age, 80 years; range, 71–91 years; $n = 40$) donors, with approval from the Ethics Committee of the Royal Free Hospital. The old volunteers in this study were not treated with any immunosuppressive drugs and retained physical mobility and social independence. All donors provided written informed consent. Paired blood and bone marrow samples (mean age, 34 years; range, 21–57 years; $n = 18$) were obtained from healthy bone marrow donors by the Department of Haematology, University College Hospital London. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Hypaque density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden).

Cell culture

The $CD4^+$ T cells were purified by positive selection using the VARIOMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. In some experiments, $CD4^+$ T cells were further sorted into CD45RA/CD27 subsets using a FACSAria flow cytometer (BD Biosciences, San Jose, CA) after staining with CD45RA and CD27 antibodies for 30 min at 4° in PBS containing 1% BSA (Sigma-Aldrich, Gillingham, UK). Cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 μg/ml gentamicin and 2 mM L-glutamine (all from Invitrogen, Eugene, OR) at 37° in a humidified 5% CO_2 incubator. Purified $CD4^+$ subsets were activated in the presence of anti-CD3 antibody (purified OKT3 0·5 μg/ml) and autologous PBMCs irradiated with 40 Gy gamma-radiation, as a source of multiple co-stimulatory ligands provided by B cells, dendritic cells and macrophages found in these populations.[28] In other experiments, cells were cultured in the presence of recombinant human (rh) IL-2 (5 ng/ml), IL-7 (10 ng/ml) or IL-15 (5 ng/ml) (all from R&D Systems, Minneapolis, MN). Cytokines were added at the beginning of the cell culture

and not replenished. These cells were harvested at different times for phenotypic and functional analyses.

Measurement of antigen-specific CD4+ T cells

The PBMCs were stimulated with 10 μg/ml of purified protein derivative (PPD; Statens Serum Institut, Copenhagen, Denmark), 1/50 dilution of varicella zoster virus (VZV) -infected cell lysate, 1/200 dilution of Epstein–Barr virus (EBV) -infected cell lysate or 1/50 dilution of herpes simplex virus (HSV) -infected cell lysate (all from Virusys, Taneytown, MD). A CMV-infected cell lysate (used at 1/10 dilution) was prepared by infecting human embryonic lung fibroblasts with the Towne strain of CMV (European Collection of Animal Cell Cultures) at a multiplicity of infection of 2. After 5 days, the infected cells were lysed by repeated freeze–thaw cycles. The PBMCs were left unstimulated or stimulated with antigenic lysates for 15 hr at 37° in a humidified CO_2 atmosphere, with 5 μg/ml brefeldin A (Sigma-Aldrich) added after 2 hr. The cells were surface stained with peridinin chlorophyll protein-conjugated (-PerCP) CD4, phycoerythrin-conjugated (-PE) CD27 and phycoerythrin-Cy7-conjugated CD45RA (BD Biosciences) on ice. After being fixed and permeabilized (Fix & Perm Cell Permeabilization kit; Caltag Laboratories, Buckingham, UK), cells were stained with allophycocyanin-conjugated (-APC) interferon-γ (IFN-γ). Samples were acquired on an LSR I flow cytometer (BD Biosciences). For bone marrow experiments, paired peripheral blood and bone marrow samples were stimulated and analysed in parallel.

Flow cytometric analysis of cell phenotype

Cells resuspended in PBS containing 1% BSA and 0·1% sodium azide (Sigma-Aldrich) were stained for 10 min at room temperature with the following anti-human monoclonal antibodies: CD45RA-FITC (clone HI100; BD Pharmingen, San Diego, CA) or CD45RA-APC (clone MEM-56; Caltag); CD45RO-PE (clone UCHL1); CD4-PerCP (clone SK3); CD27-PE (clone M-T271); CD28-FITC (clone CD28·2); CD127-PE (clone hIL-7R-M21); CCR7-PE-Cy7 (clone 3D12) (all from BD Pharmingen); CD57-PE (clone TB03, Miltenyi Biotec). Intracellular staining for Granzyme B-PE (clone GB11; eBioscience, San Diego, CA), perforin-FITC (clone δG9; BD Pharmingen), Bcl-2-FITC (clone 124; Dako, Glostrup, Denmark) and Ki67-FITC (clone B56; BD Biosciences) was performed using the Foxp3 Staining Buffer Set (Miltenyi Biotec) according to the manufacturer's instructions. Proliferation was assessed by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay. Cells were labelled with 0·5 μM CFSE (Molecular Probes-Invitrogen, Carlsbad, CA) at 37° for 15 min in the dark, quenched with ice-cold culture medium at 4° for 5 min, and washed three times before culture in the presence of 50 ng/ml IL-7. Apoptosis was assessed using an annexin V/propidium iodide (PI) detection kit (BD Biosciences). Samples were acquired on a BD FACSCalibur 2 flow cytometer (BD Biosciences) after fixation with 1% formaldehyde (Sigma-Aldrich). Data were analysed using FlowJo software (TreeStar, Ashland, OR).

Intracellular cytokine analysis using polychromatic flow cytometry

The PBMCs (2×10^6 cells/ml) were stimulated with anti-CD3 (purified OKT3 0·5 μg/ml) for 2 hr at 37°. Unstimulated samples were incubated with equivalent amounts of PBS (negative control). After the addition of brefeldin A (10 μg/ml; Sigma), samples were incubated for another 14 hr. Cells were then incubated with 2 mM EDTA at room temperature for 10 min, washed in PBS/BSA/Azide and stained for 30 min at 4° with the following surface antibodies: CD4-PerCP (clone SK3), CD8-APC-H7 (clone SK1), CD27-PE (clone L128), CD16-FITC (clone 3G8), CD56-FITC (clone NCAM16.2) (all from BD Biosciences), CD45RA Energy Coupled Dye (ECD, clone MB1; IqProducts, Groningen, The Netherlands), CD3 Quantum Dot 605 (QDot605, clone UCHT1; Invitrogen), live/dead fixable Aqua stain (Invitrogen). After washing, lysing and permeabilizing according to the manufacturer's instructions (Perm 2 and Lysis; BD Biosciences), cells were stained intracellularly for 30 min at 4° with the following antibodies: IL-2-APC (clone 5344.111), IFN-γ-PE-Cy7 (clone B27), tumour necrosis factor-α (TNF-α) -Alexa Fluor 700 (clone MAb1) (all from BD Biosciences), CD40L Pacific Blue (clone 24-31; Biolegend, San Diego, CA). Samples were acquired on a BD LSR II flow cytometer (BD Biosciences). Data were analysed using FlowJo software (TreeStar) and Pestle and Spice (kindly donated by M. Roederer).

Akt (Ser473) phosphorylation analysis by flow cytometry

After resting the PBMCs overnight in RPMI-1640 (Sigma-Aldrich) with 1% human AB serum (Sigma-Aldrich), they were starved in serum-free RPMI-1640 for 2 hr before stimulation to reduce phosphorylation background. Following surface staining with CD45RA-FITC, CD27-APC (clone O323; eBioscience) and CD4-PE-Cy7 (clone SK3; BD Pharmingen) cells were activated with anti-CD3 (purified OKT3, 1 μg/ml) on ice for 20 min. Primary monoclonal antibodies were cross-linked with anti-mouse IgG $F(ab')_2$ (20 μg/ml; Jackson ImmunoResearch, West Grove, PA) by incubating on ice for 20 min. Cells were then stimulated at 37° for 5 min. The unstimulated control cells underwent the same manipulations but without the addition of αCD3 and cross-linker. Activation was arrested by fixing the cells with warm Cytofix Buffer (BD Biosciences) at 37° for 10 min. Cells were then permeabilized with ice--

cold Perm Buffer III (BD Biosciences) at 4° for 30 min and incubated with PE mouse anti-Akt (pS473) (BD Biosciences) for 30 min at room temperature. Cells were washed in stain buffer (BD Pharmingen) and acquired on a BD FACS Calibur 2 flow-cytometer (BD Biosciences) and analysed using FlowJo software (TreeStar).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA) and $P < 0{\cdot}05$ was considered significant. Multiple linear regression was performed using PaswStatistics 18.0 (IBM-SPSS, Chicago, IL).

Results

Persistent CMV infection, but not age itself, correlates with the increase of CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T cells

Age and CMV infection have been shown to profoundly affect the overall composition of the CD8$^+$ T-cell compartment.[12] We found that the frequency of CD45RA$^+$ CD27$^+$ (naive) CD4$^+$ T cells significantly decreased with age (Fig. 1a,b; $P = 0{\cdot}0003$) whereas the frequencies of all the primed/memory subsets significantly increased with age: CD45RA$^-$ CD27$^+$ ($P = 0{\cdot}0033$), CD45RA$^-$ CD27$^-$ ($P = 0{\cdot}0321$), CD45RA$^+$ CD27$^-$ ($P = 0{\cdot}0315$). However, this analysis does not take into account the individual contribution of ageing and CMV infection in shaping the CD4$^+$ T-cell compartment. An earlier study showed that CMV infection is associated with the accumulation of highly differentiated CD4$^+$ T cells.[16] Here we extend these observations by further discriminating between highly differentiated CD4$^+$ T cells in the basis of CD45RA re-expression. We analysed the results in two ways. First, we divided the subjects into young (< 40 years) and old (> 60 years) groups and further subdivided these individuals on the basis of their CMV seropositive or negative status (Fig. 1c). Second, we performed multiple linear regression analysis to examine more closely the impact of aging and CMV in determining the T-cell subset composition during ageing.

The percentage of CD45RA$^+$ CD27$^+$ (naive) CD4$^+$ T cells decreased with age; this decrease was significant in CMV-positive ($P = 0{\cdot}003$) but not in CMV-negative donors as assessed by the Mann–Whitney *U*-test. However, when we analysed the data using multiple linear regression analysis (see Supplementary Information, Table S1) we found that age and CMV both induce a significant decrease of the CD45RA$^+$ CD27$^+$ CD4$^+$ T-cell compartment ($P < 0{\cdot}001$ and $P < 0{\cdot}045$, respectively) but age alone seems to be the main factor modulating the increased CD45RA$^-$ CD27$^+$ subset.

The frequencies of CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ subsets were significantly higher in CMV-infected donors in both young and old age groups (Fig. 1c). Furthermore, old CMV-positive donors had significantly higher proportions of these cells compared with young seropositive subjects as assessed by the Mann–Whitney *U*-test (Fig. 1c, lower panels). When the results were analysed by multiple linear regression analysis there was a highly significant impact of CMV infection on the increase of both these populations during ageing ($P < 0{\cdot}0001$ in both cases) but age itself did not have a significant role in the accumulation of these subsets (see Supplementary Information, Table S1). In conclusion, age and CMV serostatus both contribute to the decrease in CD45RA$^+$ CD27$^+$ CD4$^+$ T cells during ageing but the increase in CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ T cells in old individuals is primarily the result of CMV infection.

Identification of virus-specific CD4$^+$ T-cell populations in healthy donors of different ages

We next investigated whether the increase in CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ cells in CMV-seropositive donors only occurred within CMV-specific CD4$^+$ T cells or also in those that are specific for different persistent viruses. To do this, we first identified virus-specific populations by intracellular IFN-γ staining after stimulation with lysates of virus-infected cells for 18 hr (see Supplementary Information, Fig. S1a).[15] Background responses detected in unstimulated cells (negative control) were subtracted from those detected in stimulated samples. Only responses > 0·02% above background were considered positive. The IFN-γ secretion after stimulation with viral lysates was specific because no cytokine production was observed when CMV lysate was used to stimulate CD4$^+$ T cells from CMV-seronegative donors as described previously.[15] We found that in CMV-seropositive donors, there was a significantly higher proportion of CMV-specific CD4$^+$ T cells compared with T cells that were specific for other persistent viruses such as VZV, HSV EBV or mycobacterial antigens (tuberculin PPD) (see Supplementary Information, Fig. S1b).

We next investigated whether the increased proportion of CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T cells in CMV-seropositive donors (Fig. 1c) was only the result of changes within the CMV-specific T-cell population. We found that there were significantly more CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T cells in CMV-seropositive donors compared with CMV-seronegative donors (Fig. 2a,b). However, although the majority of CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T cells in CMV-seropositive donors were CMV-specific, there was also a higher proportion of CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T cells specific for the other viruses in CMV-seropositive subjects (Fig. 2b,c). Similar results were observed in both young and old donors (data not shown).

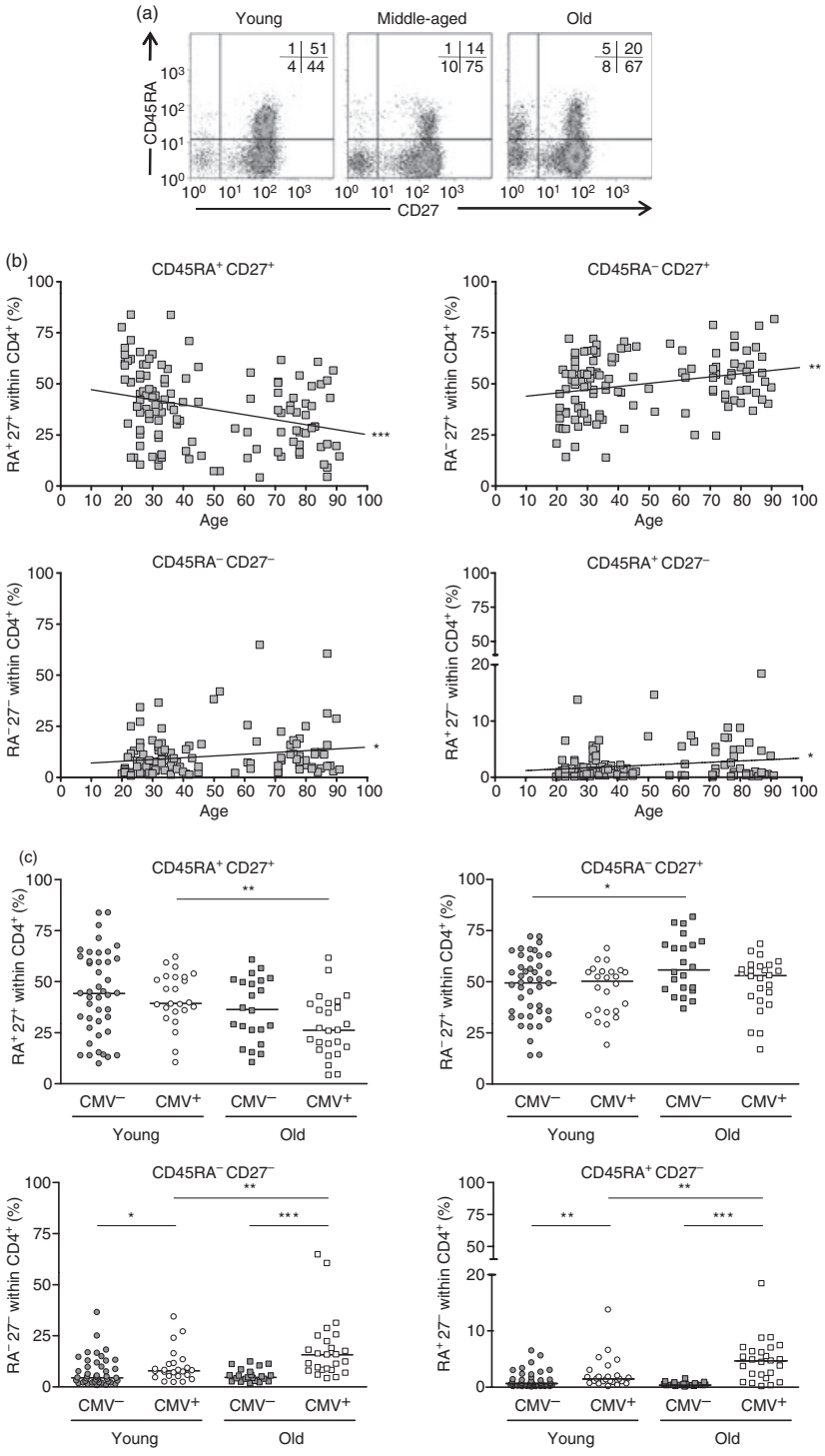

Figure 1. The inflation of CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T-cell subsets is the result of cytomegalovirus (CMV) exposure and not of age itself. (a) Phenotypic analysis of CD45RA/CD27 expression on young, middle-aged and old CD4$^+$ T cells. Peripheral blood mononuclear cells stained for CD4, CD45RA and CD27 were analysed by flow cytometry. Representative pseudocolour plots for each age-group are shown. (b) Frequencies of each of the CD45RA/CD27 populations within total CD4$^+$ T cells are represented in correlation to the age of the donors. Line of best fit was generated by linear regression and the correlation assessed by Pearson and Spearman rank (GraphPad Prism): CD45RA$^+$ CD27$^+$ (r = −0·3154, P = 0·0003), CD45RA$^-$ CD27$^+$ (r = 0·2620, P = 0·0033), CD45RA$^-$ CD27$^-$ (r = 0·1918, P = 0·0321), CD45RA$^+$ CD27$^-$ (r = 0·1924, P = 0·0315). (c) Frequencies of each of the CD45RA/CD27 populations within total CD4$^+$ T cells are represented by grouping via age (young, < 40 years; old, > 60 years) and CMV status. Horizontal lines depict median values. Statistical analysis was performed using the Mann–Whitney U-test (GraphPad Prism). * indicates P value ≤ 0·05; ** indicates P value ≤ 0·001; *** indicates P value ≤ 0·0001.

This result reinforces the idea that CMV infection influences directly the composition of the CD4$^+$ T-cell pools. Furthermore, our results indicate that CMV infection may have a global effect on driving the differentiation of other antigen-specific CD4$^+$ T cells. This confirms our previous observations where the relative expression of CD28 and CD27 instead of CD45RA and CD27 was used to identify CD4$^+$ T cells at different stages of differentiation.[15]

Highly differentiated CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T-cell subsets are multifunctional

Several reports on CD8$^+$ T cells suggest that the CD45RA$^+$ CD27$^-$ subset is terminally differentiated[17,22] with limited capacity for self-renewal. To date, few data are available on CD4$^+$ CD45RA$^+$ CD27$^-$ T cells in healthy

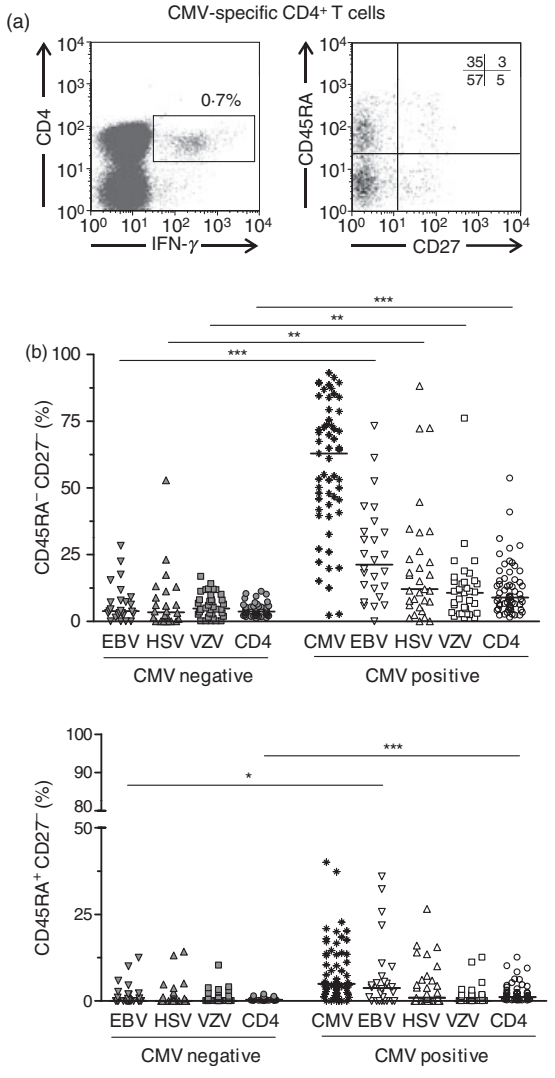

Figure 2. Frequency of CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ subsets within virus-specific cells in cytomegalovirus seronegative (CMV$^-$) and CMV$^+$ individuals. Peripheral blood mononuclear cells were stimulated with CMV, Epstein–Barr virus (EBV), herpes simplex virus (HSV) or varicella zoster virus (VZV) lysates and the phenotype of the antigen-specific CD4$^+$ T cells was assessed by flow cytometry after staining with CD4, CD45RA, CD27 and interferon-γ (IFN-γ) antibodies. Only responses $> 0{\cdot}02\%$ above background (unstimulated cells) were considered positive. The CD45RA/CD27 profile of CMV-specific CD4$^+$ T cells (CD4$^+$ IFN-γ^+) from a representative donor is shown (a). (b) The percentage of antigen-specific CD4$^+$ T cells with a CD45RA$^-$ CD27$^-$ or CD45RA$^+$ CD27$^-$ phenotype was assessed in CMV$^+$ and CMV$^-$ individuals. Horizontal lines depict median values. Statistical analysis was performed using the Mann–Whitney *U*-test (GraphPad Prism).

donors. To determine the functional characteristics of the increased CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T-cell populations in CMV-seropositive subjects we first examined their surface expression of markers that were previously shown to be associated with migration (CCR7), co-stimulation (CD28), responsiveness to cytokines (IL7-Rα) and end-stage differentiation (CD57). We found that CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T cells both showed low CCR7, CD28 and IL-7Rα but higher CD57 expression compared with naive CD45RA$^+$ CD27$^+$ and CD45RA$^-$ CD27$^+$ populations indicating that they were more differentiated (Fig. 3a). In addition, on the basis of CD28, IL-7Rα and CD57 expression, the CD45RA$^+$ CD27$^-$ subset was significantly more differentiated than the CD45RA$^-$ CD27$^-$ population (Fig. 3a).

We next investigated the functional properties of the CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ subsets of CD4$^+$ T cells. We showed that the expression of molecules associated with cytolytic potential such as granzyme B and perforin were not detectable in naïve CD45RA$^+$ CD27$^+$ and CD45RA$^-$ CD27$^+$ CD4$^+$ T cells (Fig. 3b). In contrast, both CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T cells expressed granzyme B and perforin, the levels of which were significantly higher in CD45RA$^+$ CD27$^-$ cells when these populations were compared (Fig. 3b). Other indicators of CD4$^+$ T-cell functionality include production of cytokines such as IFN-γ, IL-2 and TNF-α, and the expression of the CD40 ligand. The co-expression of more than one function in individual cells may be associated with enhanced viral control.[29] We therefore performed multiparameter flow cytometric analysis to identify simultaneously the relative expression of IFN-γ, IL-2, TNF-α and CD40 ligand in individual CD4$^+$ T cells at different stages of differentiation defined by relative expression of CD45RA and CD27 (Fig. 3c; see Supplementary Information, Fig. S2 and Table S2).

The CD45RA$^-$ CD27$^+$, CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ subsets contained more cells with three and four functions compared with the CD45RA$^+$ CD27$^+$ CD4$^+$ naive T-cell population (functions expressed are detailed in Supplementary Information, Table S2). These differences were highly significant (Wilcoxon matched pairs test; for all comparisons naive versus other subsets $P < 0{\cdot}0001$; Fig. 3c). Both CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T cells showed equivalent multifunctionality (P = ns), which was higher than in the CD45RA$^-$ CD27$^+$ and naive CD45RA$^+$ CD27$^+$ CD4$^+$ T-cell populations ($P < 0{\cdot}01$). This indicates that although CD45RA$^+$ CD27$^-$ CD4$^+$ T cells bear phenotypic characteristics of highly differentiated T cells, they are not exhausted functionally but instead are capable of potent effector function. We found no evidence for a decreased functionality of CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T cells when we compared old with young donors after activation with a polyclonal T-cell stimulus (anti-CD3 antibody); these populations were equally multifunctional in both groups of subjects (Mann–Whitney *U*-test, data not shown).

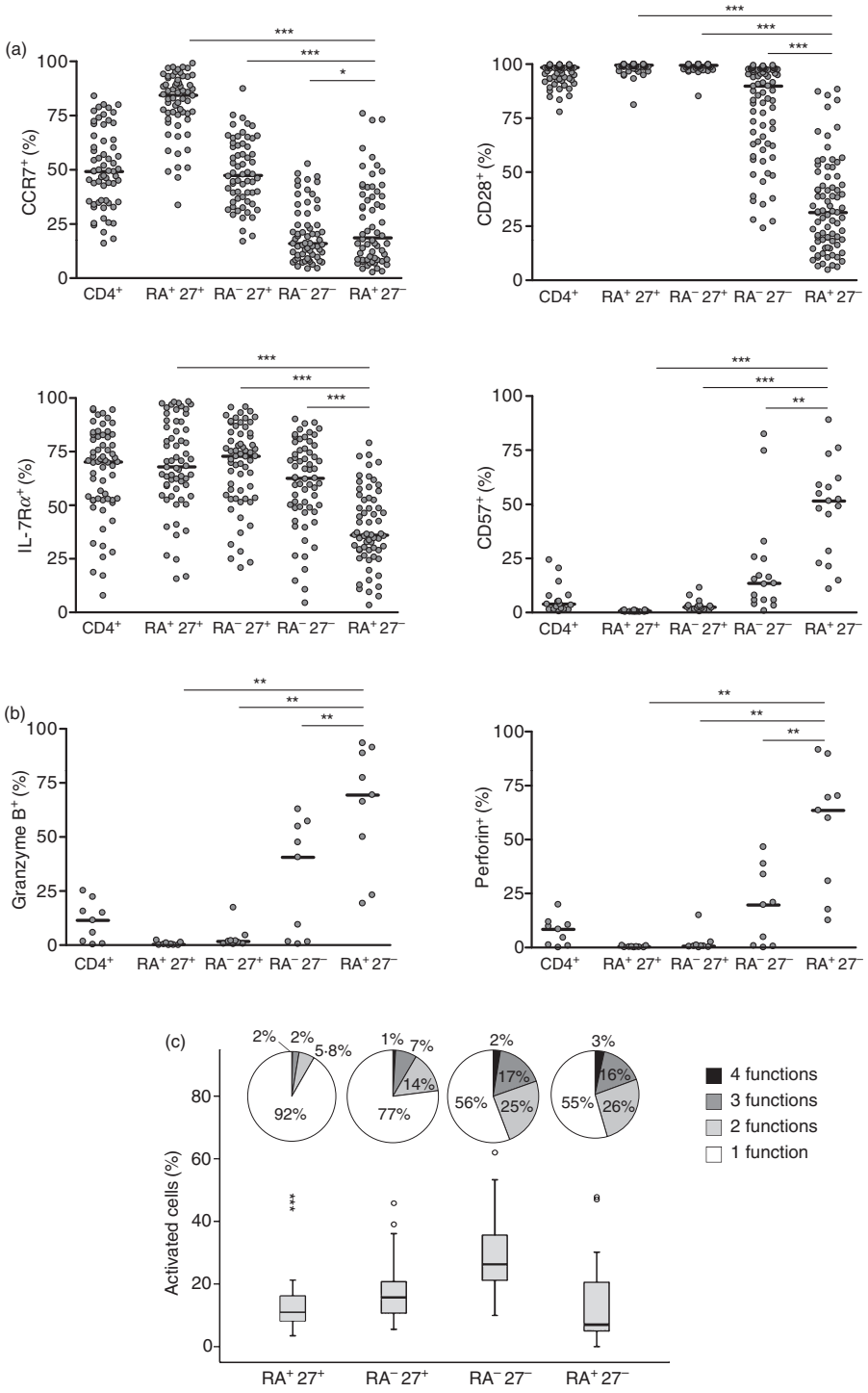

Figure 3. CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ CD4$^+$ T cells have a terminally differentiated phenotype but are multifunctional. Peripheral blood mononuclear cells (PBMCs) were stained *ex vivo* and analysed by flow cytometry. The percentage of cells expressing phenotypic (a) and functional (b) markers was determined by gating within total CD4$^+$ cells and within each of the CD45RA/CD27 subsets. Horizontal lines depict median values. Statistical analysis was performed using the Wilcoxon matched pairs test (GraphPad Prism). CD40 ligand (CD40L), interferon-γ (IFN-γ), interleukin-2 (IL-2) and tumour necrosis factor-α (TNF-α) expression by CD4$^+$ CD45RA/CD27 subsets was assessed using multiparametric flow cytometry following stimulation of PBMCs with anti-CD3 in the presence of brefeldin A for 16 hr (c). Frequency of all reacting cells within each CD45RA/CD27 subset is represented by box-plots (n = 25). Reacting cells include those that express 1, 2, 3 or 4 of the analysed activation markers. Shown are median, interquartile range (IQR) (difference between the 75th and 25th percentiles), outlier and extreme values. The pie charts show the distribution of cells showing 1, 2, 3 or 4 functions within each subset.

Survival of CD45RA$^+$ CD27$^-$ CD4$^+$ T cells following activation

Beside the ability to secrete cytokines and express cytotoxic machinery, another critical element for T-cell-mediated immune protection is their ability to proliferate and survive after activation. We observed that after T-cell receptor stimulation *in vitro* CD45RA$^+$ CD27$^+$ and CD45RA$^-$ CD27$^+$ CD4$^+$ T-cell populations expanded more than CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ subsets during culture (Fig. 4a,b; see Supplementary Information, Fig. S3a). To understand the extent to which increased cell death, rather than reduced proliferation, contributes to the decline of the CD45RA$^+$ CD27$^-$ population after *in vitro* stimulation, we measured the rate of cell death by monitoring Annexin V staining and PI incorporation after activation (Fig. 4c,d). The analysis of early apoptotic (Annexin V$^+$ PI$^-$) and late apoptotic/necrotic (Annexin V$^+$ PI$^+$) cells in the different subsets at day 3 after activation showed that CD4$^+$ CD45RA$^+$ CD27$^-$ T cells are significantly more prone to cell death than all other subsets. A time–course of Annexin V staining and PI incorporation showed that by day 15 CD4$^+$ CD45RA$^+$ CD27$^-$ T cells are almost completely dead

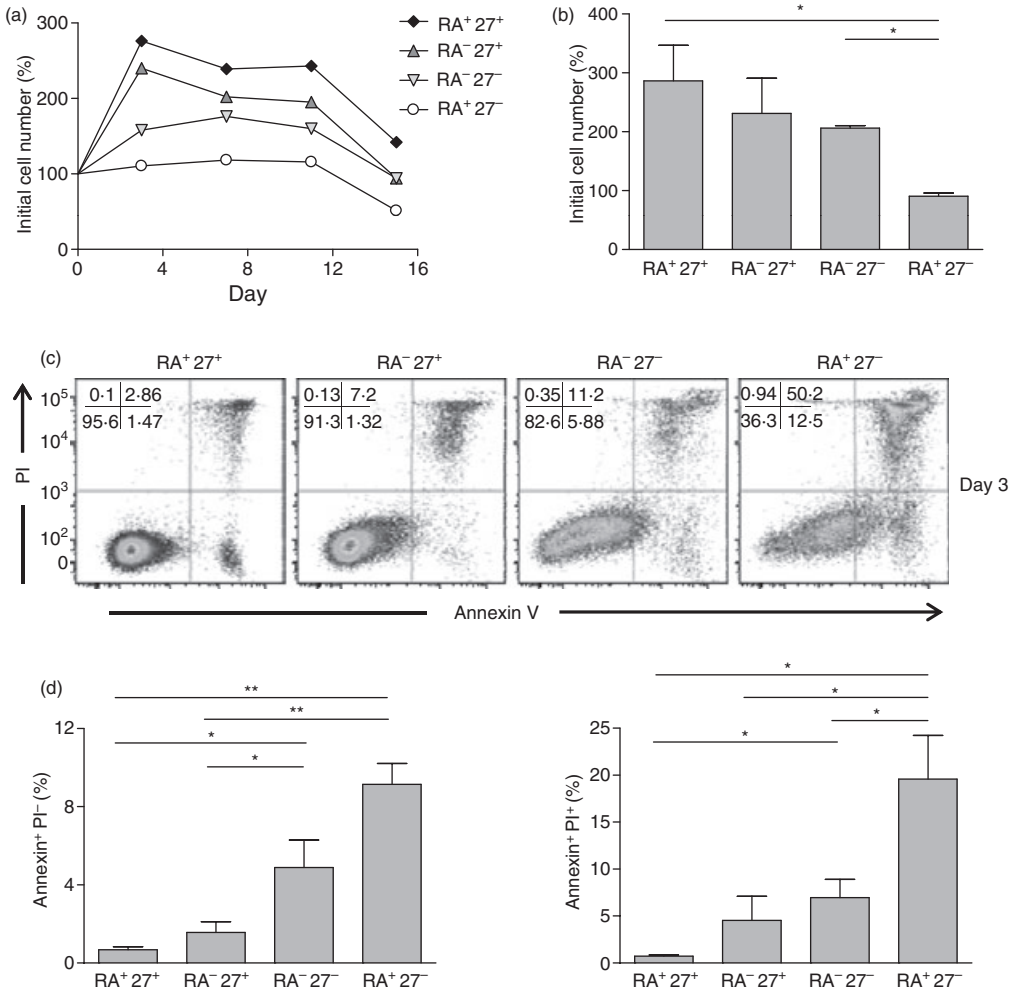

Figure 4. CD4$^+$ CD45RA$^+$ CD27$^-$ cells do not accumulate in culture following activation. (a) Purified CD45RA/CD27 CD4$^+$ T-cell subsets were activated with anti-CD3 and irradiated antigen-presenting cells. At the indicated time-points, the cell number was determined on a haemocytometer. Results are expressed as a percentage of the initial number of cells placed in culture; one representative experiment is shown (results from another donor is shown in supplementary information Fig. S3). (b) Bar graph represents cell recovery at day 3 after anti-CD3 and interleukin-2 (IL-2) activation. Error bars represent the SE from the mean of three separate experiments. Statistical analysis was performed using paired *t*-test (GraphPad Prism). (c,d) Apoptosis was assessed by Annexin V staining and propidium iodide (PI) incorporation. The percentage of early apoptotic (Annexin V$^+$ PI$^-$) and late apoptotic/necrotic (Annexin V$^+$ PI$^+$) cells was assessed after anti-CD3 and interleukin-2 (IL-2) activation on day 3. Representative pseudocolour plots are shown (c). (d) Bar graph represents early apoptotic (left panel) and late apoptotic/necrotic cells (right panel) at day 3 after anti-CD3 and IL-2 activation. Error bars represent the SE from the mean of four separate experiments. Statistical analysis was performed using paired *t*-test.

when all other subsets are still present in culture (see Supplementary Information, Fig. S3c).

To explore the possibility that pro-survival pathways are defective in CD45RA$^+$ CD27$^-$ CD4$^+$ T cells, which makes them susceptible to apoptosis, we investigated the expression of the anti-apoptotic protein Bcl-2, measured by intracellular staining of CD4$^+$ T-cell subsets directly *ex vivo* (Fig. 5a).[30] We found that Bcl-2 expression is significantly lower in CD45RA$^+$ CD27$^-$ CD4$^+$ T cells compared with all the other subsets ($P < 0{\cdot}0001$). A critical role in promoting cell survival is also ascribed to Akt, which operates by blocking the function of pro-apoptotic proteins and processes.[28,31] Akt is phosphorylated at two sites – serine 473 and threonine 308. We previously showed that there is defective phosphorylation of Akt (ser473) but not Akt(thr308) in highly differentiated CD8$^+$ T cells.[28,31] We now show that there is a decrease in pAkt(ser473) from CD45RA$^+$ CD27$^+$ (naive), CD45RA$^-$ CD27$^+$, CD45RA$^-$ CD27$^-$ and CD45RA$^+$ CD27$^-$ subsets, respectively (Fig. 5b). Therefore CD45RA$^+$ CD27$^-$ CD4$^+$ T cells have potent effector function but have decreased capacity for survival after activation, associated with decreased Bcl-2 expression and Akt(ser473) phosphorylation.

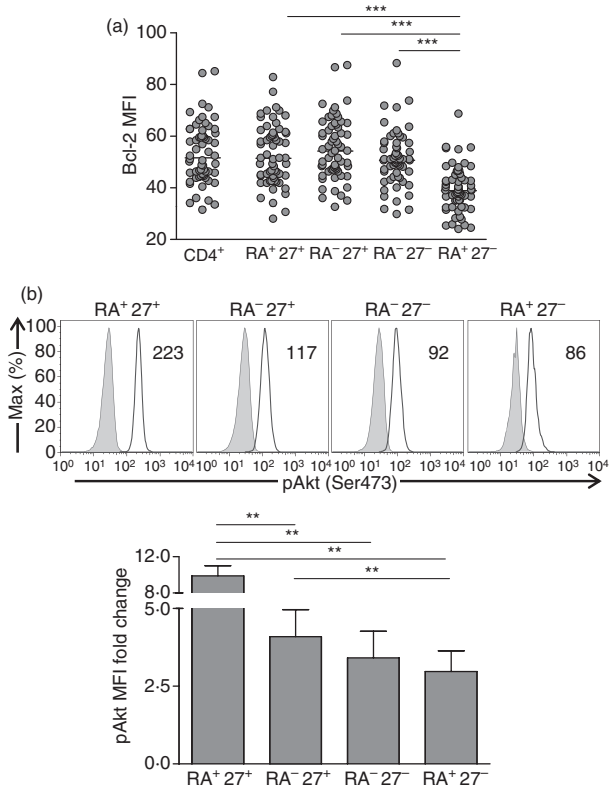

Figure 5. CD4$^+$ CD45RA$^+$ CD27$^-$ cells have altered survival signalling pathways. (a) Bcl-2 *ex vivo* mean fluorescence intensity was assessed in peripheral blood mononuclear cells (PBMCs) by gating within total CD4$^+$ T cells and within each of the CD45RA/CD27 subsets. Horizontal lines depict median values. Statistical analysis was performed using the Wilcoxon matched pairs test (GraphPad Prism). (b) Representative overlays of pAkt (Ser473) expression within CD4$^+$ CD45RA/CD27 subsets activated with anti-CD3 (solid line) and within unstimulated cells which underwent the same protocol in the absence of anti-CD3 (grey histogram) are shown. The values represent the median fluorescent intensity of pAkt (Ser473) within each subset following activation. Bar graph represents the fold change in pAkt(Ser473) mean fluorescence intensity (MFI) after activation relative to the MFI observed in unstimulated cells within the respective subset. Error bars represent the SE from the mean of five separate experiments. Statistical analysis was performed using paired *t*-test (GraphPad Prism).

CD4$^+$ memory T cells re-expressing CD45RA$^+$ derive from CD45RA$^-$ CD27$^+$ CD4$^+$ T cells by IL-7-driven homeostatic proliferation

Previous studies have shown that within CD8$^+$ T cells cytokines such as IL-15 that drive homeostatic proliferation also induce the generation of CD45RA$^+$ CD27$^-$ CD8$^+$ T cells.[21,32,33] Although the presence CD4$^+$ CD45RA$^+$ CD27$^-$ T cells has been described previously[26] the mechanism by which they are induced is not known. We showed previously that IL-7 can induce the proliferation of CD4$^+$ CD45RA$^+$ (naive) T cells without inducing CD45RO expression,[34] which was subsequently supported by other studies.[35] We therefore investigated whether this cytokine could induce CD45RA re-expression in CD45RA$^-$ CD27$^+$ or CD45RA$^-$ CD27$^-$ CD4$^+$ T cells. These cells were isolated by cell sorting then cultured in the presence of IL-2, IL-7 or IL-15 without T-cell receptor stimulation (Fig. 6; see Supplementary Information, Figs S4 and S5). After 6 days, a population re-expressing CD45RA and down-modulating CD45RO emerged from the CD45RA$^-$ CD27$^+$ cells cultured in the presence of IL-7 (Fig. 6a). T-cell receptor stimulation alone did not induce CD45RA re-expression and neither did a panel of cytokines including transforming growth factor-β, IL-10 and IFN-α (unpublished observations). We also performed a CFSE dilution assay on CD45RA$^-$ CD27$^+$ cells in the presence of IL-7 to assess whether CD45RA re-expression is accompanied by proliferation driven by IL-7. The CD45RA$^+$ cells that were generated *in vitro* from CD45RA$^-$ CD27$^+$ cells by IL-7 divided more than the cells that remained CD45RA$^-$ and CD45RO$^+$ in the same culture (Fig. 6b). Although a low level of CD45RA expression was observed in a small proportion of CD45RA$^-$ CD27$^+$ CD4$^+$ T cells that were cultured with IL-2 or IL-15 (see Supplementary Information, Fig. S4), this was considerably lower than that induced by IL-7 (Fig. 6a). The relatively weak effect of IL-15 on the induction of CD45RA in CD45RA$^-$ CD27$^+$ cells was not enhanced by a higher dose (10 ng/ml) of this cytokine (data not shown).

The CD45RA$^-$ CD27$^-$ subset cultured in the same experimental conditions did respond to IL-7 in terms of

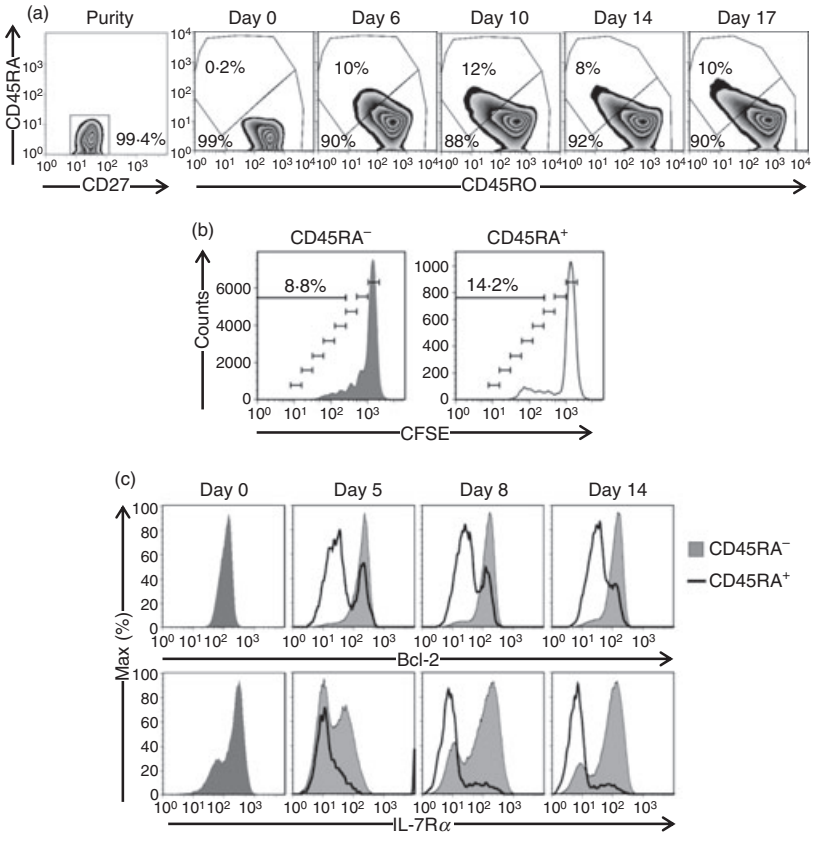

Figure 6. $CD4^+$ $CD45RA^-$ $CD27^+$ cells stably re-express CD45RA following interleukin-7 (IL-7) -driven proliferation. $CD4^+$ $CD45RA^-$ $CD27^+$ cells were purified by FACS sorting and analysed for the expression of CD45RA and CD45RO before culture. (a) Cells were stimulated with IL-7 and CD45RA/CD45RO expression was assessed by flow cytometry at the indicated time-points. The results shown are representative of 12 experiments. (b) Carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution was assessed in the cells that re-expressed CD45RA and in the population that remained $CD45RA^-$ following 14 days of culture in the presence of IL-7. Values represent the percentage of cells that underwent more than two rounds of cell division. (c) Overlays represent Bcl-2 and IL-7Rα expression before and during culture in the presence of IL-7 within $CD45RA^+$ cells (solid line) and $CD45RA^-$ cells (grey histogram). Histograms from a representative experiment out of three performed are shown.

survival (data not shown) but did not re-express CD45RA and remained $CD45RO^+$ throughout the culture period (see Supplementary Information, Fig. S5). These results suggest that IL-7-driven homeostatic proliferation can induce the re-expression of CD45RA in $CD45RA^-$ $CD27^+$ $CD4^+$ T cells but cannot induce the $CD45RA^-$ $CD27^-$ population to form the $CD45RA^+$ memory population. We next determined whether the memory $CD45RA^+$ cells that were generated *in vitro* resembled phenotypically those that are found *in vivo*. To do this we monitored the expression of CD27, Bcl-2 and IL-7Rα after different time-points of IL-7 treatment of $CD45RA^-$ $CD27^+$ $CD4^+$ T cells *in vitro*. The population that remained $CD45RA^-$ $CD45RO^+$ expressed homogeneously high levels of Bcl-2 and IL-7Rα throughout the culture period (Fig. 6c), except for the initial down-regulation of IL-7Rα (visible at day 5). In contrast the population of $CD45RA^+$ cells that emerged down-regulated both Bcl-2 and IL7-Rα over time (Fig. 6c). Interleukin-7 stimulation of $CD45RA^-$ $CD27^+$ $CD4^+$ T cells results in the generation of a population with heterogeneous expression of CD27. However, a small percentage of the CD45RA re-expressing cells are $CD27^-$ (see Supplementary Information, Fig. S6). As IL-7 induces CD45RA but not complete loss of CD27 in the timeframe of experimental protocol we acknowledge that other factors in addition to IL-7 may also be required for the generation of a $CD45RA^+$ $CD27^-$ T-cell population from $CD45RA^-$ $CD27^+$ cells.

Preferential localization of $CD45RA^+$ $CD27^-$ $CD4^+$ T cells in the bone marrow

All the results presented so far were performed using $CD4^+$ T cells from peripheral blood. The bone marrow has been known to be a source of IL-7 *in vivo*.[36] We therefore examined the possibility that there was preferential accumulation of $CD45RA^+$ $CD27^-$ $CD4^+$ T cells of a particular specificity in this lymphoid compartment. First we compared the distribution of $CD4^+$ CD45RA/CD27 subsets in paired blood and bone marrow samples from healthy donors and observed a significant increase in the percentage of $CD45RA^-$ $CD27^-$ and $CD45RA^+$ $CD27^-$ $CD4^+$ T cells in the bone marrow compared with the blood of the same individuals (Fig. 7a). We investigated next whether the specificity of T cells in the bone marrow was similar to that found in the blood of the same individuals (Fig. 7b). We found that the increased proportion of CMV-specific $CD4^+$ T cells relative to other populations was also observed in bone marrow samples, indicating that the inflation of CMV-specific T cells occurs in more than one lymphoid compartment *in vivo* (Fig. 7b). In addition, the proportion of CMV-, VZV- and EBV-specific $CD4^+$

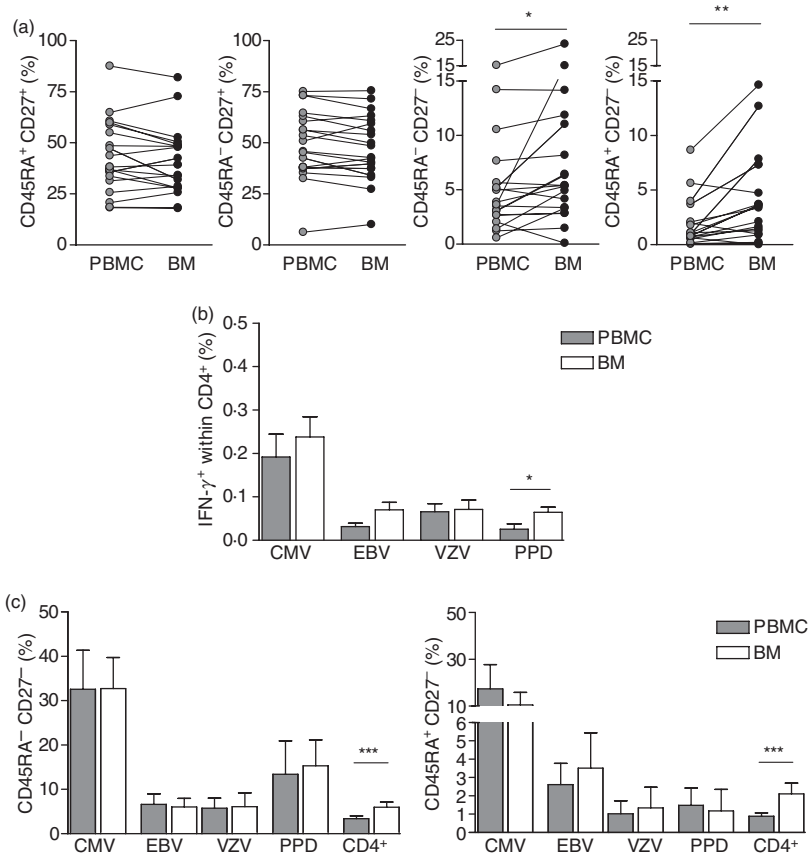

Figure 7. $CD4^+$ $CD45RA^+$ $CD27^-$ cells appear to accumulate in the bone marrow. (a) Phenotypic analysis of CD45RA/CD27 expression on paired peripheral blood mononuclear cell (PBMC) and bone marrow (BM) samples. Frequencies of each of the CD45RA/CD27 populations within total $CD4^+$ T cells are shown. (b,c) Paired PBMCs and BM cells were stimulated overnight with varicella zoster virus (VZV), cytomegalovirus (CMV) and Epstein–Barr virus (EBV) viral lysates or purified protein derivative (PPD) in the presence of brefeldin A and analysed by flow cytometry. Antigen-specific populations were identified by intracellular staining for interferon-γ (IFN-γ) production along with CD4, CD45RA and CD27 surface staining. (b) The frequency of $CD4^+$ T cells that were antigen-specific in PBMC and BM samples was determined in all donors ($n = 11$) with a positive response (> 0·02% once corrected for background). (c) The percentage of antigen specific $CD4^+$ T cells that displayed a $CD45RA^-$ $CD27^-$ or a $CD45RA^+$ $CD27^-$ phenotype was assessed in PBMCs and BM ($n = 15$). Statistical analysis was performed using the Wilcoxon matched pairs test (GraphPad Prism).

T cells was not significantly different between the two compartments. However, there were significantly more PPD-specific $CD4^+$ T cells in the bone marrow compared with the peripheral blood from the same donors, although the significance of this is not clear at present.

We next investigated whether there was preferential accumulation of $CD45RA^-$ $CD27^-$ and $CD45RA^+$ $CD27^-$ $CD4^+$ T cells of a particular specificity in the bone marrow. We found that the proportion of CMV-, VZV-, EBV- and PPD-specific populations in the bone marrow that were $CD45RA^-$ $CD27^-$ and $CD45RA^+$ $CD27^-$ was not different to that in the blood of the same individuals (Fig. 7c). Therefore it appears that $CD45RA^-$ $CD27^-$ and $CD45RA^+$ $CD27^-$ T cells of all specificities have equal propensity to accumulate in the bone marrow and that it is not a unique site for the generation of CMV-specific effector/memory $CD4^+$ T cells.

Discussion

In this study we show that whereas persistent CMV infection is mainly responsible for the increase of $CD45RA^-$ $CD27^-$ and $CD45RA^+$ $CD27^-$ $CD4^+$ T cells in older subjects, both ageing as well as CMV infection contribute to the decrease of $CD45RA^+$ $CD27^+$ $CD4^+$ T cells. This latter observation may reflect the impact of thymic involution compounded with persistent CMV infection during ageing.[1] The majority of $CD45RA^-$ $CD27^-$ and $CD45RA^+$ $CD27^-$ populations in CMV-infected subjects are CMV-specific but there are also increased numbers of these effector $CD4^+$ cells that are specific for other viruses, i.e. EBV, HSV and VZV. This suggests that CMV infection may drive a global increase in $CD4^+$ T-cell differentiation suggesting a bystander phenomenon. However, we cannot rule out the possibility that some people are particularly susceptible to the reactivation of latent viruses in general, CMV included. The bystander effect may be mediated in part by IFN-α that is secreted by CMV-stimulated plasmacytotoid dendritic cells as a result of toll-like receptor stimulation[15] or by TNF-α.[26] IFN-α and TNF-α have been shown to accelerate the loss of CD27 and CD28 in both $CD4^+$[15,37,38] and $CD8^+$[39] T cells in humans. However, the induction of IFN-α may also lead to the secondary secretion of other cytokines such as IL-15,[40,41] which may induce homeostatic proliferation and CD45RA re-expression during CMV-specific $CD8^+$ T-cell activation.[20,42–44] It is currently not known whether IFN-α can also induce IL-7 secretion by leucocytes or stromal cells but this is under investigation. These observations suggest that the accumulation of highly differentiated $CD45RA^-$ $CD27^-$ and $CD45RA^+$ $CD27^-$ $CD4^+$ T cells in CMV-infected individuals may be related in part to the

cytokines that are secreted either as a direct or indirect consequence of CMV re-activation *in vivo*.

There has been controversy about the extent to which CMV re-activation occurs in seropositive individuals. Earlier studies did not find increased CMV DNA in the blood of older humans.[45] However, a recent study confirmed that while CMV viral DNA is undetectable in the blood of healthy old volunteers, it is significantly increased in the urine of these individuals compared with a younger cohort of CMV-seropositive subjects.[46] This indicates that the ability to control CMV re-activation may be compromised during ageing and that this may lead to increased activation of CMV-specific T cells in older subjects.[46] Therefore, the increased CMV-specific T-cell re-activation together with secretion of differentiation-inducing cytokines such as IFN-α,[15,37,39] may culminate in the highly differentiated memory T-cell repertoire that is found in older CMV-infected humans.

Previous reports on $CD8^+$ T cells that re-express CD45RA have described them as terminally differentiated and exhausted.[21,22] However, we and others have shown that $CD45RA^+$ $CD27^-$ $CD8^+$ T cells can be re-activated to proliferate and exhibit effector functions *in vitro*,[20,25,32] indicating that they are functional and retain replicative potential and are an important memory subset.[47] We now extend these observations by showing that the same applies to $CD45RA^+$ $CD27^-$ cells within the $CD4^+$ T-cell population that secrete multiple cytokines as efficiently as the $CD45RA^-$ $CD27^-$ population and more efficiently than the naive $CD45RA^+$ $CD27^+$ and $CD45RA^-$ $CD27^+$ subsets after T-cell receptor activation. In addition, the $CD45RA^+$ $CD27^-$ and $CD45RA^-$ $CD27^-$ $CD4^+$ T-cell populations that accumulate in CMV-seropositive donors also have cytotoxic potential but it is not clear what their target population may be.

In addition to their functionality, the ability of $CD45RA^-$ $CD27^-$ and $CD45RA^+$ $CD27^-$ T cells to proliferate and survive after T-cell receptor or homeostatic cytokine stimulation is crucial for their role in immunity. We showed that not only $CD45RA^-$ $CD27^-$ but especially $CD45RA^+$ $CD27^-$ $CD4^+$ T cells have reduced levels of Bcl-2 and impaired Akt phosphorylation. These changes may account for the susceptibility of these cells to apoptosis after activation, which contributes to their inability to accumulate after stimulation *in vitro*. However, this does not necessarily imply that $CD45RA^-$ $CD27^-$ and $CD45RA^+$ $CD27^-$ $CD4^+$ T cells are short lived *in vivo*. It has been shown that stromal cells can promote the survival of apoptosis-prone T cells that have down-regulated Bcl-2[30,48] and that the cytokines involved are type 1 interferons (IFN-α, IFN-β).[49] In addition, IFN-α/β secreted by stromal cells can also prevent the activation-induced apoptosis of antigen-specific $CD4^+$ T-cell clones.[50] These data indicate that although $CD45RA^-$ $CD27^-$ and $CD45RA^+$ $CD27^-$ cells may appear to be susceptible to apoptosis *in vitro*, there may be soluble factors that are present *in vivo* that enable them to persist. This may explain why $CD45RA^+$ $CD27^-$ $CD8^+$ T cells from older humans show unusual kinetic properties in deuterated glucose uptake studies, where their persistence in the blood is not related to the extent to which they proliferate,[51] indicating a possible role for anti-apoptotic factors *in vivo*.

Our studies suggest that one way in which CMV-specific $CD45RA^+$ $CD27^-$ $CD4^+$ T cells may be generated is by IL-7-driven homeostatic proliferation, possibly in combination with other factors. This raises the question as to where this process may occur *in vivo*. It is widely accepted that bone marrow stromal cells are a source of IL-7 that enables the maturation and differentiation of specific progenitor cells[36] and it has been shown that professional memory $CD4^+$ T cells co-localize with IL-7-producing stromal cells *in vivo*.[52] We therefore investigated whether the bone marrow was a possible site for IL-7-driven CD45RA re-expression in memory T cells. There were significantly more $CD45RA^+$ $CD27^-$ T cells in the total $CD4^+$ compartment in the bone marrow compared with the blood of the same subjects. However, there was not a preferential accumulation of $CD45RA^+$ $CD27^-$ T cells of any particular specificity in the bone marrow. This suggests two possibilities. First, that $CD45RA^+$ $CD27^-$ T cells of all specificities preferentially migrate to the bone marrow, or alternatively IL-7 in the bone marrow may induce CD45RA re-expression on $CD4^+$ T cells irrespective of their antigen specificity. Our current experimental system does not allow us to discriminate between these possibilities.

Collectively our results suggest that cytokine secretion may have a largely ignored role in shaping the highly differentiated T-cell repertoire in older humans. Although it is currently unclear why the increase in highly differentiated T cells that are largely CMV-specific is detrimental during ageing,[5] the manipulation of the cytokines that may be involved in their generation may be a possible strategy to prevent their accumulation.

Acknowledgements

This work was supported by grants from the Biotechnological and Biological Sciences Research Council (to A.N.A.). R.I.A. received a scholarship from Fundação para a Ciência e a Tecnologia (FCT) co-financed by Programa Operacional Ciência e Inovação 2010 (POCI 2010) and FSE. We thank Professor Caroline Sabin and Doctor Pedro Coutinho for support in statistical analysis. We are also grateful to all the blood donors who took part in this study.

Disclosures

The authors declare no financial conflicts of interest.

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Figure S1. High frequency of cytomegalovirus (CMV) - specific $CD4^+$ T cells. Peripheral blood mononuclear cells were stimulated with CMV, Epstein–Barr virus (EBV), herpes simplex virus (HSV), varicella zoster virus (VZV) or purified protein derivative (PPD) lysate and the percentage of interferon-γ (IFN-γ) secreting antigen-specific $CD4^+$ T cells was assessed by flow cytometry (a). The frequency of $CD4^+$ T cells that were specific for CMV, EBV, HSV, VZV or PPD was determined in individuals who were seropositive for these agents (b). Only responses $> 0.02\%$ above background (unstimulated cells) were considered positive. Horizontal lines depict median values. Significantly increased frequency of CMV specific $CD4^+$ T cells relative to the other antigens is indicated (Wilcoxon rank test, GraphPad Prism).

Figure S2. Multiparameter flow cytometric analysis. Representative dot plots from one donor show the distribution of stimulated CD4 T cells within each CD45RA/CD27 subset. Panels show CD4 plotted against: CD40 ligand (CD40L; upper right), interferon-γ (IFN-γ; upper left), interleukin-2 (IL-2; lower right) and tumour necrosis factor-α (TNF-α; lower left), each for unstimulated and anti-CD3 stimulated T cells.

Figure S3. Cell recovery. Purified CD45RA/CD27 $CD4^+$ T-cell subsets were activated with anti-CD3 and irradiated antigen-presenting cells and irradiated antigen-presenting cells. At the indicated time-points, the cell number was determined on a haemocytometer. Results are expressed as a percentage of the initial number of cells placed in culture; results for one donor are shown. (b,c) Apoptosis was assessed by Annexin V staining and propidium iodide (PI) incorporation. The percentage of early apoptotic (Annexin V^+ PI^-) and late apoptotic/necrotic (Annexin V^+ PI^+) cells was assessed in the indicated days. Representative pseudocolour plots are shown (b).

Figure S4. $CD4^+$ $CD45RA^-$ $CD27^+$ cells were purified by FACS sorting and analysed for the expression of CD45RA and CD45RO before culture. Cells were stimulated with interleukin-2 (IL-2) or IL-15 and CD45RA/CD45RO expression was assessed by flow cytometry at the indicated time-points. The results shown are representative of four experiments.

Figure S5. $CD4^+$ $CD45RA^-$ $CD27^-$ cells were purified by FACS sorting and analysed for the expression of CD45RA and CD45RO before culture. Cells were stimulated with interleukin-7 (IL-7), IL-2 or IL-15 and CD45RA/CD45RO expression was assessed by flow cytometry at the indicated time-points. The results shown are representative of three experiments.

Figure S6. $CD4^+$ $CD45RA^-$ $CD27^+$ cells were purified by FACS sorting. Cells were stimulated with interleukin-7 (IL-7), or IL-15 and CD45RA/CD27 expression was assessed by flow cytometry at the indicated time-points.

Table S1. Results from multiple linear regression fitting age and cytomegalovirus (CMV) status as co-variates. Table shows the unstandardized coefficient, significance and 95% confidence interval from the output of SPSS software for each CD45RA/CD27 subset. Unit of age is equal to 1 year.

Table S2. Mean frequencies and the standard error of the mean of CD40 ligand (CD40L), interferon-γ (IFN-γ), interleukin-2 (IL-2) and tumour necrosis factor-α (TNF-α) in all possible combinations in each CD45RA/CD27 subset.

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CD45RA Re-Expressing CD4^{+} Memory T Cells Exhibit p38 MAP kinase Regulated Telomere Independent Senescence

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Word Count: 3349

Abstract

In this study we demonstrate that memory $CD4^+$ T cells that re-express CD45RA ($CD27^-CD45RA^+$; EMRA) express high levels of surface KLRG-1, reduced replicative capacity, decreased survival, low telomerase activity and high expression of nuclear γH2AX after T cell receptor (TCR) activation. Paradoxically, despite exhibiting these characteristics of senescence, this population has significantly longer telomeres then central memory-like ($CD45RA^-CD27^+$) and effector memory-like ($CD45RA^-CD27^-$) $CD4^+$ T cells in the same individuals. The p38 mitogen activated protein kinase (MAPK) has been shown to regulate telomere independent senescence in fibroblasts and we found that the expression of both total and phosphorylated forms of this molecule were highest in the EMRA population. Furthermore the inhibition of p38 signaling after TCR activation significantly reduced apoptosis and enhanced both the survival and telomerase activity in $CD27^-CD45RA^+$ T cells. We conclude therefore that EMRA $CD4^+$ T cells exhibit telomere independent senescence. Furthermore, this senescence programme is maintained by active p38 signaling and is reversible.

Introduction

Human T cell memory is mainly maintained throughout life by episodes of proliferation induced by antigenic challenge and to a much lesser extent by continued generation of new cells, as the thymus involutes early in life. However there is a finite limit to the proliferative capacity of T memory cells that is set by the erosion of repeating hexameric sequences of DNA at the ends of chromosomes known as telomeres, that are lost with each replicative cycle (Goronzy et al., 2006). Without compensatory mechanisms such as the induction of the enzyme telomerase, telomeres attrition in cycling cells results in growth arrest or replicative senescence (Hayflick and Moorhead, 1961). This process was first described in fibroblasts but also occurs in human T cells after activation and is known as telomere-dependent senescence (Akbar and Vukmanovic-Stejic, 2007; Effros, 2004; Plunkett et al., 2005). Proliferative arrest that is independent of telomere length can also occur in cells (telomere independent senescence) (Toussaint et al., 2002). This process occurs when DNA is damaged by reactive oxygen species, by ionizing radiation, chromatin perturbation and activation of p53 and stress pathways (Campisi and d'Adda di Fagagna, 2007; Toussaint et al., 2000). Telomere independent senescence has been extensively investigated in fibroblasts (von Zglinicki et al., 2005) however it is not clear whether this process also occurs in human T cells.

Since the original use of CD45RA and CD45RO antibodies to identify unprimed and primed/memory subsets of T cells (Akbar et al., 1988; Merkenschlager and Beverley, 1989; Sanders et al., 1988) it has become clear that some primed/memory T cells can re-express the CD45RA molecule (Bell and Sparshott, 1990; Faint et al., 2001; Hamann et al., 1997; Pilling et al., 1996). These cells were subsequently shown to be $CCR7^-$, $CD27^-$ and $CD28^-$, and therefore have an effector memory-like phenotype (EMRA; effector memory-like cells that re-express CD45RA) (Appay et al., 2008; Harari et al., 2004; Sallusto et al., 2004). Initially, it was thought that the EMRA population were end-stage T cells (Champagne et al., 2001) however subsequent studies indicated that this population can be induced to exhibit effector functions and to proliferate to a limited extent provided that they are activated under optimal conditions (Barber et al., 2006;

Dunne et al., 2005; Waller et al., 2007). These EMRA T cells are found in both CD4 and CD8 populations (Akbar and Fletcher, 2005; Koch et al., 2008; Okada et al., 2008; Romero et al., 2007) and are the dominant memory population that persists after some forms of vaccination (Akondy et al., 2009). However the exact nature of these T cells is not clear.

When human T cells differentiate from a naïve to an effector memory phenotype they lose their capacity to upregulate telomerase activity and this is associated with progressive telomeres reduction (Effros et al., 2005; Fletcher et al., 2005; Plunkett et al., 2005; Weng et al., 1995). However the telomere length or telomerase activity of $CD4^+CD27^-CD45RA^+$ (EMRA) T cells has never been investigated. In this study we made the unexpected observation that although $CD4^+$ EMRA T cells have many phenotypic and functional characteristics of a senescent population, they have significantly longer telomeres than $CD27^+$ $CD45RA^-$ (central memory; CM) and $CD27^-$ $CD45RA^-$ (effector memory; EM) cells from the same donor. We found that the senescence characteristics of this population was mediated in part by active p38 signaling and was reversible. This is the first report that $CD4^+$ $CD27^-CD45RA^+$ EMRA T cells exhibit characteristics of senescence that is not due to excessive telomere erosion. This identifies a new functional constraint on the memory T cell pool of older humans (Hong et al., 2004), subjects with persistent viral infections (Hislop et al., 2005; Khan et al., 2002; Libri et al., 2010; Wills et al., 2002) and autoimmune diseases (Lindstrom and Robinson, 2010; Thewissen et al., 2005; Weyand et al., 2003) that all contain increased proportions of these cells.

Results and Discussion

CD4$^+$ CD27$^-$CD45RA$^+$ (EMRA) T cells exhibit phenotypic and functional characteristics of cellular senescence.

CD4$^+$ T cells can be subdivided into 4 populations on the basis of their relative expression of CD27 and CD45RA (Fig.1A). In previous studies, on the basis of surface receptor expression, functional activity and telomere length, undifferentiated populations have been shown to express CD27$^+$CD45RA$^+$, those that are at an early stage of differentiation are CD27$^+$CD45RA$^-$ while highly differentiated CD4$^+$ T cells are CD27$^-$ CD45RA$^-$ (Appay et al., 2008; Libri et al., 2010). These subsets are analogous to those identified in other reports where surface CCR7 together with CD45RA expression were used (Appay et al., 2008; Harari et al., 2004). In addition, both sets of markers identify a fourth subset that is CD27$^-$CD45RA$^+$ (EMRA) and the proportion of these cells are increased in older humans (Harari et al., 2004; Libri et al., 2010) and patients with chronic viral infections (Dunne et al., 2002; Faint et al., 2001; Hislop et al., 2005; Khan et al., 2002; Wills et al., 2002) and may therefore represent an end-stage population. The observation that these cells expressed high levels of surface KLRG1 that has been shown to identify senescent T cells (Ouyang et al., 2003; Voehringer et al., 2001), supported this possibility (Fig. 1B). However the CD27$^-$CD45RA$^-$ population also expressed high levels of this molecule indicating that they may also have characteristics of senescence. The phosphorylation of the histone protein H2AX can be used to identify DNA damage foci in senescent fibroblasts (Passos et al., 2010; Tanaka et al., 2007). We found that after TCR activation, both the CD27$^-$CD45RA$^-$ and the CD27$^-$CD45RA$^+$ populations expressed significantly higher levels of γH2AX than the other subsets (Fig 1C). This was not due to the identification of replicating instead of damaged DNA as we only included non-proliferating T cells in our analysis (Supplementary Fig. A). Therefore both CD27$^-$CD45RA$^-$ and CD27$^-$CD45RA$^+$ T cells have the phenotype of senescent populations.

Previous studies showed that highly differentiated CD4$^+$ T cells can be identified by the loss of CD27 and CD28 expression (CD27$^-$CD28$^-$) and these cells have low telomerase activity and reduced replicative potential compared to less differentiated

populations in the same subjects (Fletcher et al., 2005; Romero et al., 2007). The CD27$^-$ CD28$^-$ population is heterogeneous and consists of both CD27$^-$CD45RA$^-$ and CD27$^-$ CD45RA$^+$ T cells (Appay et al., 2008). We found that telomerase activity was significantly reduced in the CD27$^-$CD45RA$^+$ cells compared to the other populations (Fig. 1C). Therefore although both CD27$^-$CD45RA$^-$ and CD27$^-$CD45RA$^+$ T cells exhibit characteristics of senescence, the loss of telomerase activity is more pronounced in the latter population.

CD4$^+$CD27$^-$CD45RA$^+$ T cells have relatively long telomeres.

The low telomerase activity in CD27$^-$CD45RA$^+$ T cells prompted us to investigate whether they had very short telomeres. The relatively low numbers of these cells *in vivo* precluded the use of conventional DNA isolation and electrophoresis methods to analyze their telomere lengths (Fletcher et al., 2005; Libri et al., 2010; Roth et al., 2005). We therefore investigated the telomere length of MACS isolated CD4$^+$ T cells by 3 colour fluorescence *in situ* hybridization coupled to flow cytometry using CD45RA, CD27 and a fluorescence labeled telomere probe (flow-FISH; Fig. 2A). We confirmed that relatively undifferentiated T cells (CD27$^+$CD45RA$^+$) have longer telomeres than the early differentiated, central memory-like subset (CD27$^+$CD45RA$^-$), which in turn have significantly longer telomeres than the effector memory-like (CD27$^-$CD45RA$^-$) T cell population (Fig. 2B). However an unexpected observation was that CD27$^-$CD45RA$^+$ T cells that have significantly longer telomeres than the CD27$^+$CD45RA$^-$ and the CD27$^-$ CD45RA$^-$ memory subsets but shorter telomeres than the undifferentiated/naive CD27$^+$CD45RA$^+$ T cells (Fig. 2B). This suggested that although both CD27$^-$CD45RA$^-$ and CD27$^-$CD45RA$^+$ T cells have the characteristics of pre-senescent T cells, the senescence in the latter population was associated with telomerase inhibition but paradoxically, not excessive telomere erosion.

CD4$^+$CD27$^-$CD45RA$^+$T cells express high levels of p38 MAP kinase activity.

The activation of p38 MAP kinase has an essential role in both telomere dependent and telomere independent senescence of fibroblasts (Iwasa et al., 2003; Maruyama et al., 2009). Furthermore telomere-independent senescence can be induced in fibroblasts

by the constitutive activation of p38 (Davis and Kipling, 2009; Haq et al., 2002; Passos et al., 2010). We therefore investigated whether p38 signaling regulated the senescence in CD27$^-$CD45RA$^+$ T cells that resembled telomere independent senescence in fibroblasts. Although several studies have been performed on the role of p38 in the development and cytokine secretion of T lymphocytes (Berenson et al., 2006; Dodeller and Schulze-Koops, 2006; Rincon and Pedraza-Alva, 2003), it's involvement with lymphocyte differentiation is unclear. When we examined either the total level of p38 or the phosphorylated form of this molecule we found that the highest expression of both was found in CD27$^-$CD45RA$^+$ T cells (Fig. 3A, 3B). However the CD27$^-$CD45RA$^-$ subset also showed significantly higher levels of this molecule than the CD27$^+$CD45RA$^+$ and CD27$^+$CD45RA$^-$ populations (Fig. 3A, 3B). Therefore both the CD27$^-$CD45RA$^-$ and CD27$^-$CD45RA$^+$ T cells upregulate the p38 MAP kinase that is a characteristic of senescence in fibroblasts. However, the senescence in the latter population is unlikely to be related to telomere erosion.

p38 Map kinase signaling regulates senescence associated functional changes in CD4$^+$CD27$^-$CD45RA$^+$ T cells.

Previous studies showed that CD4$^+$CD27$^-$CD45RA$^+$ T cells had diminished capacity to expand in culture and were highly susceptible to apoptosis compared to the other subsets (Libri et al., 2010). We investigated whether this defect as well as the decrease in telomerase induction in these cells was mediated by p38 signaling. To do this we blocked p38 signaling in activated T cell by the addition of BIRB796 (BIRB), a p38 inhibitor that blocks the activation of all four of the isoforms of p38 (Bain et al., 2007). We first showed that this inhibitor was specific as it blocked the phosphorylation of p38 (pThr180/pTyr182) but not JNK (pThr183/pTyr185) in activated T cells (Fig. 4A) in accordance with others (Bagley et al., 2010; Davis et al., 2010). We next confirmed that CD27$^-$CD45RA$^+$ T cells were impaired in their ability to expand in culture after TCR activation compared to the other subsets (Fig. 4B). The addition of BIRB to these cells during activation however significantly increased the cell recovery after activation (Fig. 4B). In addition we showed that the inability of CD27$^-$CD45RA$^+$ T cells to expand after activation was due to increased levels of apoptosis (Fig. 4C, Supplementary Fig. B) and

not decreased capacity to enter cell cycle identified by ki67 staining (Fig. 4D). A fundamental difference in the manifestation of senescence in T cells and fibroblasts is that senescence fibroblasts can persist without cell division for extended periods *in vitro* while senescent T cells are very susceptible to apoptosis (Campisi and d'Adda di Fagagna, 2007; Feldser et al., 2003). An important observation was that the increased recovery of CD27$^-$CD45RA$^+$ T cells after blocking p38 signaling was due to the significant reduction of apoptosis (Fig. 4C, supplementary Fig. B) and not increased cell cycling (Fig. 4D). The inhibition of apoptosis by p38 blockade was due in part to the upregulation of the anti-apoptotic molecule Bcl-2 in these cells (Supplementary Fig.C, D).

Although several studies have highlighted the pivotal role of p38 signaling in cellular senescence, the relationship between the activation of this molecule and telomerase activity has not been investigated. We therefore questioned whether the low telomerase activity in the CD27$^-$CD45RA$^+$ T cell population was linked to increased p38 signaling in these cells. We found that the low telomerase activity in the CD27$^-$CD45RA$^+$ T cell population was significantly enhanced by up to 3.5 fold in these cells by blocking p38 compared to the cells without the inhibitor (Fig. 4D). Although telomerase activity in the CD27$^-$CD45RA$^-$ population was also increased by blocking p38 signaling, this result was not significant. This is the first demonstration that the low telomerase activity that has previously been found in highly differentiated human T cells is due mainly to decreased induction in the CD27$^-$CD45RA$^+$ T cell population (Effros et al., 2005; Fletcher et al., 2005; Plunkett et al., 2005; Weng et al., 1995). Collectively these data suggest that p38 signaling actively shapes the senescence characteristics of human CD4$^+$ lymphocytes and its effects are most striking in CD27$^-$CD45RA$^+$ T cells that express the highest levels of this molecule after activation.

A fundamental characteristic of CD27$^-$CD45RA$^+$ T cells is that they upregulate a senescence programme after activation that is mediated in part by p38 signaling and is not due to excessive telomere erosion. In contrast, on the basis of their significantly short telomeres, the CD27$^-$CD45RA$^-$ population may be more susceptible to telomere dependent senescence. One key unanswered question is how are the CD27$^-$CD45RA$^+$ T cells generated *in vivo?* Indirect observations suggest that proinflammatory cytokines

such as TNF-α may have a role since the treatment of T cells with this cytokine upregulates p38 expression (Raingeaud et al., 1995) while the inhibition of TNF-α signaling enhances telomerase activity in cultured T cells (Parish et al., 2009). In addition, homeostatic cytokines like IL7 and IL-15 can also induce re-expression of CD45RA by primed T cell populations (Geginat et al., 2001; Libri et al., 2010) however it is not clear if they also have a role of initiating a senescence programme in T cells. Our preliminary observations suggest that p38 also has a similar role in regulating telomerase activity and senescence in CD8$^+$ T cells however there are some differences compared to the CD4$^+$ population. Interestingly, telomerase can also be enhanced in human CD8$^+$ T cells by activating the ERK pathway (Fauce et al., 2008) with a small molecule telomerase activator (TAT2) however the potential interplay between the ERK and p38 MAP kinase pathways in the regulation of telomerase activity requires further investigation. Immunity declines during ageing and the identification of ways to boost the activity of the immune system is crucial. Understanding the signaling processes that regulate T cell senescence may be important in diverse clinical situations for example during chronic viral infection and ageing. The identification that some of the senescence related changes are reversible raises the possibility of identifying a safe therapeutic window for blocking T cell senescence to improve immunity in certain situations.

Materials and methods

Blood sample collection and isolation

Heparinized peripheral blood was collected from healthy volunteers between the ages of 26 and 60 (median age 39), with approval from the Ethics Committee of the Royal Free Hospital. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). CD4$^+$ T cells were purified by positive selection using the VARIOMACS system (Miltenyi Biotec) according to the manufacturer's instructions. In some experiments, CD4$^+$ T cells were further sorted into CD45RA/CD27 subsets using a FACSAria flow cytometer (BD Biosciences, San Jose, CA) after staining with CD4, CD45RA and CD27 antibodies for 30 minutes at 4°C in 1% phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma-Aldrich).

Cell culture and use of inhibitors

Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 mg/mL streptomycin, 50 µg/ml Gentamicin and 2 mM L-glutamine (all from Invitrogen) at 37°C in a humidified 5% CO_2 incubator. Purified CD4$^+$ subsets were activated in the presence of anti-CD3 antibody (purified OKT3, 0.5µg/ml) and PBMCs irradiated with 40 Gy gamma-radiation, as a source of multiple co-stimulatory ligands provided by B cells, dendritic cells, and macrophages found in these populations. In other experiments, cells were cultured in the presence of recombinant human (rh) IL-2 (5 ng/ml) (R&D Systems). In some experiments the p38 inhibitor BIRB796 was added to the culture. BIRB796 was obtained from David Kipling already dissolved in DMSO at the concentration of 50mM (Bagley et al., 2006). It has been diluted in 0,1% DMSO and used at a final concentration of 500nM. Cells were pretreated with the inhibitor for 30 minutes. A solution of 0.1% DMSO was used as control.

Flow cytometric analysis of cell phenotype

Isolated T cells were re-suspended in PBS containing 1% BSA and 0.1% sodium azide (Sigma-Aldrich) then stained for 10 minutes at room temperature with the

following anti–human monoclonal antibodies: CD45RA (Allophycocyanin; clone MEM56, Abcam); CD27 (Phycoerythrin; clone M-T271, BD Pharmingen); CD4 (Phycoerythrin-Cy7; clone SK3, BD Pharmingen); KLRG1 (AlexaFluro 488, kind gift from Prof P. Pircher, University of Freiburg). Intracellular staining was performed for Bcl-2 (Phycoerythrin; clone Bcl-2/100, BD Pharmingen), Ki67 (Fluorescein isothiocyanate; clone B56, BD Pharmingen) and p38 (rabbit polyclonal anti-p38, Cell Signaling; Alexa Fluor 488 goat anti-rabbit Ig, Invitrogen). The intracellular staining was performed using the Foxp3 Staining Buffer Set (Miltenyi Biotec) according to the manufacturer's instructions. Apoptosis was assessed using an Annexin V/ Propidium Iodide (PI) detection kit (BD Pharmingen). Samples were acquired on a BD LSR II flow-cytometer (BD Biosciences) after fixation with 1% formaldehyde (Sigma-Aldrich). Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Staining of phosphorylated proteins by flow cytometry

The analysis of p38 (pT180/pY182) was performed directly ex vivo. Following surface staining for CD45RA, CD27 and CD4, PBMCs were fixed with warm Cytofix Buffer (BD Biosciences) at 37°C for 10 minutes. Cells were then permeabilized with ice-cold Perm Buffer III (BD Biosciences) at 4°C for 30 minutes and incubated with the anti-p38 antibody (pT180/pY182) (Alexa Fluor 488; clone 36/p38, BD Pharmingen) for 30 minutes at room temperature. Cells were washed in Stain Buffer (BD Pharmingen). For the detection of γH2AX (pSer139) (Alexa 488, clone 2F3, Biolegend), purified subsets were activated with 0.5 µg/ml of immobilized anti-CD3 and 5 ng/ml of rhIL-2 for 4 days. Intracellular staining was performed using the BD Phosflow buffers above mentioned. Samples were acquired on a BD LSR II flow-cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR).

Telomere length measurement by flow fluorescent in situ hybridization coupled to flow cytometry (flow-FISH)

Telomere length of MACS-sorted CD4+ T cell populations defined by expression of CD45RA and CD27 were measured using a modified version of the flow-FISH method that was previously described (Henson et al., 2009; Plunkett et al., 2007). In brief, CD4+

cells were surface stained using surface antibodies, washed in PBS then fixed in 1 mM BS^3 (Perbio Science). The reaction was quenched with 50 mM Tris (pH 7.2) in PBS. After washing in PBS followed by hybridization buffer, cells were incubated with the protein nucleic acid telomeric probe $(C_3TA_2)_3$ conjugated to Cy5 (Panagene). After heating for 10 minutes at 82°C, samples were left to hybridize. Samples were washed in post-hybridization buffer followed by PBS and analyzed immediately by flow cytometry. All samples were run in triplicate alongside cryopreserved PBMCs with known telomere fluorescence to ensure consistency of results. Kilobase length was determined from mean fluorescence intensity values using a standard curve. The standard curve was constructed using samples of varying telomere length analyzed both by flow-FISH and telomeric restriction fragment analysis (Plunkett et al., 2007).

Measurement of telomerase activity

Telomerase activity was determined using a modified version of the telomeric repeat amplification protocol (Oncor, Gaithersburg, MD) by Holt et al (Holt et al, 1996). In brief, purified subsets were activated with anti-CD3 (0.5µg/ml) and irradiated APCs for 4 days. Cell extracts from equivalent numbers of Ki67$^+$ cells were used for telomeric elongation, using a [γ-^{32}P] ATP-end-labelled telomerase substrate (TS) primer. These samples were then amplified by PCR amplification, using 25 to 28 cycles of 30 s at 94°C and 30 s at 59°C. The PCR products were run on a 12% polyacrylamide (Sigma-Aldrich) gel that was then exposed to an autoradiography film (Hyperfilm MP, Amersham). Telomerase activity was calculated as a ratio between the optical density of the telomeric repeat bands and of the internal standard band (IS). As a negative control lysis buffer was used in place of cell extract. A control template containing the same sequence as the TS primer plus 8 telomeric repeats was used as a PCR positive control.

RT-PCR analysis of Bcl-2 mRNA

Expression of Bcl-2 mRNA was analyzed by semiquantitative reverse transcription (RT)-PCR amplification. CD4$^+$ cells were cultured with anti-CD3 (0.5µg/ml) and rhIL-2 (5 ng/ml) in the presence or absence of BIRB796 for 3 days. Total RNA was isolated using

RNeasy kit (Qiagen), and cDNA was synthesized. Bcl-2 expression was evaluated by RT-PCR on an ABI PRISM 7500 (Applied Biosystems) with the following primers: forward 5'-TTGCTTTACGTGGCCTGTTTC-3'; reverse 5'-GAAGACCCTGAAGGACAGCCAT-3'. The housekeeping 18S mRNA, used as an external standard, was amplified from the same cDNA reaction mixture using specific primers. The level of Bcl-2 was expressed as a ratio to the level of 18S to control for differing levels of cDNA in each sample.

Western blot analysis

CD4$^+$ T cells were activated with PMA (0.5 μg/ml, Sigma-Aldrich) and ionomycin (0.5 μg/ml, Sigma-Aldrich) in the presence or absence of BIRB796. Cells were harvested after 30 minutes of stimulation and lysates were obtained by sonicating cells in 50 mM Tris-HCl (pH 7.5), 2 mM EGTA, 0.1% Triton X-100 buffer. Lysates from 2 x10^6 cells were fractionated on SDS-polyacrylamide electrophoresis gels and analyzed by immunoblotting with either anti-phospho-p38 (pThr180/pTyr182, Cell Signaling), anti-pJNK (pThr183/pTyr185, BD Biosciences) or anti-β-actin (Abcam) using the ECL Advanced Western Blotting Detection kit (Amersham Biosciences), according to the protocol provided by the manufacturer.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). Data are presented as mean plus or minus standard error of mean (SEM). *P* less than 0.05 was considered significant.

Acknowledgements

We thank Mark Bagley (School of Chemistry, Cardiff University) for generous gifts of reagents and Terry Davis (School of Medicine, Cardiff University) for useful comments.

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Figure 1. CD4$^+$ revertants express high levels of KLRG-1 and γ-H2AX following activation and are defective for telomerase activity . (A) Phenotypic analysis of CD27/CD45RA expression on CD4$^+$ T cells. PBMCs stained for CD4, CD27 and CD45RA were analysed by flow cytometry. Representative pseudo-color plots are shown. (B) Bar graph shows the cumulative data for the percentage of KLRG-1 expressed on CD4$^+$ CD27/CD45RA T cell subsets. Error bars represent the SE from the mean of sixteen donors. Statistical analysis was performed using the two-tailed Student's *t* test (GraphPad Prism). (C) Purified CD27/CD45RA CD4$^+$ T cell subsets were activated with anti-CD3 and rhIL-2. On day 4, expression of the DNA damage marker γ-H2AX was assessed by flow cytometry. The analysis has been performed on non-proliferating lymphocytes (gate A). Bar graph shows the percentage of γ-H2AX positive cells within each subset. Error bars represent the SE from the mean of three separate experiments. Statistical analysis was performed using the two-tailed Student's *t* test (GraphPad Prism). Pseudo-color plots from a representative experiment out of three are shown. (D) Telomerase activity was determined by telomeric repeat amplification protocol assay. Purified subsets were activated with anti-CD3 and irradiated APCs for 4 days. Graph represents telomerase activity normalized for the activity observed in the Naive subset. Error bars represent the SE from the mean of five separate experiments. Statistical analysis was performed using the two-tailed Student's *t* test (GraphPad Prism). Autoradiography of a TRAP assay acrylamide gel from a representative experiment is shown. Control template consists of PCR mix and telomeric template with no cell extract added. As a negative control, lysis buffer was used instead of cell extract.

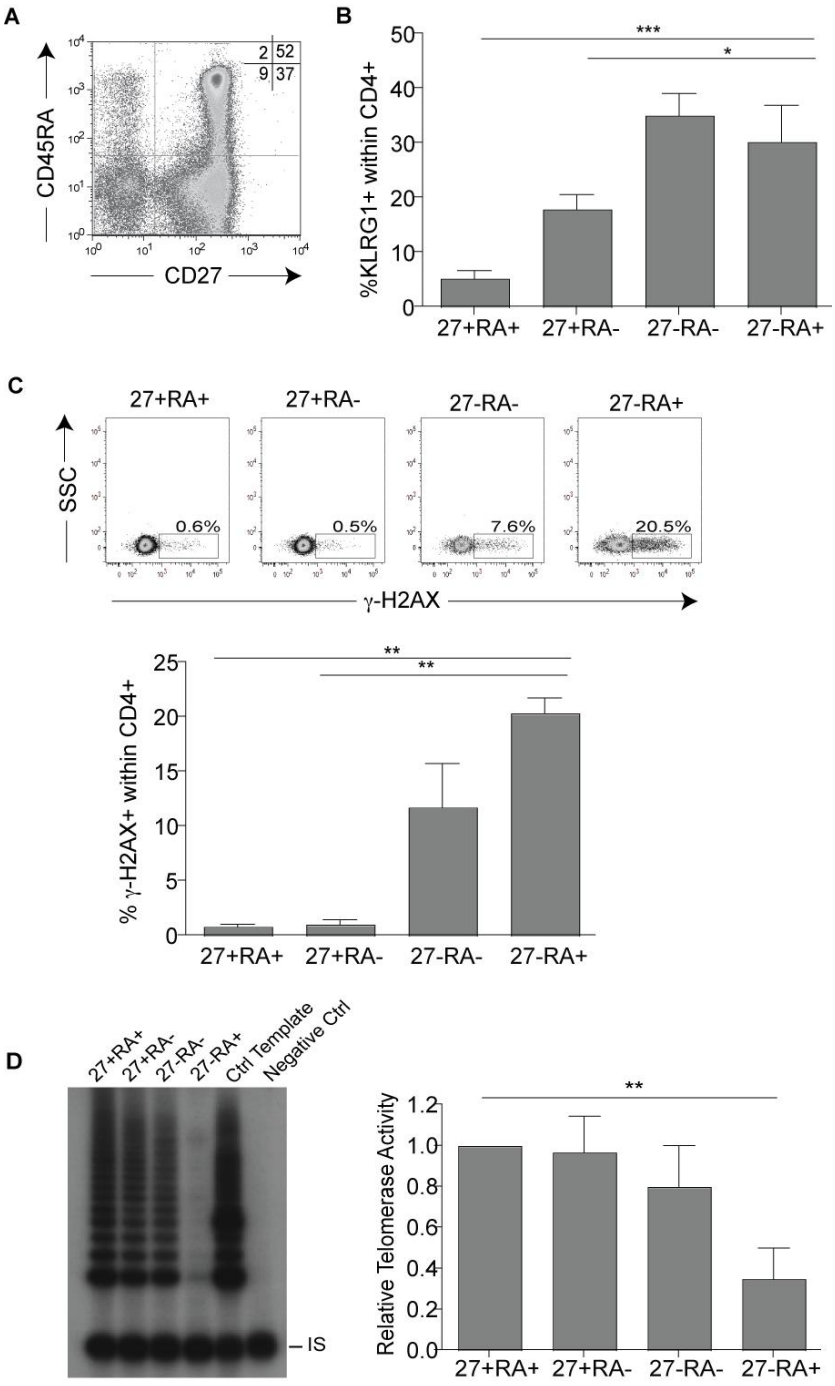
Figure 2. CD4$^+$ revertants do not have the shortest telomeres (A) Representative confocal microscopy image showing CD4$^+$ cells (red) hybridized with a quantitative fluorescent PNA telomere probe (blue), the intensity of which is proportional to telomere length. (B) Telomere length was determined by Flow-FISH. Each circle represents one individual with the mean telomere length shown as a horizontal bar. Statistical analysis was performed using the two-tailed Student's *t* test (GraphPad Prism).

Figure 3. CD4$^+$ 27$^-$RA$^+$ express higher levels of total and phosphorylated p38 *ex*

***vivo*.** The median fluorescence intensity of total p38 (A) and of phosphorylated p38 (B) was assessed *ex vivo* in PBMCs by gating within total CD4$^+$ T cells and within each of the CD27/CD45RA subsets. Overlays of total p38 (A) and of phospho-p38 (B) within the respective subsets are shown. The values represent the median fluorescent intensity of p38 within each subset. Bar graphs represent the *ex vivo* mean fluorescence intensity of total p38 (A) and phospho-p38 (B) normalized for the levels of expression in the naive population. Error bars represent the SE (A n=7, B n=10). Statistical analysis was performed using the two-tailed Student's *t* test (GraphPad Prism).

Figure 4. p38 inhibition improves cell survival and increases telomerase activity on CD4$^+$ revertants. (A) Western blot showing the effects of the p38 inhibitor BIRB796 on p38 and JNK phosphorylation. β-actin was used as a loading control. (B) Effects of BIRB796 on cell recovery. On day 4, the cell number was determined on a hemocytometer. Bar graph represents the number of cells recovered normalized for the initial number of cells placed in culture. Error bars represent the SE (n=3). (C) Bar graph shows the percentage of apoptotic cells (Annexin V$^+$ PI$^-$) within each subset in the presence or absence of BIRB796 treatment. Purified CD27/CD45RA CD4$^+$ T cell subsets were activated with anti-CD3 and IL-2, with (white bars) or without (grey bars) BIRB796. On day 4, apoptosis was assessed by Annexin V staining and PI incorporation. Error bars represent the SE (n=3). (D) Bar graph shows the percentage of Ki67$^+$ proliferating cells in presence and absence of BIRB796. Purified CD45RA/CD27 CD4$^+$ T cell subsets were activated with anti-CD3 and rhIL-2, with (white bars) or without (grey bars) BIRB796. On day 4, proliferation was assessed by Ki67 staining. Results from 5 experiments are shown. (E) Autoradiography of a TRAP assay acrylamide gel from a representative experiment is shown. Telomerase activity was determined by telomeric repeat amplification protocol assay. Purified subsets were activated with anti-CD3 and irradiated APCs for 4 days in absence and presence of BIRB796. Bar graph shows the fold change of telomerase activity following treatment with BIRB796. As a negative control, lysis buffer was used instead of cell extract. Results have been normalized for the telomerase activity of each population in the absence of inhibitor. Error bars represent the SE (n=4). Statistical analysis was performed using the two-tailed Student's *t* test (GraphPad Prism).

Figure 1.


A
CD45RA
CD27
2
52
9
37
B
%KLRG1+ within CD4+
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27+RA+
27+RA-
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C
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27+RA-
27-RA-
27-RA+
SSC
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27+RA+
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27-RA-
27-RA+
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Relative Telomerase Activity
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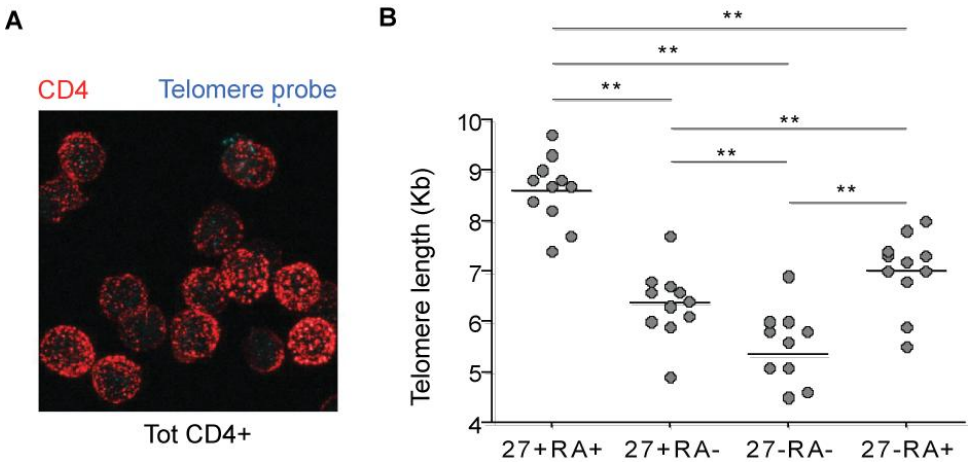
Figure 2.


A
CD4
Telomere probe
Tot CD4+
B
Telomere length (Kb)
10
9
8
7
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4
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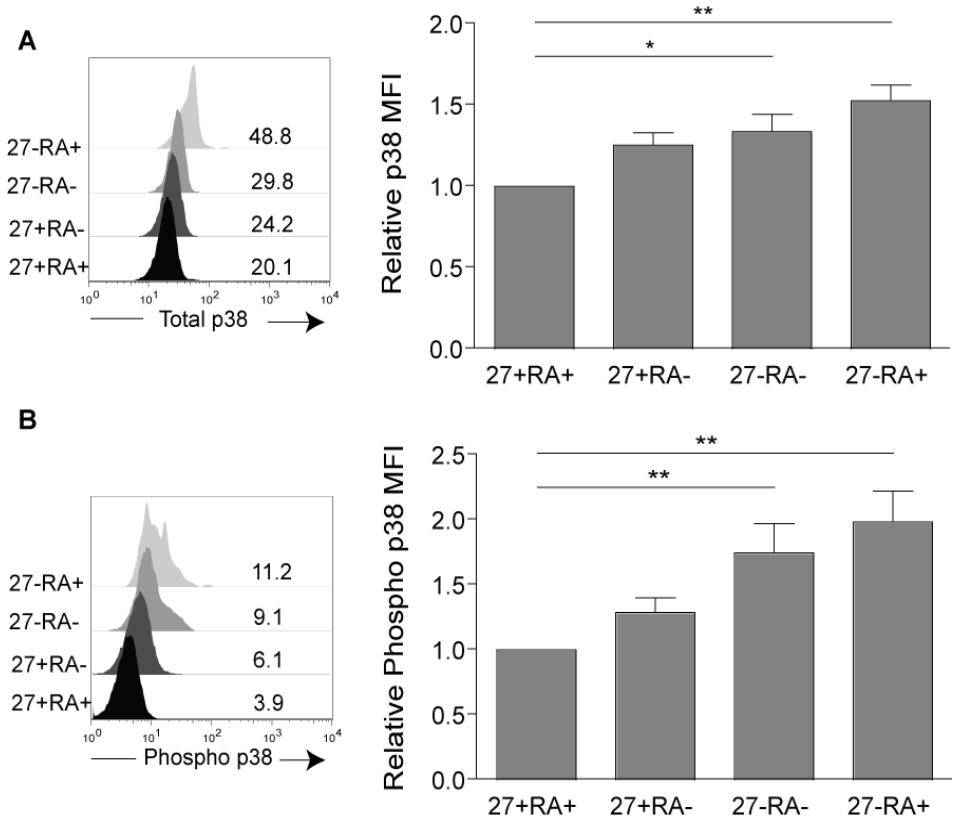
27+RA+
27+RA-
27-RA-
27-RA+

Figure 3.


A
27-RA+
27-RA-
27+RA-
27+RA+
48.8
29.8
24.2
20.1
Total p38
Relative p38 MFI
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*
2.0
1.5
1.0
0.5
0.0
27+RA+
27+RA-
27-RA-
27-RA+
B
27-RA+
27-RA-
27+RA-
27+RA+
11.2
9.1
6.1
3.9
Phospho p38
Relative Phospho p38 MFI
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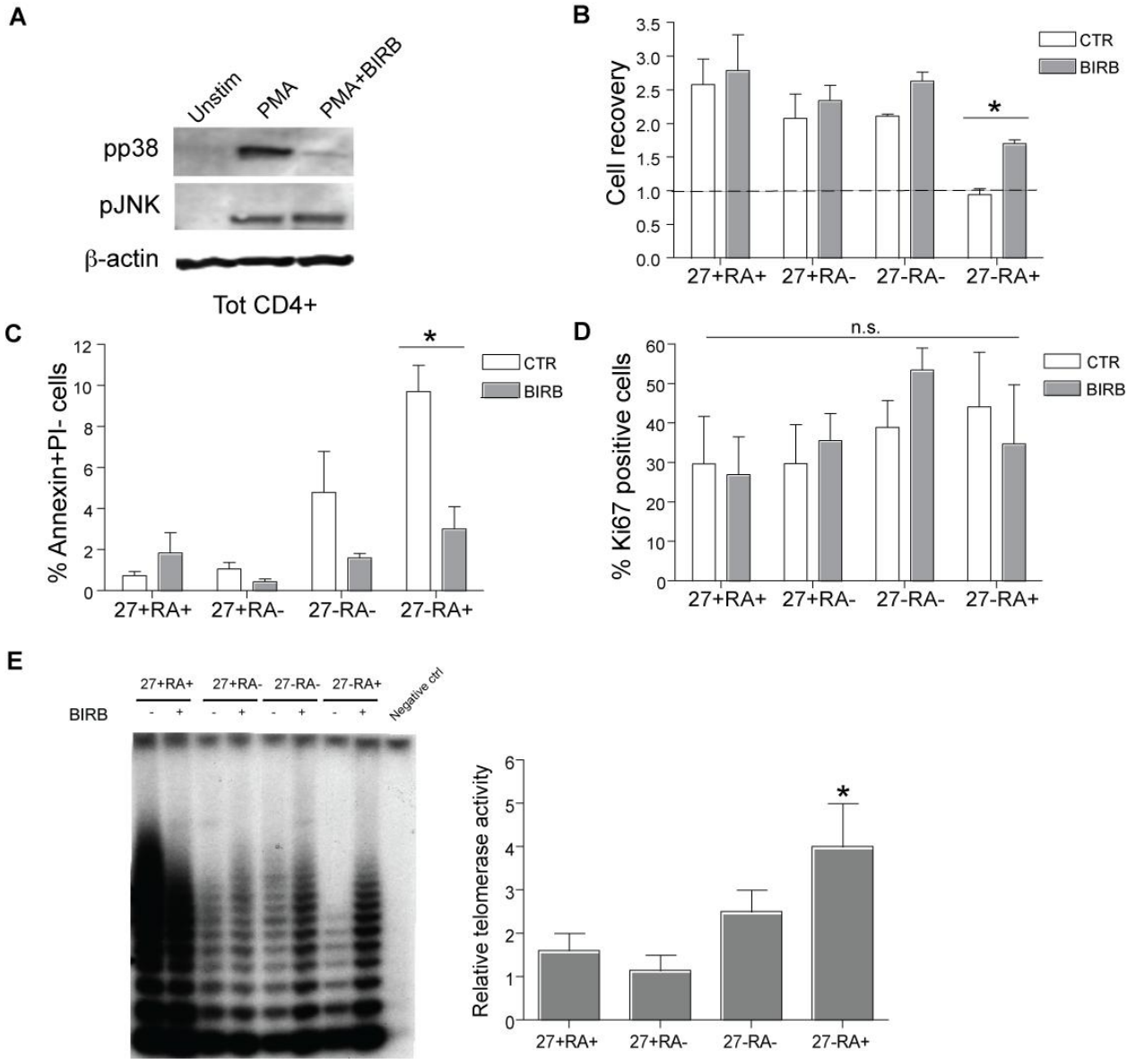
**
2.5
2.0
1.5
1.0
0.5
0.0
27+RA+
27+RA-
27-RA-
27-RA+

Figure 4.


A
Unstim
PMA
PMA+BIRB
pp38
pJNK
β-actin
Tot CD4+
B
Cell recovery
CTR
BIRB
*
27+RA+
27+RA-
27-RA-
27-RA+
C
% Annexin+PI- cells
*
CTR
BIRB
27+RA+
27+RA-
27-RA-
27-RA+
D
% Ki67 positive cells
n.s.
CTR
BIRB
27+RA+
27+RA-
27-RA-
27-RA+
E
BIRB
27+RA+
27+RA-
27-RA-
27-RA+
Negative ctrl
Relative telomerase activity
*
27+RA+
27+RA-
27-RA-
27-RA+

Supplementary data. (A) Purified CD27/CD45RA CD4+ T cell subsets were activated with anti-CD3 and rhIL-2. On day 4, expression of the DNA damage marker γ-H2AX was assessed by flow cytometry. The analysis has been performed on non-proliferating lymphocytes (gate A). (B) Purified CD27/CD45RA CD4$^+$ T cell subsets were activated with anti-CD3 and rhIL-2, with (lower panel) or without (upper panel) BIRB796. On day 4, apoptosis was assessed by Annexin V staining and PI incorporation. Representative pseudocolour plots are shown. (C) Bcl-2 expression was assessed at the protein level by flow-cytometry. Total CD4 were stimulated for four days with anti-CD3 and IL2 with and without BIRB796. A representative plot from three donors is shown. (D) Bar graph shows Bcl-2 mRNA levels measured in total CD4 stimulated for three days with anti-CD3 and rhIL2 in the presence or absence of BIRB796 treatment. The mRNA levels have been measured by Real Time PCR. The results from three different experiments are represented in the graph.

Supplementary Figure.