# Characterization of Effector-Memory CD8+ T cells and their Association with Human Cognitive Function

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Tese para obtenção do Grau de Doutor em
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# Preface

*It's the possibility of having a dream come true that makes life interesting.*
Paulo Coelho

The interactions between the immunological system (IS) and the central nervous system (CNS) have been extensively studied over the last few decades. In fact, the importance of T cells for CNS homeostasis is very well-documented in both human and murine models. Human effector-memory CD8+ T cells comprise highly differentiated cells characterized by the loss of the co-stimulatory receptor CD28 and the expression of functional natural killer cell receptors (NKRs), including inhibitory receptors, that are known to be expanded in healthy elderly people, including centenarians, but also in various chronic diseases. These cells are highly heterogeneous and multifunctional, suggesting that they can exert different roles, including cytotoxic, suppressor and regulatory functions. Importantly, the role of human effector-memory CD8+ T cells, particularly those re-expressing the tyrosine phosphatase isoform CD45RA (i.e., CD8+ TEMRA cells), remains poorly characterized in the context of cognition and neurodegeneration. While CD8+ TEMRA cells are often described as detrimental, recent studies have challenged this view, underscoring the need for more detailed phenotypic, functional, and transcriptional analyses. Hence, this thesis aims to contribute to a better understanding of the potential beneficial role of CD8+ TEMRA cells, identifying novel markers of discrete subsets of highly differentiated CD8+ T cells, and to explore the factors involved in their generation. Importantly, this work provides new insights into the biology of human effector-memory CD8+ T cells and paves the way to future research that may further elucidate the interactions between CD8+ TEMRA cells and the CNS.

# Resumo

Os linfócitos T CD8+ efectores de memória humanos são células altamente diferenciadas que diferem na expressão da isoforma tirosina fosfatase CD45RA, sendo designados como linfócitos CD8+ TEM e CD8+ TEMRA. Estas células são altamente heterogéneas e polifuncionais, possuindo propriedades citotóxicas, reguladoras e supressoras, sendo capazes de migrar para tecidos e órgãos não-linfóides, incluindo o cérebro em determinadas circunstâncias. Expansões de linfócitos CD8+ TEM e CD8+ TEMRA foram já descritas em doenças crónicas inflamatórias, tumores e infecções virais, assim como em idosos saudáveis, nos quais podemos incluir centenários. Nas últimas décadas, o papel dos linfócitos T CD8+, nomeadamente os linfócitos CD8+ TEMRA, tem sido amplamente estudado no contexto do envelhecimento, cognição e neurodegeneração, sendo geralmente descritos como prejudiciais para o sistema nervoso central (SNC), embora investigações recentes têm vindo a questionar esta visão. Estes linfócitos T CD8+ altamente diferenciados podem surgir por mecanismos dependentes e independentes do TCR, tais como a diferenciação induzida por citocinas como a interleucina (IL)-15. Curiosamente, foi demonstrado que a exposição crónica a antigénios leva à geração de linfócitos T CD8+ que expressam níveis reduzidos da cadeia CD8β, embora mecanismos independentes de estimulação antigénica permaneçam ainda pouco compreendidos. Neste estudo, realizámos uma caracterização extensiva de células mononucleares do sangue periférico (PBMC), assim como de moléculas do antigénio leucocitário humano (HLA) numa coorte de idosos voluntários com diferentes estados cognitivos. A análise detalhada dos níveis de expressão de CD45RA neste compartimento revelou a presença de duas populações distintas: linfócitos CD8+ TEMRA$^{low}$ e linfócitos CD8+ TEMRA$^{high}$. Os linfócitos CD8+ TEMRA$^{high}$ formaram uma população bem definida e significativamente expandida nos idosos com declínio cognitivo, enquanto os voluntários sem declínio cognitivo apresentavam níveis elevados de linfócitos CD8+ TEMRA$^{low}$. A análise da expressão de CD8α e CD8β revelou também a existência de duas populações distintas de linfócitos T CD8+ com base na expressão de CD8β: linfócitos T CD8αβ$^{low}$ e CD8αβ$^{High}$, sendo a primeira mais prevalente em indivíduos sem declínio cognitivo. A estimulação das células com recurso a PMA e Ionomicina revelou um aumento significativo da produção de IFN-γ nos linfócitos T CD4+ de idosos com declínio cognitivo. Importa ainda referir que todos os voluntários estudados, excepto um, eram seropositivos para citomegalovírus (CMV). Por fim, verificou-se uma maior prevalência do serotipo HLA-B8, pertencente ao haplótipo ancestral HLA-A1, Cw7, B8, DR3, DQ2, entre os idosos com declínio cognitivo. Adicionalmente, avaliámos o impacto da IL-15 na expressão da cadeia CD8β à superfície celular, utilizando linfócitos T CD8+ *naïve* purificados que foram

marcados com CFSE e cultivados com IL-15 durante 12 dias. A IL-15 induziu uma robusta proliferação e diferenciação dos linfócitos T CD8+ naïve, levando a uma diminuição da expressão de CD8β à superfície celular, enquanto a expressão da cadeia CD8α se manteve estável ou aumentou ligeiramente. Isto resultou na geração de linfócitos T CD8αβ$^{low}$ e CD8αβ$^{-}$ (ou seja, CD8αα). Por outro lado, nem a IL-2 nem a IL-7 foram capazes de reproduzir este efeito. A determinação dos níveis de ARNm das isoformas de CD8β e de CD8α por qPCR revelou que a IL-15 promoveu uma redução significativa dos níveis de ARNm da isoforma M-4, aumentando os níveis das isoformas M-1 e M-2, juntamente com os níveis de CD8α. Importa ainda assinalar, que a análise dos níveis totais de tirosina quinase Lck demonstrou um aumento significativo nos linfócitos T CD8+ após cultura com IL-15, quando comparadas com os linfócitos T CD8+ ao início da cultura. Os nossos resultados sugerem que os linfócitos T CD8+ podem desempenhar um papel protector em termos de cognição e neurodegeneração, ao identificar novos marcadores que definem subpopulações discretas de linfócitos T CD8+ altamente diferenciadas que se encontram expandidas em idosos sem declínio cognitivo, assim como ao identificar a IL-15 como um factor envolvido na geração destas subpopulações. Estudos adicionais, nomeadamente fenotípicos, funcionais e transcriptómicos de linfócitos T CD8+ obtidos *ex vivo* e *in vitro*, são necessários para elucidar as suas propriedades funcionais únicas.

# Palavras-chave

Linfócitos T CD8+;Efetores de Memória;CD45RA;CD8β;IFN-γ;Idosos;Cognição;IL-15.

# Resumo alargado

Os linfócitos T CD8+ efectores de memória humanos consistem em células altamente diferenciadas que diferem na expressão da isoforma tirosina fosfatase CD45RA, sendo designados como linfócitos CD8+ TEM e CD8+ TEMRA. Estas células são altamente heterogéneas e polifuncionais, possuindo propriedades citotóxicas, reguladoras e supressoras, tendo a capacidade de migrar para tecidos e órgãos não-linfóides, incluindo o cérebro, onde podem permanecer como linfócitos T de memória residentes (TRM). Expansões de linfócitos CD8+ TEM e CD8+ TEMRA já foram descritas em doenças crónicas inflamatórias, tumores, infecções virais, mas também em idosos saudáveis, incluindo centenários, estando associados ao conceito de envelhecimento saudável. Nas últimas décadas, o papel dos linfócitos T CD8+ no contexto do envelhecimento, cognição e neurodegeneração tem sido amplamente estudado, sendo descritos tanto como prejudiciais quanto benéficos. Deste modo, a presença e expansão de linfócitos T CD8+, nomeadamente linfócitos CD8+ TEMRA, no sangue, líquido cefalorraquidiano (LCR) e cérebro têm sido geralmente considerados prejudiciais para o sistema nervoso central (SNC), embora investigações recentes têm vindo a questionar esta visão. Estes linfócitos T CD8+ altamente diferenciados podem surgir por mecanismos dependentes e independentes do TCR, tais como a diferenciação induzida por citocinas como a interleucina (IL)-15. Curiosamente, foi demonstrado que a exposição crónica a antigénios leva à geração de linfócitos T CD8+ que expressam níveis reduzidos da cadeia CD8β, embora mecanismos independentes de estimulação antigénica ainda não tenham sido devidamente estudados. Adicionalmente, os linfócitos CD8+ TEM e CD8+ TEMRA são descritos como linfócitos CD8+ T oligoclonais, que perderam a expressão do receptor CD28 e que expressam uma variedade de receptores *natural killer* (NKRs), incluindo receptores inibidores. Neste estudo, realizámos uma caracterização abrangente de células mononucleares do sangue periférico (PBMC), assim como de moléculas do antigénio leucocitário humano (HLA) numa coorte de 86 idosos voluntários que foram classificados com base no estado cognitivo. Estes idosos foram avaliados por uma equipa especializada que realizou a avaliação com base na Escala de Deterioração Global (GDS), no teste revisto de Exame Cognitivo de Addenbrooke (ACE-R) e num teste de marcha de três metros, tendo sido classificados em três categorias distintas: Sem Declínio Cognitivo (NCI, n=13), Declínio Cognitivo Ligeiro (MCI, n=15) e Declínio Cognitivo (CI, n=22). Um total de 34 voluntários foram excluídos do estudo. Os critérios de exclusão utilizados incluíam a desistência do estudo, outras infecções para além de citomegalovírus (CMV), diagnóstico prévio de AVC, doença de Parkinson, neoplasias, perturbações psiquiátricas como epilepsia, trauma ou falta de dados clínicos. A análise inicial não revelou diferenças significativas nas percentagens de linfócitos CD8+ TEMRA entre os diferentes grupos. No

entanto, uma análise detalhada dos níveis de expressão de CD45RA neste compartimento revelou a presença de duas populações distintas: linfócitos CD8+ TEMRA$^{low}$ e linfócitos CD8+ TEMRA$^{high}$. Este padrão de expressão evidenciou a presença e o aumento da percentagem de uma população bem definida de linfócitos CD8+ TEMRA$^{high}$ nos idosos com declínio cognitivo, enquanto os voluntários sem declínio cognitivo apresentavam níveis elevados de linfócitos CD8+ TEMRA$^{low}$. A análise da expressão de CD8α e CD8β revelou a existência de duas populações distintas de linfócitos T CD8+ com base na expressão de CD8β: linfócitos T CD8αβ$^{low}$ e CD8αβ$^{high}$. Os indivíduos sem declínio cognitivo apresentavam percentagens mais elevadas de linfócitos T CD8αβ$^{low}$. Importa referir, que a existência de uma correlação robusta e positiva entre os linfócitos CD8+ TEMRA$^{low}$ e os linfócitos T CD8αβ$^{low}$ representa uma forte indicação de que se trata de populações similares de linfócitos T CD8+. Além disso, a produção de IFN-γ por linfócitos T activados com PMA e Ionomicina encontrava-se significativamente aumentada nos linfócitos T CD4+ de idosos com declínio cognitivo. Importa ainda referir que todos os voluntários estudados, excepto um, eram seropositivos para citomegalovírus (CMV). Por fim, verificou-se uma maior prevalência do serotipo HLA-B8, pertencente ao haplótipo ancestral HLA-A1, Cw7, B8, DR3, DQ2, entre os idosos com declínio cognitivo. Adicionalmente, avaliámos o impacto da IL-15 na expressão da cadeia CD8β à superfície celular, utilizando linfócitos T CD8+ *naïve* purificados que foram marcados com CFSE e cultivados com IL-15 durante 12 dias. De salientar, a IL-15 induziu uma robusta proliferação e diferenciação dos linfócitos T CD8+ naïve, levando a uma diminuição da expressão de CD8β à superfície celular que se revelou dependente da divisão celular, resultando na geração de linfócitos T CD8αβ$^{low}$ e CD8αβ$^{-}$ (ou seja, CD8αα), enquanto a expressão da cadeia CD8α se manteve estável ou aumentou ligeiramente. Importa referir que a cultura de linfócitos T CD8+ naïve com IL-2 ou IL-7 não foi capaz de reproduzir o efeito observado com IL-15. A utilização combinada destas citocinas demonstrou que IL-15+IL-2 foi capaz de gerar linfócitos CD8αα de uma forma semelhante à IL-15 isoladamente, embora a combinação IL-15+IL-7 tenha inibido ligeiramente a formação destas células. A determinação dos níveis de ARNm das isoformas de CD8β e de CD8α por qPCR revelou que a IL-15 promoveu uma redução significativa dos níveis de ARNm da isoforma M-4, enquanto os níveis das isoformas M-1 e M-2 aumentaram juntamente com os níveis de CD8α. Importa ainda assinalar, que a análise dos níveis totais de tirosina quinase Lck demonstrou um aumento significativo nos linfócitos T CD8+ após cultura com IL-15, quando comparadas com os linfócitos T CD8+ ao início da cultura. Os nossos resultados sustentam a hipótese de que os linfócitos T CD8+ podem desempenhar um papel protector em termos de cognição e neurodegeneração, ao identificar novos marcadores que definem subpopulações

discretas de linfócitos T CD8+ altamente diferenciados que se encontram expandidas em idosos sem declínio cognitivo, assim como ao identificar a IL-15 como um factor envolvido na geração destas subpopulações. É fundamental realizar uma caracterização fenotípica, funcional e transcriptómica de linfócitos T CD8+ obtidos *ex vivo* e *in vitro*, de modo a elucidar as suas características funcionais únicas.

# Abstract

Human effector-memory CD8+ T cells consist of highly differentiated cells that differ in the expression of the tyrosine phosphatase isoform CD45RA, being designated as CD8+ TEM and CD8+ TEMRA cells. These highly heterogeneous and polyfunctional cells possess cytotoxic, regulatory, and suppressive features, and are capable of migrating to non-lymphoid tissues and organs, including the brain under certain conditions. Expansions of CD8+ TEM and CD8+ TEMRA cells have been described in chronic inflammatory diseases, tumors and viral infections, as well as in healthy elderly individuals, including centenarians. Over the past few decades, the role of CD8+ T cells, particularly CD8+ TEMRA cells, has been the subject of several studies in the context of aging, cognition, and neurodegeneration, where they have been generally regarded as detrimental to the central nervous system (CNS), though recent investigations have challenged this view. These highly differentiated CD8+ T cells are known to arise through TCR-dependent and TCR-independent mechanisms, such as cytokine-driven proliferation via interleukin (IL)-15. Intriguingly, chronic antigenic stimulation has been shown to drive the generation of CD8+ T cells expressing low levels of the CD8β chain, though antigen-independent mechanisms remain poorly understood. Herein, we have performed a comprehensive characterization of peripheral blood mononuclear cells (PBMC) as well as of human leukocyte antigen (HLA) molecules in a cohort of elderly volunteers differing in their cognitive status. A detailed analysis of the level of expression of CD45RA in the CD8+ TEMRA compartment revealed the presence of two distinct populations: CD8+ $TEMRA^{low}$ and CD8+ $TEMRA^{high}$ cells. Notably, CD8+ $TEMRA^{high}$ cells formed a well-defined and sharply delineated population that was significantly expanded in cognitively impaired volunteers, whereas cognitively unimpaired volunteers were enriched in CD8+ $TEMRA^{low}$ cells. Further analysis of CD8α and CD8β expression also identified the existence of two distinct CD8+ T cells subsets based on the expression of CD8β: $CD8\alpha\beta^{low}$ and $CD8\alpha\beta^{high}$ T cells, with the former being more prevalent among cognitively unimpaired individuals. Moreover, stimulation with PMA and Ionomycin revealed significantly increased IFN-γ production by CD4+ T cells from cognitively impaired elderly. Noteworthy, all but one of the volunteers studied were cytomegalovirus (CMV) seropositive. Finally, a higher prevalence of the HLA-B8 serotype, which belongs to the ancestral haplotype HLA-A1, Cw7, B8, DR3, DQ2, was found among the cognitively impaired elderly. Additionally, we assessed the impact of IL-15 on the cell surface expression of CD8β using CFSE-labeled purified human naïve CD8+ T cells cultured for 12 days. IL-15 induced a robust proliferation and differentiation, resulting in a cell cycle-dependent down-modulation of CD8β from the cell surface, while CD8α expression remained stable or increased slightly. This led to the generation of $CD8\alpha\beta^{low}$ and CD8αβ–

(i.e., CD8αα) T cells. In contrast, IL-2 and IL-7 alone were unable to replicate this effect. Determination of mRNA levels for CD8α and CD8β isoforms by qPCR revealed that IL-15 promoted a significant decrease in mRNA levels of the CD8β M-4 isoform, while increasing the levels of the M-1 and M-2 isoforms alongside with CD8α. Remarkably, analysis of the level of the tyrosine kinase Lck showed a significant increase in CD8+ T cell blasts after culture of CD8+ T cells with IL-15, when compared to CD8+ T cells at the beginning of the culture. Our findings show an association with certain CD8+ T cell subsets that is compatible with a protective role in cognition and neurodegenerative diseases by identifying novel markers that define discrete subsets of highly differentiated CD8+ T cells expanded in cognitively unimpaired elderly individuals and identify IL-15 as a factor involved in the generation of these subsets. In-depth phenotypic, functional, and transcriptomic characterization of *ex vivo* and *in vitro* obtained CD8+ T cell subset is warranted to further elucidate their unique functional properties.

# Keywords

CD8+ T cells;Effector-Memory;CD45RA;CD8β;IFN-γ;Elderly;Cognition;IL-15.

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# List of Abbreviations

| | |
|---|---|
| ACE-R | Addenbrooke's Cognitive Examination - Revised |
| AD | Alzheimer's disease |
| AIDS | Acquired immunodeficiency syndrome |
| ANOVA | Analysis of variance |
| APC | Allophycocyanin |
| APC | Antigen-presenting cell |
| AREG | Amphiregulin |
| ART | Antiretroviral therapy |
| BBB | Blood-brain barrier |
| Bcl-2 | B-cell lymphoma 2 |
| Bcl-xL | B-cell lymphoma-extra large |
| BCSFB | Blood-cerebrospinal fluid barrier |
| BMI | Body mass index |
| BSA | Bovine serum albumin |
| CCR7 | C-C chemokine receptor 7 |
| CD | Cluster of differentiation |
| cDNA | Complementary deoxyribonucleic acid |
| CDR3 | Complementarity-determining region 3 |
| CFSE | Carboxyfluorescein succinimidyl ester |
| CI | Cognitive impairment |
| CMV | Cytomegalovirus |
| CNS | Central nervous system |
| COPD | Chronic obstructive pulmonary disease |
| COS-7 | CV-1 (simian) origin, substrate 7 |
| CSF | Cerebrospinal fluid |
| CST-C | Centro de Sangue e da Transplantação - Coimbra |
| CTLA-4-Ig | Cytotoxic T-lymphocyte antigen 4 – Immunoglobulin fusion protein |
| CXCL16 | C-X-C motif chemokine ligand 16 |
| CXCR6 | C-X-C motif chemokine receptor 6 |
| DC | Dividing cells |
| DNA | Deoxyribonucleic Acid |
| EBIcohort | Elderly of Beira Interior cohort |
| EBNA | Epstein-Barr virus nuclear antigen |
| EBV | Epstein-Barr virus |
| EDTA | Ethylenediaminetetraacetic acid |
| Fas | First apoptosis signal receptor |

| | |
|---|---|
| FBS | Fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| Foxp3 | Forkhead box P3 |
| FSC | Forward scatter |
| FSC-A | Forward scatter - area |
| FSC-H | Forward scatter - height |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GDS | Global deterioration scale |
| GMFI | Geometric mean fluorescence intensity |
| HCMV | Human cytomegalovirus |
| HER2 | Human epidermal growth factor receptor 2 |
| HFE | High Fe |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigen |
| IELs | Intraepithelial lymphocytes |
| IFN-γ | Interferon gamma |
| IgG | Immunoglobulin G |
| IgG1 | Immunoglobulin G1 |
| IgG2a | Immunoglobulin G2a |
| IgG2b | Immunoglobulin G2b |
| IL-10 | Interleukin-10 |
| IL-12 | Interleukin-12 |
| IL-15 | Interleukin-15 |
| IL-15Rα | Interleukin-15 receptor alpha |
| IL-15Rβ | Interleukin-15 receptor beta |
| IL-1β | Interleukin-1 beta |
| IL-2 | Interleukin-2 |
| IL-21 | Interleukin-21 |
| IL-2Rα | Interleukin-2 receptor alpha |
| IL-4 | Interleukin-4 |
| IL-6 | Interleukin-6 |
| IL-7 | Interleukin-7 |
| IL-7Rα | Interleukin-7 receptor alpha chain |
| IL-9 | Interleukin-9 |
| Iono | Ionomycin |
| IPST | Portuguese Institute of Blood and Transplantation |
| IS | Immunological system |

| | |
|---|---|
| $k_a$ | Association constant |
| KIR | Killer-cell immunoglobulin-like receptor |
| KIR2DL1 | Killer-cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 1 |
| KIR2DL2/3 | Killer-cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 and 3 |
| KIR2DL4 | Killer-cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4 |
| KIR3DL1 | Killer-cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1 |
| MAIT | Mucosal-associated invariant T cells |
| MCI | Mild cognitive impairment |
| MFI | Mean fluorescence intensity |
| MHC | Major histocompatibility complex |
| MHC-I | Major histocompatibility complex class I |
| MIP-1β | Macrophage inflammatory protein-1 beta |
| mRNA | Messenger ribonucleic acid |
| MtSCI | Moderate to severe cognitive impairment |
| $NaN_3$ | Sodium azide |
| NCI | No cognitive impairment |
| NDC | Non-dividing cells |
| $NH_4Cl$ | Ammonium chloride |
| NK | Natural killer cells |
| NKG2A | Natural killer group 2 member A |
| NKG2D | Natural killer group 2 member D |
| NKp46 | Natural killer cell p46-related protein |
| NKRs | Natural killer receptors |
| NKT | Natural killer T cells |
| NS | Not significant |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate-buffered saline |
| PD-1 | Programmed cell death protein 1 |
| PE | Phycoerythrin |
| PE-Cy7 | Phycoerythrin-Cyanine |
| PerCP-Cy5.5 | Peridinin-chlorophyll protein–Cyanine 5.5 |
| PerCP | Peridinin-chlorophyll-protein |
| pH | Potential of hydrogen |

| | |
|---|---|
| PMA | Phorbol 12-myristate 13-acetate |
| qPCR | Quantitative polymerase chain reaction |
| RA | Rheumatoid arthritis |
| RBC | Red blood cells |
| RC | Resting cells |
| RNA | Ribonucleic acid |
| RPMI | Roswell Park Memorial Institute medium |
| RPMI-1640 | Roswell Park Memorial Institute 1640 medium |
| RPS18 | Ribosomal protein S18 |
| RT | Room temperature |
| SEM | Standard error of the mean |
| SLE | Systemic lupus erythematosus |
| SSC | Side scatter |
| STAT3 | Signal transducer and activator of transcription 3 |
| STAT5 | Signal transducer and activator of transcription 5 |
| TCM | Central-memory T cells |
| TCR | T cell receptor |
| TCRVβ | T cell receptor variable beta |
| TEM | Effector-memory T cells |
| TEMRA | Effector-memory CD45RA+ T cells |
| TGF-β | Transforming growth factor beta |
| TIM-3 | T cell immunoglobulin and mucin-domain 3 |
| TN | Naïve T cells |
| TNF-α | Tumor necrosis factor alpha |
| Treg | Regulatory T cell |
| TRM | Tissue-resident memory T cells |
| TSCM | Stem-cell memory T cells |
| UBI | Universidade da Beira Interior |
| γc | Common gamma chain |
| γδ | Gamma delta cells |

# List of Scientific Publications

## Publications included in this doctoral thesis:

**Esgalhado AJ**, Reste-Ferreira D, Albino SE, Sousa A, Amaral AP, Martinho A, Oliveira IT, Verde I, Lourenço O, Fonseca AM, Cardoso EM, Arosa FA. CD45RA, CD8β, and IFNγ Are Potential Immune Biomarkers of Human Cognitive Function. *Front Immunol* (2020) 11:592656. doi: 10.3389/fimmu.2020.592656

**Esgalhado AJ**, Reste-Ferreira D, Weinhold S, Uhrberg M, Cardoso EM, Arosa FA. *In vitro* IL-15-activated human naïve CD8+ T cells down-modulate the CD8β chain and become CD8αα T cells. *Front Immunol* (2024) 15:1252439. doi: 10.3389/fimmu.2024.1252439

## Publications not included in this doctoral thesis:

Cardoso EM, **Esgalhado AJ**, Patrão L, Santos M, Neves VP, Martinez J, Patto MAV, Silva H, Arosa FA. Distinctive CD8+ T cell and MHC class I signatures in polycythemia vera patients. *Ann Hematol* (2018) 97:1563–1575. doi: 10.1007/s00277-018-3332-7

Arosa FA, **Esgalhado AJ**, Reste-Ferreira D, Cardoso EM. Open MHC Class I Conformers: A Look through the Looking Glass. *Int J Mol Sci* (2021) 22:9738. doi: 10.3390/ijms22189738

Furtado A, **Esgalhado AJ**, Duarte AC, Costa AR, Costa-Brito AR, Carro E, Ishikawa H, Schroten H, Schwerk C, Gonçalves I, Arosa FA, Santos CRA, Quintela T. Circadian rhythmicity of amyloid-beta-related molecules is disrupted in the choroid plexus of a female Alzheimer's disease mouse model. *J Neurosci Res* (2023) 101:524–540. doi: 10.1002/jnr.25164

Cardoso EM, Lourenço-Gomes V, **Esgalhado AJ**, Reste-Ferreira D, Oliveira N, Amaral AS, Martinho A, Gama JMR, Verde I, Lourenço O, Fonseca AM, Buchli R, Arosa FA. HLA-A23/HLA-A24 serotypes and dementia interaction in the elderly: Association with increased soluble HLA class I molecules in plasma. *HLA* (2023) 102:660–670. doi: 10.1111/tan.15149

Duarte LH, Peixoto HA, Cardoso EM, **Esgalhado AJ**, Arosa FA. IL-10 and TGF-β, but Not IL-17A or IFN-γ, Potentiate the IL-15-Induced Proliferation of Human T Cells: Association with a Decrease in the Expression of β2m-Free HLA Class I Molecules Induced by IL-15. *Int J Mol Sci* (2024) 25:9376. doi: 10.3390/ijms25179376

# Chapter 1

## Introduction

## Human CD8+ T cells: a heterogenous and multifunctional pool

There is now a broad consensus that the CD8+ T cell compartment is constituted by five major pools: naïve cells (CD8+ TN), stem-cell memory cells (CD8+ TSCM), central-memory cells (CD8+ TCM), effector-memory cells (CD8+ TEM) and effector-memory CD45RA+ cells (CD8+ TEMRA) (1–6). The characterization of CD8+ T cell populations has been possible based on the expression of CD45, CCR7, CD28, and CD27 (among others). Accordingly, CD8+ TN cells are comprised by polyclonal T cells that recently emigrated from the thymus that are yet to encounter an antigen, and are characterized by the expression of CD45RA, CCR7, CD28, and CD27. This subset of CD8+ T cells is able to recirculate between blood and secondary lymphoid organs due to the expression of CCR7 and CD62L. The CD8+ TSCM pool comprises antigen-experienced cells displaying a similar phenotype and gene expression profile of CD8+ TN cells, while expressing the CD95 receptor and having the functional attributes of memory cells. CD8+ TCM cells follow CD8+ TSCM cells in the differentiation pathway and are characterized by the loss of the expression of CD45RA, while retaining the expression of CCR7, CD28 and CD27. Finally, CD8+ TEM and CD8+ TEMRA cells differ on the expression of the tyrosine phosphatase isoform CD45RA and are characterized by the loss of expression of CCR7, CD28 and CD27. Though, the expression of the latter marker can sometimes be observed in the CD8+ TEM subset (1–3). The investigations performed over the last decades have contributed to the view that CD8+ TEMRA cells are highly heterogeneous and polyfunctional, and endowed with cytotoxic, suppressor and regulatory features. Due to their particular set of characteristics, highly differentiated CD8+ TEM and CD8+ TEMRA cells have the ability to migrate to non-lymphoid tissues and organs, including the brain under certain conditions (4). A fraction of these cells may stay as non-recirculating tissue-resident CD8+ T cells (CD8+ TRM), due to the expression of CD69 and CD103 that contribute to their retention in the organ and/or tissue (5). Moreover, it has been suggested that the presence of these cells in such locations may allow a faster response to stressful/harmful situations, through the secretion of cytokines, such as IFN-γ, IL-10 and/or TGF-β, and factors like amphiregulin (AREG). These, activate pathways leading to tissue repair and regeneration (6).

## CD8+ T cells in Health and Disease

Over the years, the expansion of highly differentiated CD8+ T cells with polyfunctional properties has been extensively reported across various disease settings and in healthy individuals. The presence of a subset of CD8+ T cells lacking the costimulatory molecule

CD28 in healthy individuals was first described in a seminal study that highlighted their distinct phenotypic and functional characteristics (7). Shortly after, Posnett et al. reported the oligoclonal expansion of CD8+CD28- T cells in healthy elderly individuals (8), an observation that was corroborated and subsequently extended to centenarians (9,10), with Fagnoni et al. demonstrating a clear positive correlation between aging and the peripheral expansion of CD8+CD28- T cells (10). Notably, the progressive loss of CD28 expression was also observed in long-term *in vitro* T cell cultures (9). Interestingly, these CD8+CD28- T cells are phenotypically heterogenous with regard to the tyrosine phosphatase CD45 isoform expression, encompassing both CD45RO+ and CD45RA+ subsets (10,11), and producing high levels of IFN-γ, IL-4, and IL-10 (11).

The association between expansions of CD8+ TEM and CD8+ TEMRA (namely CD8+CD28- T cells) has been particularly explored in people with cytomegalovirus (CMV) seropositivity (12). However, this association has always been controversial, and a number of reports have not found any association between CMV seropositivity and CD8+ T cell expansions. The accumulation and persistence of CMV-specific CD8+ T cells in the elderly, a phenomenon known as memory CD8+ T cell inflation (13), has historically led to the view that these cells are dysfunctional, due to their association with a restricted TCR repertoire, and weak TCR-mediated responses. This, may impair responses to novel antigens, increase susceptibility to chronic inflammatory diseases, and cause a higher risk of mortality (14–22), besides an ineffective response to vaccines (23,24). However, the accumulated body of evidence in recent years has challenged the notion that these cells are dysfunctional. Studies have demonstrated that CMV-specific CD8+ T cells exhibit polyfunctionality, including the expression of functional inhibitory receptors and cytokine secretion (25–29), retaining the ability to proliferate and sustain long-term survival (30–32). Notably, a higher degree of polyfunctionality has been observed in CMV-specific CD8+ T cells from young and middle-aged CMV-seropositive individuals (32,33). Moreover, these cells have been shown to contribute to immune responses to pathogens and viral infections, underscoring their potential protective role (34–37). Longitudinal studies suggest that CMV serology may not be the most accurate indicator of a relationship between CMV infections and CD8+ T cells expansions (38,39), warranting further investigations for a better understanding of these complex interactions (40). Furthermore, studies have consistently demonstrated that the frequency of CD8+CD28- T cells increases markedly during Human Immunodeficiency Virus (HIV) infection and correlates positively with plasma viral load and inversely with CD4+ T cell counts (41–46). Notably, persistent expansions of CD8+CD28- T cells have also been observed in HIV-infected children receiving antiretroviral therapy (ART),

suggesting that viral suppression does not fully normalize T cell homeostasis in this population (47). A substantial proportion of CD8+CD28- T cells are HIV-specific CD8+ T cells, suggesting that repeated antigenic stimulation contributes to their differentiation and expansion (48–53). Despite their reduced proliferative capacity (54), these cells retain potent cytotoxic capabilities, exhibiting high levels of perforin and cytokines such as IFN-γ and TNF-α (55–58). The accumulation of CD8+CD28- T cells in HIV-infected individuals has been linked with worsening of clinical outcomes, including faster disease progression, impaired immune recovery, and development of non-Acquired Immunodeficiency Syndrome (AIDS) comorbidities, even in patients receiving early ART (59–68). Indeed, elevated proportions of CD8+CD28-CD57+ T cells have been independently associated with subclinical carotid artery disease in HIV-infected women (69). Interestingly, a contrasting association has also been observed, i.e., low frequencies of CD8+CD28-CD57+ T cells during HIV infection correlated with increased mortality, which was shown to be mitigated by ART (61). Moreover, a distinct subset of CD8+CD28- regulatory T cells, characterized by low expression of CD127 and expression of CD39, has been shown to expand during chronic HIV infection with evidence suggesting a pathogenic role in both AIDS-related and non-AIDS comorbidities (64). Finally, the increase of the CD8+CD28- T cell subset has also been observed in other chronic viral infections, namely Epstein–Barr virus (EBV) (70–72), hepatitis C virus (73), human parvovirus $B_{19}$ (74), and in children after natural acute measles infection (but not after vaccination) (75). Interestingly, this increase has also been observed in non-viral infections (76–79).

Expansions of CD8+CD28- T cells have also been reported in a broad spectrum of hematological (80–87) and non-hematological cancers (88–107), being often correlated with adverse clinical outcomes. Filaci et al. demonstrated that CD8+CD28- regulatory T cells infiltrate a wide range of human tumors and, together with CD4+CD25+ T cells, potently suppress T cell proliferation and cytotoxic function, thereby exerting significant immunosuppressive activity. Importantly, this infiltration was shown to be tumor-associated and correlated with advanced disease stages and reduced overall survival (97). In addition, CD8+ T cells expressing Foxp3 were found to infiltrate human gastric tumors and were likewise associated with tumor progression (103). Elevated peripheral levels of CD8+CD28- T suppressor cells in metastatic breast cancer patients have been linked to decreased progression-free survival and increased risk of death, both during chemotherapy (101) and following adoptive T cell therapy (100). Similar observations have been described in patients with non-small cell lung cancer (96) and nasopharyngeal carcinoma (108) after chemoradiotherapy and radiotherapy, respectively. Conversely,

Montagna et al. described a population of HLA-unrestricted CD8+CD28- T cells involved in immune surveillance against autologous leukemic blasts in pediatric patients with acute leukemia (84). In solid tumors, a beneficial role for CD8+CD28- T cells has also been reported. For instance, infiltration of CD8+CD28- T cells in human colorectal cancer has been associated with prolonged patient survival (106), whereas in HER2-positive metastatic breast cancer patients, elevated levels of circulating CD8+CD28- T cells were associated with longer progression-free survival and an overall favorable prognostic (95). These observations suggest that under certain contexts, CD8+CD28- T cells may exert potent antitumor activity. Remarkably, a distinctive immune signature characterized by the expansion of CD8+ TEMRA cells and a unique CD3+CD8$^{int}$CD28$^{int}$ T cell population has been identified in patients with Polycythemia Vera, a myeloproliferative neoplasm (109).

Altered frequencies of highly differentiated CD8+ T cells have been described in several autoimmune and autoimmune-related diseases such as rheumatoid arthritis (RA) (110–113), systemic lupus erythematosus (SLE) (114–117), multiple sclerosis (118), Wegener's granulomatosis (119,120), Sjögren's syndrome (121), Ankylosing Spondylitis (122), Grave's disease (123), Pars Planitis (124), Dermatomyositis and Polymyositis (125). In RA, expansions of oligoclonal CD8+ T cells in the peripheral blood have been reported (111), along with increased frequencies of differentiated subsets such as CD8+CD28- and CD8+CD57+ T cells, which have been associated with disease activity (110,126). Notably, treatment with abatacept, a fusion protein (CTLA-4-Ig) that acts as a costimulation blocker binding to CD80 and CD86 on antigen-presenting cells (APCs), and blocking the engagement of CD28 on T cells, thus preventing full T cell activation, led to a significant reduction in circulating CD8+CD28- T cells, which correlated with clinical improvement, thereby suggesting a potential pathogenic role of these cells (127). Within the synovial fluid, the role of differentiated CD8+ T cell subsets appears more complex. Cho et al. identified a population of effector-memory IL-10-producing CD8+ suppressor T cells, which were inversely correlated with disease activity (112), indicating a potential regulatory function. In contrast, Ajam et al. reported an enrichment of CD8+CD28- T cells expressing high levels of PD-1 in the inflamed synovium of RA patients, particularly those with relapsing disease, linking this phenotype to chronic activation and disease progression (128). In SLE, the role and characteristics of CD8+CD28- T cells have been explored with varying findings. Early studies observed that while CD8+CD28- T cells are present in SLE patients, their frequencies did not significantly differ from those in healthy controls (114,116). However, functional impairments in these cells have been documented. CD8+CD28- T cells from active SLE patients exhibited reduced

suppressive activity, potentially linked to an altered cytokine secretion profile, specifically decreased secretion of IL-6 and increased secretion of IL-12 by these cells (129). Additionally, Tulunay et al. reported a reduction in CD8+CD28- T cell numbers in SLE patients, accompanied by diminished IL-10 production, further supporting the notion of compromised suppressive function (117). Interestingly, an increase in CD8+CD28- T cells has been observed in patients with active lupus nephritis, suggesting a potentially distinct behavior of this subset in organ-specific involvement (130). More recently, an elevated CD8+CD28-/CD8+CD28+ T cell ratio was shown to positively correlate with disease activity and renal damage, suggesting that an imbalance between these subsets may contribute to SLE pathogenesis (131). In the majority of autoimmune diseases, namely Ankylosing Spondylitis, Grave's disease, Pars Planitis, Sjögren's syndrome, Wegener's granulomatosis, Dermatomyositis and Polymyositis expansions of CD8+CD28- T cells have been documented, often correlating with cytotoxic effector functions and disease severity (119–125). By contrast, organ-specific autoimmune diseases such as multiple sclerosis and type 1 diabetes mellitus exhibit a numerical defect of CD8+CD28- T suppressor cells, implicating a failure of this regulatory subset in those conditions (118).

Expansions of CD8+CD28- T cells have been reported in various conditions associated with elevated oxidative stress, such as HFE hemochromatosis (132), chronic alcohol consumption (133), hemodialysis (134), β-thalassemia (135), and even acute physical exercise (136–140). Noteworthy, the high numbers of CD8+CD28- T cells in the peripheral blood of heavy alcohol drinkers were shown to be associated with low levels of liver enzymes, suggesting a protective role for this subset (133).

CD8+CD28- T cells producing high levels of intracellular perforin and expressing reduced levels of IFN-γ were found to be increased in the induced sputum of individuals with severe asthma (141), whereas a link between the expansion of CD8+CD57+ T cells in pulmonary lymphoid follicles of patients with chronic obstructive pulmonary disease (COPD) and local immune dysfunction has been proposed (142). Overall, the role of these cells in chronic respiratory disorders needs further studies (143,144).

In transplantation, expansions of highly differentiated CD8+ T cells, particularly the CD8+CD28- T cell subset, have been well documented (145–153) and accumulating evidence from liver, kidney, intestinal and hematopoietic stem cell transplantation suggests that elevated frequencies of these cells are associated with reduced need for immunosuppression, decreased risk of graft loss, rejection, and relapse, as well as long-

term survival (152–160). However, recent studies in kidney transplants recipients have raised concerns about the role of CD8+CD28- T and CD8+ TEMRA cells, with some reports linking their expansion to an increased risk of graft rejection (161–164). In fact, CD8+ TEMRA cells have been found to accumulate in the peripheral blood and kidney graft tissues of kidney transplant recipients (165), including in situations of chronic kidney failure. They are also associated with decreased effective response to infections and other pos-transplant malignancies (166), leading to the hypothesis that these highly differentiated CD8+ T cells may serve not only as biomarkers but also as potential therapeutic target in kidney transplant recipients (167).

Overall, highly differentiated CD8+ T cells exhibiting different profiles and mechanisms of action have been identified in almost all chronic diseases, being associated with both positive and negative outcomes, underlining the need for further characterization that can allow the development and improvement of vaccine strategies and immunotherapies (168,169).

## CD8+ T cells in Neurodegeneration

CD8+ T cells have historically been regarded as detrimental to the central nervous system (CNS), particularly in the context of neurodegenerative diseases. Indeed, early studies implicated these cells in CNS injury, cognitive decline, and neurodegeneration, portraying them as pathogenic mediators (170–179). It is now well established that CD8+ T cells infiltrate the brain not only in the course of neurodegenerative diseases but also during normal aging, as demonstrated in both human post-mortem samples (180–184) and animal models (185–188). Among these brain-infiltrating lymphocytes, CD8+ TEM, CD8+ TEMRA, and CD8+CD69+ TRM cells, expressing or lacking CD103, have been identified (182,189). Interestingly, the CD103+ subset often exhibits reduced cytolytic activity and has been shown to display polyfunctionality upon stimulation, including IFN-γ and TNF-α secretion (189). Transcriptomic profiling of CD8+ T cells from the peripheral blood and brain of transgenic Alzheimer's disease (AD) mice has revealed a gene expression signature characteristic of TRM cells (190). These cells have been shown to have upregulated the expression of inhibitory receptors such as PD-1 and TIM-3 and to closely interact with choroid plexus epithelial cells (191). Furthermore, CD8+ TRM cells have been shown to exacerbate inflammation following ischemic brain injury or after ex vivo stimulation (192), and elevated frequencies have been reported in the cerebrospinal fluid (CSF) of patients with neurodegenerative diseases (193). The accumulated evidence from various murine models has demonstrated that both

peripheral and brain-infiltrating CD8+ T cells contribute to axon degeneration, memory deficits, cognitive and motor decline, as well as plaque and tangle-like deposition, thereby driving progressive neurodegeneration (186,194–198). RNA sequencing analysis has also implicated CD8+ T cells residing in the brain in modulating neuronal- and synapse-related gene expression, which may contribute to neuronal dysfunction (188). Notably, a three-dimensional human neuroimmune axis model recently demonstrated that CD8+ T cell infiltration worsens AD pathology (199), with effector-memory CD8+ T cells being identified as the predominant lymphocyte population enriched in the brains of individuals with AD dementia (200). Gate et al. reported increased numbers of clonally expanded CD8+ TEMRA cells in the peripheral blood and CSF of AD patients, which was associated with cognitive impairment (201). Moreover, activated human CD8+ T cells and CD8+CD69+CD103+ TRM-like cells have also been observed in the CSF of patients with mild AD and other neurodegenerative diseases, respectively (193,202). Of note, the CXCL16-CXR6 pathway has recently been identified as a key mechanism mediating CD8+ T cell trafficking to the CSF (203). The expansion of these highly differentiated CD8+ T cells in the peripheral blood and CSF has been documented by others (204), with peripheral CD8+ TEMRA cells being associated with neuronal injury and neuroinflammation in aging and during the progression of AD or AD-related dementia (205). Intriguingly, the accumulation of peripheral CD8+ TEMRA cells have recently been detected in AD patients even prior to clinical onset (206).

## CD8+ T cells in Neuroregeneration

Despite the previously described detrimental roles of CD8+ T cells in cognition and neurodegenerative diseases, studies have also demonstrated their protective and beneficial roles. Early studies in mouse models have shown that CD8+ T cells are essential for efficient remyelination of CNS axons (207), and for the control and clearance of viral infections, such as the West Nile Virus and murine CMV in neonates (208,209). Notably, Zarif et al. reported that CD8+ T cells play a pivotal role in hippocampal-behavioral improvement, neurogenesis and synaptic plasticity (210). Moreover, brain-resident TRM cells induced in the periphery have been shown to rapidly secrete cytokines upon activation, contributing to neuroprotection (211). This finding is in agreement with previous reports that described CD8+ TRM cells as key players in CNS immunosurveillance under homeostatic conditions (192,212). In addition, regulatory CD8+ T cells have been shown to suppress autoreactive CD4+ T cells in an experimental autoimmune encephalomyelitis model (213). Remarkably, CD8+ T cells expressing CXCR6 and PD-1 were identified in the brain and found to mitigate AD pathologies,

including β-amyloid deposition and cognitive decline. CXCL16-CXCR6 intracellular communication between CD8+ T cells and microglia is thought to be a key part of the process (214). Importantly, the dichotomous roles attributed to CD8+ T cells warrant careful interpretation. Thus, the expansion of CD8+ TEMRA cells in the peripheral blood, CSF and brain does not imply, *per se*, that these cells are deleterious or protective (215–217), since this compartment is composed by a heterogenous pool of highly differentiated CD8+ T cells. They express overlapping markers, and are endowed with cytotoxic, suppressor and regulatory subsets (6). Therefore, in-depth phenotypic, functional and transcriptomic analyses of CD8+ TEMRA cells may allow the identification of discrete subpopulations and novel markers that can distinguish protective from pathogenic subsets (215–217). Furthermore, it is also important to consider that the brain microenvironment may impact the development and function of these cells, making it very important to identify factors that can modulate them (217).

## General Features of Human Effector-Memory CD8+ T cells

The number of naïve T cells emerging from the thymus progressively decreases during the lifespan of humans, due to thymic involution. This leads to an increase and expansion of memory cells, in particular highly differentiated human CD8+ TEM and CD8+ TEMRA cells (218,219). Expansions of such subsets of CD8+ T cells have been shown to be related to chronic antigenic stimulation (i.e., TCR-dependent stimulation), but also the result of encounters with cytokines (i.e., TCR-independent stimulation), such as IL-15 as it has been reported in HIV patients (220), reviewed by (6). Generation of human CD8+ TEM and CD8+ TEMRA cells has also been demonstrated *in vitro* upon culture of CD8+ T cells with IL-15 (221,222), including functional CD8+ TEMRA cells (222). While unnoticed, antigen-driven human effector-memory CD8+ T cells expressing low levels of the CD8β chain have been previously described in healthy individuals (223–225), though a possible antigen-independent stimuli may also be involved. Human CD8+ TEM and CD8+ TEMRA cells display a range of characteristics that distinguish them from conventional CD8+ T cells, which has been suggested to be a compensation for functional deficits of conventional immune cells (219). One of the hallmarks of human CD8+ TEM and CD8+ TEMRA cells is the progressive loss of CD28 receptor expression with chronological aging, which has been shown to be generally irreversible, due to the direct inactivation of the gene promoter (226,227). Interestingly, CD8+ T cells have a higher rate of CD28 loss when compared to CD4+ T cells (228). In opposition to humans, mouse T cells do not lose CD28 expression with chronological aging, which may actually increase (229). Another feature of human CD8+ TEM and CD8+ TEMRA cells is related to

the expression of receptors commonly expressed by other cells, in particular NK cells. Indeed, expression of both activating (e.g., CD56, CD16, NKG2D, CD161, activation KIR) and inhibitory receptors (e.g., CD94, NKG2A, inhibitory KIR), among others, has been illustrated by several studies contributing to the view that these cells may display multiple functions (222,230–238). Interestingly, the inhibitory receptors were shown to transmit survival signals to the CD8+ T cells, thus augmenting their lifetime (239–241). Oligoclonality of the TCR with deficient signaling (8,242) and shortened telomeres, consistent with replicative senescence (54,243), are also characteristics of CD8+ TEM and CD8+ TEMRA cells. Moreover, human CD8+ TEM and CD8+ TEMRA cells have a very limited proliferative capacity after stimulation via the TCR/CD3 complex (244,245), though they are resistant to apoptosis. This may explain their survival rate and persistence on circulation and accumulation with increasing aging (240,246). Taken together, human CD8+ TEM and CD8+ TEMRA cells display unique characteristics that enable them to scrutinize and respond to TCR-independent signals, indicating a critical role for the homeostasis of the human system (6,247–249).

## Interleukin-15: its importance in CD8+ T cell biology

Interleukin-15 (IL-15) is a pleiotropic cytokine that plays a pivotal role in the survival, proliferation, and differentiation of NK and CD8+ T cells, besides having a deep impact in the biology of NKT cells and intraepithelial lymphocytes (IELs) (250–253). In fact, it has been shown to contribute to the induction of CD8+ TEM and CD8+ TEMRA cells *in vitro* (222,254,255).

IL-15 is a member of the common cytokine receptor γ chain (γc) family of cytokines that includes IL-2, IL-4, IL-7, IL-9 and IL-21 (256). It is believed to act in *trans* with the low affinity IL-15 receptor expressed by CD8+ T cells (see below) after *trans*-presentation by the private IL-15 receptor, IL-15Rα. Regarding IL-15Rα, eight isoforms generated by alternative splicing of the various exons encoding for the mature protein have been identified. One splicing variant includes the deletion of exon 2, which comprises the sushi domain, thereby resulting in a defective IL-15Rα that is unable to bind IL-15 (257). IL-15Rα mRNA is expressed in a variety of immune and non-immune cells and tissues.

The IL-15 receptor complex consists of a heterotrimeric structure composed by three subunits: a private high-affinity receptor, IL-15Rα, the IL-2/IL-15Rβ (CD122) shared with IL-2 and the γc (CD132) (258–260). The IL-2/IL-15Rβ and the γc do not possess intrinsic enzymatic activity, thus cytokine binding induces their oligomerization with signaling through these subunits requiring the association/recruitment of kinases to the cytoplasmic domains of these molecules. Indeed, IL-15Rα is structurally similar to IL-

2Rα, containing a short cytoplasmic tail, a transmembrane domain, a stalk region, a hinge region and a sushi domain, which is required for cytokine binding, whereas IL-2Rα has two sushi domains (260,261). IL-15 binds to IL-15Rα with high-affinity ($K_a$ greater or equal to $10^{11}$ $M^{-1}$), even in the absence of IL-2/IL-15Rβ and the γc. However, in the absence of IL-15Rα, the IL-15Rβγc receptor complex binds IL-15 only with intermediate affinity. Binding of IL-15 to IL-15Rα is 1000-fold higher than IL-2 binding to IL-2Rα. Upon binding IL-15, IL-15Rα undergoes some conformational changes, though the extent to which this occurs, and its consequences remain unknown (262–264). Interestingly, the affinity of the βγc complex for IL-15 is not further increased by the presence of IL-15Rα (260,261).

Early studies demonstrated that memory T cells were able to respond to IL-15 stimulation and proliferate (265). IL-15 also enhanced their cytotoxic profile through the increase of perforin, granzymes A and B, and IFN-γ mRNA expression (266) and contributed to their survival *in vitro* (267). The impact of IL-15 on CD8+ T cell biology is described below.

Dubois and colleagues introduced the concept of trans-presentation for the first time, providing explanations for several unanswered questions regarding IL-15 biology. Consistent with the reported high-affinity between IL-15 and IL-15Rα, these two molecules were shown to form stable complexes at the cell surface of activated monocytes (257). This observation was consistent with previous reports showing the expression of active IL-15 at the cell surface of human monocytes (268,269). Furthermore, this elegant study demonstrated that IL-15 and IL-15Rα associate intracellularly and could be followed from the endoplasmic reticulum to the cell surface. Thus, trans-endosomal recycling of IL-15/IL-15Rα complexes promotes their constant existence at the cell surface. Most important, cell surface IL-15Rα can present IL-15 in trans to neighboring cells that express IL-2/IL-15Rβ and γc, but not IL-15Rα, such as CD8+ T cells (257). These observations explain the results from a previous study showing that normal CD8+ T cells fail to proliferate when adoptively transferred into IL-15Rα-/- mice, whereas CD8+ T cells lacking the IL-15Rα were able to proliferate in a normal host environment. Hence, IL-15 responses were dependent on IL-15Rα expression by surrounding cells (270). Following *trans*-presentation, membrane-bound IL-15/IL-15Rα complexes are cleaved from presenting cells and internalized by responding cells, which can subsequently recycle these complexes to support their individual proliferation and survival (271). Additionally, *trans*-presentation of IL-15 has been confirmed by biophysical evidence/assays (272).

The importance of IL-15 for CD8+ T cell activation, proliferation, differentiation and survival is very well documented in human and animal models. Studies performed in IL-15$^{-/-}$ and IL-15Rα$^{-/-}$ mice indicated a role for IL-15 in the expansion and maintenance of memory CD8+ T cells (273,274). In this sense, IL-15Rα$^{-/-}$ mice were shown to exhibit deficient numbers of CD8+ T both in the thymus and in the periphery, with a reduced number of memory CD8+ T cells. In contrast, IL-15$^{-/-}$ mice displayed reduced numbers of memory CD8+ T cells that were reversible with the *in vivo* administration of exogenous IL-15 (274). Importantly, homeostatic proliferation of naïve and memory CD8+ T cells is driven by IL-15, as demonstrated by *in vitro* (220–222,236,275–277) and *in vivo* (278–284) studies, which is fundamental for the maintenance of this subset in the absence of antigenic or TCR-mediated stimulation. The levels of expression of IL-2/15Rβ are low on CD8+ TN cells, intermediate on CD8+ TCM cells and high on CD8+ TEM and CD8+ TEMRA cells (276,278), accounting for the observed selective expansion and maintenance of memory CD8+ T cells. Several studies have also demonstrated the effects of IL-15 on CD8+ T cells differentiation, namely in the *in vitro* generation of memory CD8+ T cells from their naïve counterparts (221). Moreover, IL-15 induces the expression of both activation and inhibitory NKRs and increases the cytotoxic profile of CD8+ T cells through an elevated production of perforin and granzyme B, with secretion of the cytokines IFN-γ and TNF-α also being observed (220–222,236,275). A role for IL-15 in the generation of CD8+CD28- T cells has been demonstrated (255,285), and includes subsets of suppressor cells (286). Additionally, IL-15 contributes to the homeostasis (284,287,288) and survival of naïve and memory CD8+ T cells, namely through the upregulation of Bcl-2, Bcl-xL and the inhibition of Fas-induced apoptosis (222,277,289–291).

Our research group has contributed to the body of knowledge with elegant studies demonstrating the differentiation of CD8+ T cells upon *in vitro* culture with IL-15 (221,222,236). Accordingly, IL-15 was shown to be able to differentiate CD8+ TN into different subsets of memory CD8+ T cells, namely CD8+ TEM and CD8+ TEMRA cells presenting several phenotypic and functional modifications (222). Indeed, culture of CD8+ T cells with IL-15 induces the expression of several natural killer receptors (NKRs), such as CD56, NKG2D, NKG2A, KIR2DL2/3, KIR2DL4 and NKp46 (222,236). Interestingly, these CD8+ T cells have increased levels of Bcl-2, suggesting that they are more resistant to apoptosis. They also displayed an MHC-independent cytotoxicity against MHC-I negative target cells by secreting perforin and granzyme B (222). Together with the expression of different anti-inflammatory (IL-10) and pro-inflammatory (IFN-γ, TNF-α, IL-1β, and MIP-1β) cytokines upon crosslinking of the expressed NKRs, these findings suggest a potential regulatory/suppressor role for IL-15-

induced CD8+ T cells with IL-15 being able to modulate the phenotypic and functional profile of these cells enabling them to respond to both TCR-dependent and TCR-independent stimuli (221,222,236).

## The CD8 receptor

The CD8 receptor is a glycoprotein that can be expressed at the cell surface of CD8+ T cells as a disulfide-linked CD8αα homodimer or a CD8αβ heterodimer (292). NK cells, γδ T cells and certain subsets of IELs only express the CD8αα homodimer (293). CD8β requires the association of CD8α for its stable expression at the cell surface, though expression of human CD8ββ homodimers at the cell surface in the absence of CD8α has been observed upon transfection of the COS-7 cell line and murine lymphocytes. The same was not observed for mouse CD8β (294). These CD8ββ homodimers can be assembled intracellularly but were shown to be unstable and to degrade rapidly.

In 1987, Pauline Johnson was able to identify the genomic sequence of human CD8β (295), which was fundamental for the demonstration that CD8β could be expressed at the cell surface of human CD8+ T cells (296–298). At that time, expression of CD8β was thought to be restricted to rodents.

Overall, the CD8αβ heterodimer has long been considered a "better" coreceptor when compared to the CD8αα homodimer (299), namely due to its ability to broaden the antigen recognition capacity of CD8+ T cells, to increase the efficiency of the interaction between the TCR and MHC class I molecules, and to enhance the activation of the CD8α-associated tyrosine kinase Lck (300–305). On the other hand, CD8αα is not considered a functional homolog of CD8αβ and has been implicated as a negative regulator of T cell activation (306,307). Indeed, CD8αα homodimers can be transiently induced in CD8+ T cells, promoting survival and differentiation into memory CD8+ T cells (308) and have been proposed to endow these cells with suppressor functions (309).

Expansions of CD8+ T cells expressing lower levels of the CD8β chain have been described in the peripheral blood of healthy individuals and patients with various diseases (223–225,310–315). Specifically, populations of $CD8\alpha\beta^{low}$ and CD8αα T cells have been identified following bone marrow transplantation (313), and in conditions such as Wiskott-Aldrich Syndrome (311), SLE (314), psoriasis (315), and viral infections, including EBV (225) and HIV (225,312).

Phenotypic and functional characterization of $CD8\alpha\beta^{low}$ and CD8αα T cells has revealed that these cells exhibit an effector-memory phenotype, expressing both activation and inhibitory receptors, while secreting perforin, granzymes, as well as cytokines like IFN-γ

(223–225). An elegant study by Werwitzke et al. further categorized CD8αβ$^{low}$ T cells based on the expression of CD28, demonstrating that CD8αβ$^{low}$CD28- T cells frequently re-express CD45RA, while expressing CD16, CD56, CD94, NKB1 and CD158a. These cells were shown to contain intracellular perforin, granzyme A and IFN-γ (224). Further studies extended these findings by identifying and characterizing CD8αα T cells, suggesting distinct origins. Konno et al. proposed that CD8αα T cells arise from clonally expanded CD8αβ$^{high}$ T cells (223), whereas Walker et al. suggested that these cells were mucosal-associated invariant T (MAIT) cells (225). CDR3 spectratyping analyses revealed that CD8αβ$^{high}$ T cells are highly polyclonal, whereas CD8αβ$^{low}$ and CD8αα T cells are oligoclonal, supporting the view that CD8β downregulation was associated with antigenic stimulation (223). The expression of CD8αα homodimers has been shown to promote survival and differentiation into memory CD8+ T cells (308). Other studies showed that CD8αα was a coreceptor for KIR3DL1, implicating it in the regulation of CD8+ T cells responses (309).

The human *CD8B* gene is duplicated and consists of the *CD8B-1* and *CD8B-2* genes, located on chromosome 2. The *CD8B-1* gene (2p11.2) is constituted by 8 exons and localizes 25-35 kb upstream and in the same transcriptional orientation as the *CD8A* gene. On the other hand, the *CD8B-2* gene (2q12.2) consists of 7 exons and is unlinked to *CD8B-1* (316–318). Interestingly, four membrane-bound (M-1, M-2, M-3 and M-4) and two secreted (S-1 and S-2) isoforms of the *CD8B* gene generated by alternative splicing have been identified (317–319). The M-1, M-2, M-3 and M-4 CD8β isoforms differ in the length of their cytoplasmic tails (3, 39, 14 and 36 amino acids, respectively) and are differentially expressed in thymocytes and various subsets of CD8+ T cells (319). In this sense, the M-1 isoform is the most widely expressed in thymocytes and peripheral blood CD8+ T cells, namely in CD8+ TN cells. The expression of the M-2 isoform is controlled by ubiquitination of a lysine residue, K215, in its cytoplasmic tail. Indeed, this isoform was found to be located in a lysosomal compartment in resting cells, though upon stimulation, the mRNA levels increased, and the M-2 protein localized to the cell surface together with the TCR complex, suggesting a role in CD8+ T cell activation. Of note, the M-3 isoform is barely detected, and its physiological role is currently unknown (319). The M-4 isoform is predominantly expressed in CD8+ TEM and CD8+ TEMRA cells and has a cytoplasmic tail with distinctive characteristics (319,320). In fact, the M-4 cytoplasmic tail is characterized by a dihydrophobic leucine-based receptor internalization motif that regulates its cell surface expression and down-modulation. Additionally, the cytoplasmic tail is able to associate with ubiquitinated proteins and it was found to be itself mono-ubiquitinated on a lysine residue in human cell lines. Finally,

human peripheral blood T cells expressing the M-4 isoform were shown to secrete increased levels of MIP-1β, indicating enhanced antigen recognition (320).

In summary, the precise role of human effector-memory CD8+ T cells in cognition and neurodegenerative diseases remains unclear, despite most studies portraying these cells as detrimental. Identifying novel markers that discriminate protective from harmful CD8+ T cell subsets, along with elucidating the factors involved in their generation and differentiation, is fundamental and may pave the way for new therapeutic strategies.

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# Chapter 2

## Aims of the thesis

# Aims of the thesis

Despite increasing evidence supporting a dynamic interaction between the immunological system (IS) and the central nervous system (CNS), the role of the adaptive branch of the IS, namely human effector-memory CD8+ T cells in cognitive function remains poorly understood. Although these CD8+ T cells have frequently been associated with deleterious effects, emerging studies suggest that they may also exert beneficial function, which can be explained by their heterogeneity and multifunctionality. A deeper characterization of these highly differentiated CD8+ T cells is therefore essential to identify (bio)markers that distinguish beneficial from detrimental subsets, and to elucidate how factors such as IL-15 influence their generation.

In this context, the aims of the thesis were:

1. To perform a comprehensive immunological characterization of peripheral blood mononuclear cells as well as the expression of HLA class I molecules in a cohort of elderly volunteers differing in their cognitive status.

2. To investigate the impact of IL-15 on the expression of CD8β at the cell surface of IL-15-cultured naïve CD8+ T cells as well as on the mRNA levels of CD8α and CD8β isoforms.

3. To determine the expression levels of the tyrosine kinase Lck following culture of CD8+ T cells with IL-15.

# Chapter 3

## CD45RA, CD8β, and IFNγ Are Potential Immune Biomarkers of Human Cognitive Function

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# CD45RA, CD8β, and IFNγ Are Potential Immune Biomarkers of Human Cognitive Function


## Abstract

There is increasing evidence that in humans the adaptive immunological system can influence cognitive functions of the brain. We have undertaken a comprehensive immunological analysis of lymphocyte and monocyte populations as well as of HLA molecules expression in a cohort of elderly volunteers (age range, 64–101) differing in their cognitive status. Hereby, we report on the identification of a novel signature in cognitively impaired elderly characterized by: (1) elevated percentages of CD8+ T effector-memory cells expressing high levels of the CD45RA phosphate receptor (TEMRA$^{hi}$); (2) high percentages of CD8+ T cells expressing high levels of the CD8β chain (CD8β$^{hi}$); (3) augmented production of IFNγ by *in vitro* activated CD4+ T cells. Noteworthy, CD3+CD8+ TEMRA$^{hi}$ and CD3+CD8β$^{hi}$ cells were associated with impaired cognition. Cytomegalovirus seroprevalence showed that all volunteers studied but one were CMV positive. Finally, we show that some of these phenotypic and functional features are associated with an increased frequency of the HLA-B8 serotype, which belongs to the ancestral haplotype HLA-A1, Cw7, B8, DR3, DQ2, among cognitively impaired volunteers. To our knowledge, this is the first proof in humans linking the amount of cell surface CD45RA and CD8β chain expressed by CD8+ TEMRA cells, and the amount of IFNγ produced by *in vitro* activated CD4+ T cells, with impaired cognitive function in the elderly.



## Keywords

Effector-memory CD8+ T cells, elderly, brain cognition, HLA class I, healthy aging, CD4+ IFNγ+.


## Introduction

A possible role for the immunological system in maintaining central nervous system (CNS) homeostasis has long been a matter of debate. Generally, it has been considered harmful in the context of neurodegenerative disorders with an autoimmune etiology (1). However, the accumulated evidence from clinical and experimental studies has consolidated the view that the innate and adaptive components of the immunological

system are crucial players in neuronal homeostasis and cognitive function by fine-tuning the balance between neuroprotection and neurodegeneration (2–4). Thus, early studies in mice showed that CD4+ and CD8+ T cell deficiency was associated with cognitive dysfunction and deficient remyelination after spinal-cord injury, and that thymus-derived CD4+CD25+ Treg cells specific for myelin self-antigens were necessary to confer protection after injury to the CNS (5–7). Although similar studies in humans are lacking, there is recent evidence suggesting that innate and adaptive immunological cells modulate hippocampal neurogenesis and behavior through not well understood mechanisms (8). In this respect, recent reports have shown that the human brain is inhabited by a large fraction of effector-memory CD8+ T cells expressing or not the CD45RA isoform, with some becoming tissue-resident upon expression of CD69 and CD103 (9). Resident CD8+CD69+ T cells in the human brain show increased expression of tissue homing and inhibitory receptors, and are low producers of granzymes (9). Some of these brain-resident CD8+ T cell features are shared by certain populations of peripheral blood effector-memory CD8+ T cells (10, 11), and are in accordance with experimental evidence suggesting that peripheral blood circulating CD8+ TEMRA cells migrate to the brain parenchyma (12, 13). In this respect, a number of clinical and experimental studies have shown that migration of peripheral blood effector-memory CD4+ and CD8+ T cells into the CNS may occur via the blood-cerebrospinal fluid barrier (BCSFB) and *via* the blood-brain barrier (BBB) (12–14) and that brain-resident T cells may leave and reenter the blood circulation (15). In this regard, a recent seminal study in Alzheimer's disease patients has identified an immune signature that consists of expansions of CD8+ TEMRA cells in peripheral blood as well as in the cerebrospinal fluid produced by the choroid plexus (16).

In humans, the concept of cognitive function is closely linked with a successful aging process and both have been associated with expansions of NK-like CD8+ T cells, a heterogeneous pool of CD8+ T cells that include CD8+ TEMRA cells (17–19). A fraction of the expanded CD8+ TEMRA cells found in peripheral blood of aged healthy people, including centenarians, are claimed to be driven by human cytomegalovirus, a wide-spread virus found in young and aged people (20, 21). Yet, the CD8+ TEMRA cell expansions seen in peripheral blood may be caused by other factors, such as aging itself (18, 22), anomalies in signaling molecules (23, 24), inflammatory environments (25), physical exercise (26), and homeostatic cytokines (19, 27). Moreover, exceptional aging is not necessarily associated with high levels of disability (28), bringing up the question of which factors, endogenous and/or exogenous, may contribute to better cognitive function in the elderly (17). In this respect, recent human studies have provided evidence that expression of certain NK receptors (CD56 and NKG2D), but not others (NKG2A and

KIR2DL1), by human NK-like CD8+ T cells is associated with better cognitive and physical function among elderly people (18). In line with these results, Serra-Miranda et al., described an immunological signature associated with better cognitive performance in healthy senior people that is characterized by low numbers of effector-memory CD4+ T cells and high numbers of B cells in peripheral blood (29). Although the molecular mechanisms used by the CD8+ T cells, and their receptors, to modulate cognitive, behavioral and physical functions in the elderly are presently uncertain, the involvement of secreted cytokines and other factors in response to the sensing of the inhabited environment are likely candidates in mediating this important biological function (19, 30, 31).

To further explore an association between immunological cells and cognitive function in humans, we undertook a comprehensive analysis of peripheral blood lymphocyte and monocyte populations in a cohort of elderly volunteers. The results revealed that the expression of CD45RA and CD8β by CD8+ T cells, the production of IFNγ by activated CD4+ T cells, and the presence of the HLA-B8 molecule constitute a novel immunological signature that discriminates between cognitively impaired and unimpaired elderly.

# Material and Methods

## Subjects and Classification Criteria

A total of 86 volunteers were recruited from elderly people from retirement homes and day care centers of the Beira Interior region of Portugal (EBIcohort, https://icon-cics.weebly.com/ebicohort-english.html). Blood from each volunteer was collected in EDTA tubes (10mL, for phenotype studies) and heparin tubes (4mL, for IFNγ studies) and assigned a double identification code, according with the Ethics Committee approved proposal. Coded samples were processed within hours of collection to obtain peripheral blood mononuclear cells (PBMC) and plasma. One mL aliquots of whole blood and plasma were cryopreserved in a −80°C freezer for later studies. PBMC were immediately phenotyped and functionally characterized.

Volunteers were evaluated by a trained team which assessed the volunteers using the Global Deterioration Scale (GDS), a revised Addenbrooke's Cognitive Examination (ACE-R) test (32) and also performed physical activity analysis. For physical activity, a three-meter walking test was performed following previous described guidelines (33). The gait speed was determined as the distance traversed (3-meters) by the time between the first and the last step. The volunteers were classified in groups using the GDS, in which cognitive status is defined by GDS, ACE-R and clinical information. The groups

are the following: A) Volunteers with no cognitive impairment (NCI) were those with GDS stages 1 and 2, having an ACE-R value indicative of normal cognitive impairment (106.49 ± 3.05% of minimal normal score) and did not have clinical indications of disease involving cognitive impairment; B) volunteers with mild cognitive impairment (MCI) were those with GDS stage 3, and having an ACE-R score slightly lower than minimal considered as normal (media of 73.6 ± 3.61% of minimal normal cognitive impairment level); C) volunteers with moderate to severe cognitive impairment (MtSCI, thereafter CI) were those with GDS stages 4 or above (34, 35), that include 14 volunteers that performed ACE-R tests (47.51 ± 4.91% of minimal normal cognitive impairment level), and 8 volunteers with clinical information indicating existence of dementia or Alzheimer's disease, for which there was not possible to apply ACE-R test. All obtained data from volunteers (personal, clinical and evaluation data) were stored according with data protection regulation and legal directives.

Of the total cohort, a total of 34 volunteers were excluded from analysis. The exclusion criteria included withdrawal from the study, infection other than CMV, diagnosis of previous stroke, Parkinson's disease, neoplasia, psychiatric disorders such as epilepsy, trauma or absence of clinical data. The remaining 50 volunteers included 13 volunteers with no cognitive impairment (NCI, 8 males and 5 females), 15 volunteers with mild cognitive impairment (MCI, 3 males and 12 females) and 22 volunteers with cognitive impairment (CI, 4 males and 18 females). For HLA typing two additional volunteers were studied, one male NCI and one male CI. This study was approved by the local Ethics Committee in accordance with the Declaration of Helsinki (Ref. Number CE-UBI-Pj-2017-012). All the participants or their legal representatives gave their written informed consent.

## Cells and Flow Cytometry Studies

Peripheral Blood Mononuclear Cells (PBMC) were obtained from a cohort of elderly volunteers differing in their cognitive status after centrifugation over Lymphoprep (STEMCELL Technologies). Contaminating red blood cells (RBC) were lysed in RBC lysis solution (10 mM TRIS, 155 mM $NH_4Cl$, pH 7.4) for 10 min at 37°C. For cell surface staining of lymphocytes, approximately $0.5x10^6$ PBMC were incubated in 96-well round-bottom plates at 4°C in the dark for 45 min with combinations of the different fluorochrome-conjugated antibodies, previously diluted in staining solution (Phosphate-Buffered Saline (PBS), 0.2% BSA, and 0.1% $NaN_3$). For monocyte labeling, cells were first incubated with Human TruStain FcX (BioLegend) for 10 min at room temperature prior to cell surface staining. Appropriate combinations of fluorochrome-conjugated monoclonal antibodies against CD3, CD4, CD8α, CD8β, CD14, CD16, CD19, CD28,

CD45RA, CD56, CD202b, CCR7, NKG2D, KIR2DL1, and IFNγ, together with irrelevant isotypes were used (**Supplemental Table 3.1**). After staining, cells were washed, acquired in a BD Accuri C6 (BD Biosciences) and analyzed using BD Accuri C6 software (BD Biosciences) or FlowJo software (FlowJo, LLC, for GMFI calculations). For lymphocytes, a minimum of 10,000 and a maximum of 20,000 events were acquired within the CD3+CD4+ and CD3+CD8+ T cell regions after gating on the lymphocyte region as determined by FSC and SSC. For monocytes, 20,000 events were acquired on the monocyte region as determined by FSC and SSC.

## Cell Activation and IFNγ Production

For IFNγ detection by activated T cells, 100µL of heparinized blood per test were diluted with 400µL of RPMI medium (Merck Millipore) and placed in 24-well plates. The diluted whole blood was stimulated by adding Cell Activation Cocktail containing PMA, Ionomycin and Brefeldin A (BioLegend) for 4 h in an incubator at 37°C and 5% CO2. After stimulation, cells were harvested and RBC were lysed twice in RBC lysis solution for 10 min at 37°C. Cells were then labeled with fluorochrome-conjugated monoclonal antibodies against cell surface receptors CD3, CD4, CD8 and CD28 in staining buffer in 96-well round-bottom plates for 45 min at 4°C in the dark. After extracellular labelling, cells were fixed for 30 min and permeabilized using eBioscience™ Intracellular Fixation & Permeabilization Buffer Set. After washing, cells were stained with FITC-conjugated anti-IFNγ or mouse IgG1-FITC (BioLegend, **Supplemental Table 3.1**) for 30 min at room temperature. After the washing steps, cells were resuspended in PBS, and whenever possible between 10-20 thousand events within the CD3+CD4+ and CD3+CD8+ T cell regions acquired using BD Accuri C6 flow cytometer (BD Biosciences) and analyzed using BD Accuri C6 software (BD Biosciences).

## Cytomegalovirus Seropositivity

For CMV detection, cryopreserved plasma samples were thawed and anti-CMV IgG antibodies detected by using 96-well microplate ELISA kits (Demeditec), according to manufacturer instructions. Tests were performed in duplicate and the amount of CMV-specific IgG antibody bound calculated using a BioRad xMark™ Microplate Absorbance Spectrophotometer. The concentration of IgG antibodies was calculated by comparing to a reference curve obtained with calibrators (i.e., human serum diluted with PBS, with 1, 10, 30, 90 U/mL of anti-CMV IgG antibodies) following manufacturer instructions.

## HLA Determination

DNA was extracted from peripheral blood using the MagAttract® DNA Blood Midi M48 kit or QIAamp DNA Stool Mini Kit (QS). HLA typing was accomplished using One

Lambda® LABTypeSSO kits at low resolution level (serology equivalent) for HLA-A, -B, -C, -DRB1, -DQA1 and -DQB1 loci followed by Luminex® xMAP® technology. Data were deduced in Fusion v4.2 software and are presented at serological equivalent or at antigen allele level when there is no serological equivalent.

### Statistical analysis

Statistical analysis was performed using SPSS software (version 26, IBM) and statistical significance was defined as $p<0.05$. Graphs were done using GraphPad Prism 7 software. Continuous variables were expressed as the mean ± standard error of the mean (SEM). Differences in means among the three cognitive status' groups were analyzed using one-way analysis of variance (ANOVA). Two-way ANOVA was used to examine the influence of two different categorical independent variables on one continuous dependent variable. When ANOVA showed significant differences, pairwise comparisons between means were tested using *post hoc* Bonferroni multiple comparisons test. Comparison between the percentage of CD8+IFNγ T and CD4+IFNγ+ T cells and the percentage of CD28– cells among $CD8\beta^{lo}$ and $CD8\beta^{hi}$ T cells was assessed using paired samples T-test. Fisher's Exact test was used to evaluate differences in the frequencies of categorical variables (e.g., HLA antigens/serotypes, gender and CMV seropositivity) of unrelated samples (cognitive status' groups). Z-test was used to evaluate differences within cognitive status' groups, and adjusted p-values were calculated using Bonferroni method. Spearman's ρ correlation coefficient was used to analyze the correlation between cognitive status' groups and continuous variables. Pearson correlation was used to analyze the correlation between two continuous variables. MetaboAnalyst (version 4.0) was used for cluster analysis and data visualization (36). Heatmap was created using Euclidean distance measure, Ward clustering algorithm, normalized data, and showing only group averages.

## Results

### Main Peripheral Blood Mononuclear Cell Populations Among Elderly Differing in Their Cognitive Status

Relevant clinical data of the different volunteers studied are shown in **Table 3.1**. The groups were age-matched, but the number of males in the NCI group (8 out of 13, 61%) was overrepresented in relation to the CI group (4 out of 22, 18%). However, two-way analysis of variance (ANOVA) to examine the influence of gender and cognition on the results obtained, showed that the results obtained are influenced by cognitive impairment ($p<0.05$) but not by gender. On the contrary, physical activity (as measured by three-meters walking) was statistically significantly different among groups (One-way Anova with Bonferroni's correction, $p=0.003$, **Table 3.1**). Also shown in **Table 3.1** are

the results of CMV seropositivity, which showed that 49 out of the 50 volunteers were IgG seropositive for CMV, as determined by ELISA. A thorough characterization of the different mononuclear cell populations present in peripheral blood samples was performed following the gating strategy illustrated in **Figure 3.1**. This strategy allowed us to characterize the three main populations of monocytes according to the expression of CD14 and CD16 into classical, intermediate and non-classical monocytes. Likewise, this strategy permitted to characterize T, B and NK populations as well as T cell subpopulations, according to the presence or absence of naïve and differentiation markers, such as CD28, CCR7, CD45RA, CD56, NKG2D, and KIR2DL1. In addition, we extended further our analysis by including antibodies against the CD8β chain, besides the widely used against the CD8α chain. While antibodies against CD8α identify both CD8+ T cells and CD8+ NK cells, antibodies against CD8β exclusively detect CD8+ T cells. The results showed that elderly people differing in their cognitive status have no significant differences in the percentage of the different monocyte populations present in peripheral blood nor in the percentages of CD3+ T cells, CD4+ T cells, CD8+ T cells, CD19+ B cells, CD3−CD56+ NK cells, and CD3+CD56+ NKT cells (**Supplemental Figure 3.1**). We could also not detect any other statistically significant difference in the percentage of naive (TN), central-memory (TCM), effector-memory (TEM) and effector-memory CD45RA+ (TEMRA) in CD4+ and CD8+ T cells among the three groups. Likewise, we found no differences in the expression of CD28, CD56, NKG2D and KIR2DL1 among CD4+ and CD8+ T cells (see **Supplemental Figure 3.1**).

**Table 3.1.** Relevant data of the volunteers under study.

| **Clinical data** | | | | ***P*-value[g/h]** |
|---|---|---|---|---|
| Cognitive Status (number)[a] | NCI (n=13) | MCI (n=15) | CI (n=22) | – |
| Age, Mean ± SEM (range) | 83.7 ± 2.4 (69-96) | 82.5 ± 2.1 (64-96) | 84.7 ± 1.9 (67-101) | NS[g] |
| Gender (M/F) | 8/5 | 3/12 | 4/18 | 0.023[h*] |
| Gait Speed (m/s) | 0.60 ± 0.05 | 0.41 ± 0.06 | 0.33 ± 0.04 | 0.003[g**] |
| Body Mass Index (BMI) | 25.4 ± 2.6 | 23.1 ± 2.7 | 20.9 ± 3.4 | NS[g] |
| CMV Seropositivy | 12/13 | 15/15 | 22/22 | NS[h] |

| **Lymphocytes (Mean ± SEM)** | | | | ***P*-value[g]** |
|---|---|---|---|---|
| % CD19+ (B cells)[b] | 4.6 ± 0.8 | 5.1 ± 1.0 | 3.7 ± 0.6 | NS |
| % CD3−CD56+ (NK cells)[b] | 24.5 ± 3.4 | 25.0 ± 3.0 | 25.4 ± 3.2 | NS |
| % CD3+CD56+ (NKT cells)[b] | 8.1 ± 1.5 | 8.3 ± 1.5 | 5.3 ± 0.9 | NS |
| % CD3+ T cells (T cells)[b] | 66.6 ± 2.9 | 67.0 ± 2.4 | 67.9 ± 3.3 | NS |
| % CD3+CD4+ T cells[b] | 34.8 ± 4.1 | 40.3 ± 3.6 | 39.6 ± 2.3 | NS |
| % CD4+ TN[c] | 40.9 ± 6.5 | 34.6 ± 6.3 | 48.8 ± 3.4 | NS |
| % CD4+ TCM[c] | 31.6 ± 3.2 | 37.7 ± 4.2 | 28.7 ± 2.5 | NS |
| % CD4+ TEM[c] | 20.1 ± 4.8 | 23.4 ± 3.7 | 16.6 ± 2.1 | NS |
| % CD4+ TEMRA[c] | 7.3 ± 2.6 | 4.4 ± 1.4 | 5.9 ± 1.7 | NS |
| % CD3+CD8+ T cells[b] | 31.4 ± 4.9 | 26.0 ± 2.9 | 27.7 ± 2.5 | NS |
| % CD8+ TN[d] | 11.9 ± 2.5 | 14.3 ± 2.6 | 14.3 ± 1.9 | NS |
| % CD8+ TCM[d] | 6.8 ± 1.2 | 9.2 ± 2.0 | 7.5 ± 1.3 | NS |
| % CD8+ TEM[d] | 19.5 ± 4.1 | 20.2 ± 3.7 | 17.4 ± 3.0 | NS |
| % CD8+ TEMRA[d] | 61.8 ± 5.1 | 56.4 ± 4.5 | 60.8 ± 4.3 | NS |
| **Monocytes (Mean ± SEM)** | | | | |
| % CD14+CD16− (classical)[e] | 85.5 ± 1.8 | 82.1 ± 3.6 | 81.4 ± 2.4 | NS |
| % CD14+CD16+ (intermediate)[e] | 4.4 ± 0.7 | 5.8 ± 0.9 | 6.8 ± 1.2 | NS |
| % CD14−CD16+ (non-classical)[e] | 6.0 ± 0.9 | 7.8 ± 2.9 | 7.0 ± 1.4 | NS |

| | | | | |
|---|---|---|---|---|
| % CD14+CD202b+[e] | 3.5 ± 0.9 | 3.1 ± 0.4 | 4.7 ± 1.0 | NS |
| % CD16+CD202b+[e] | 3.0 ± 0.7 | 2.2 ± 0.5 | 3.5 ± 0.9 | NS |
| **HLA Serological Antigens (%)[f]** | NCI (n=14) | MCI (n=15) | CI (n=23) | ***P*-value[h]** |
| HLA-A03 | 14.3 | 23.3 | 4.3 | 0.044*** |
| HLA-B08 | 0.0 | 6.7 | 17.4 | 0.034* |
| HLA-C12 | 17.9 | 26.7 | 6.5 | 0.045*** |
| HLA-DQB1_6 | 14.3 | 26.7 | 6.5 | 0.045*** |

[a]*Cognitive status determined by Global Scale Deterioration (see Materials and Methods section). NCI, No Cognitive Impairment; MCI, Mild Cognitive Impairment; CI, Cognitive Impairment.*

[b]*Percentages determined after gating in the lymphocyte gate.*

[c]*Percentages determined after gating in CD3+CD4+ T cells.*

[d]*Percentages determined after gating in CD3+CD8+ T cells.*

[e]*Percentages determined after gating in the monocyte gate.*

[f]*Percentages determined after applying the following formula: # of seropositive antigens × 100 /(# of volunteers per group × 2). In the serological analysis two additional volunteers (one NCI and one CI) without flow cytometry data were included.*

[g]*P-values determined by One-way ANOVA test; NS, Not significant.*

[h]*P-values determined by Fisher's Exact test with post hoc z-test to compare column proportions with p-values adjusted using Bonferroni's method.*

**Statistically significantly different between NCI and CI.*

***Statistically significantly different between NCI vs. MCI (p=0.045) and NCI vs. CI (p=0.003).*

****Statistically significantly different between MCI and CI.*

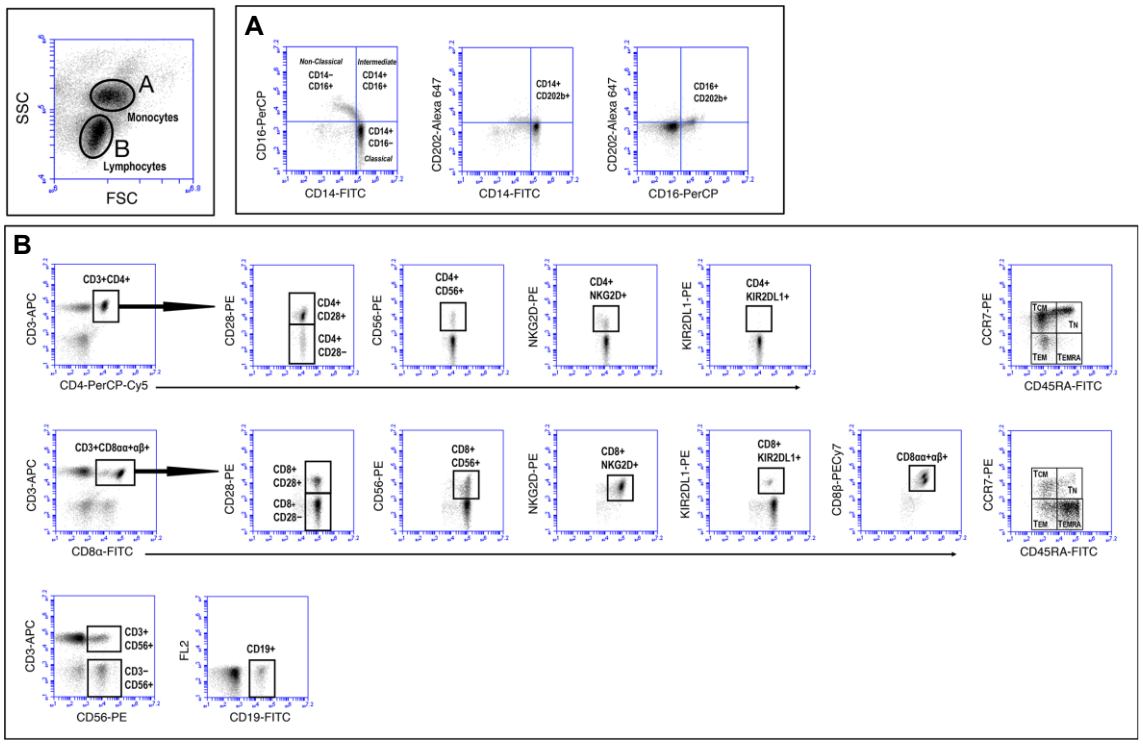



**Figure 3.1.** Gating strategy to study monocyte and lymphocyte populations. Peripheral blood mononuclear cells (PBMC) were isolated and stained as described in the Material and Methods section. Labeled cells were acquired in a BD Accuri C6 flow cytometer and monocytes and lymphocytes discriminated according to FSC and SSC characteristics (top dot-plot). Markers were analyzed after creating an electronic gate around monocytes **(A)** and lymphocytes **(B)**. The different T cell markers expressed (y axes) were analyzed after creating electronic gates in CD3+CD4+ T cells (**B**, upper row) and CD3+CD8+ T cells (**B**, middle row). NK (CD3−CD56+), NKT (CD3+CD56+) and B (CD19+) cells were calculated as indicated (**B**, lower row).

## The Level of Expression of CD45RA and CD8β in CD8+ T Cells Discriminates Between Cognitively Unimpaired and Cognitively Impaired Elderly

Even though we could not detect any significant difference in the percentages of TEMRA cells among the three volunteer groups, a thorough analysis of the level of expression of CD45RA among CD8+ TEMRA cells revealed differences in the amount of CD45RA. **Figure 3.2A** shows that expression of CD45RA at the cell surface of CCR7−CD8+ TEMRA cells, as determined by mean fluorescence intensity (MFI) values, was statistically significantly lower in no-cognitively impaired (NCI) volunteers when compared to both mild cognitively impaired (MCI) volunteers (31314 ± 3667 vs. 49489 ± 4996, mean ± SEM, p=0.034) and cognitively impaired (CI) volunteers (31314 ± 3667 vs. 50017 ± 4233, mean ± SEM, p=0.016). In order to avoid the influence of outliers in these results, we also compared geometrical mean fluorescence intensity values (GMFI). The results of this analysis were identical to the results of MFI. Thus, there were statistically significant differences between the 3 groups (One-way ANOVA, p= 0.008), with the CI group

presenting higher GMFI values. Moreover, after application of the Bonferroni test for multiple comparisons, statistically significant differences were found between the NCI and MCI groups (p=0.029), and between NCI and CI groups (p=0.011). This finding prompted us to perform an in-deep analysis of the CD8+ TEMRA population. The results showed the presence of two distinct CD45RA subpopulations (**Figure 3.2B**). One population expressing high levels of CD45RA (henceforth designated as CD8+ TEMRA$^{hi}$) and another population expressing lower levels of CD45RA (henceforth designated as CD8+ TEMRA$^{lo}$). When analyzed individually, this pattern of expression revealed marked differences between the NCI volunteers and the MCI and CI volunteers, with the two later showing a noticeable and sharp CD8+ TEMRA$^{hi}$ population (**Figure 3.2C**). Determination of the relative percentage of CD8+ TEMRA$^{hi}$ and CD8+ TEMRA$^{lo}$ cells within CD8+ TEMRA revealed marked differences between the NCI and CI groups (**Figure 3.2D**). Thus, in CI volunteers the relative percentage of CD8+ TEMRA$^{hi}$ cells was statistically significantly increased by two-fold when compared to NCI volunteers (35.2 ± 5.3 vs. 17.1 ± 2.7, mean ± SEM, p=0.041). Accordingly, the relative percentage of CD8+ TEMRA$^{lo}$ cells was markedly reduced (64.8 ± 5.3 vs. 82.9 ± 2.7, mean ± SEM, p=0.041). Similar results were observed when the percentage of CD8+ TEMRA$^{hi}$ and CD8+ TEMRA$^{lo}$ cells within CD8+ T cells were compared (data not shown). Importantly, cross-correlation studies revealed that the relative percentage of CD8+ TEMRA$^{hi}$ and CD8+ TEMRA$^{lo}$ cells were positively (p=0.289, p=0.042) and negatively (p=−0.289; p=0.042), respectively, associated with cognitive status.

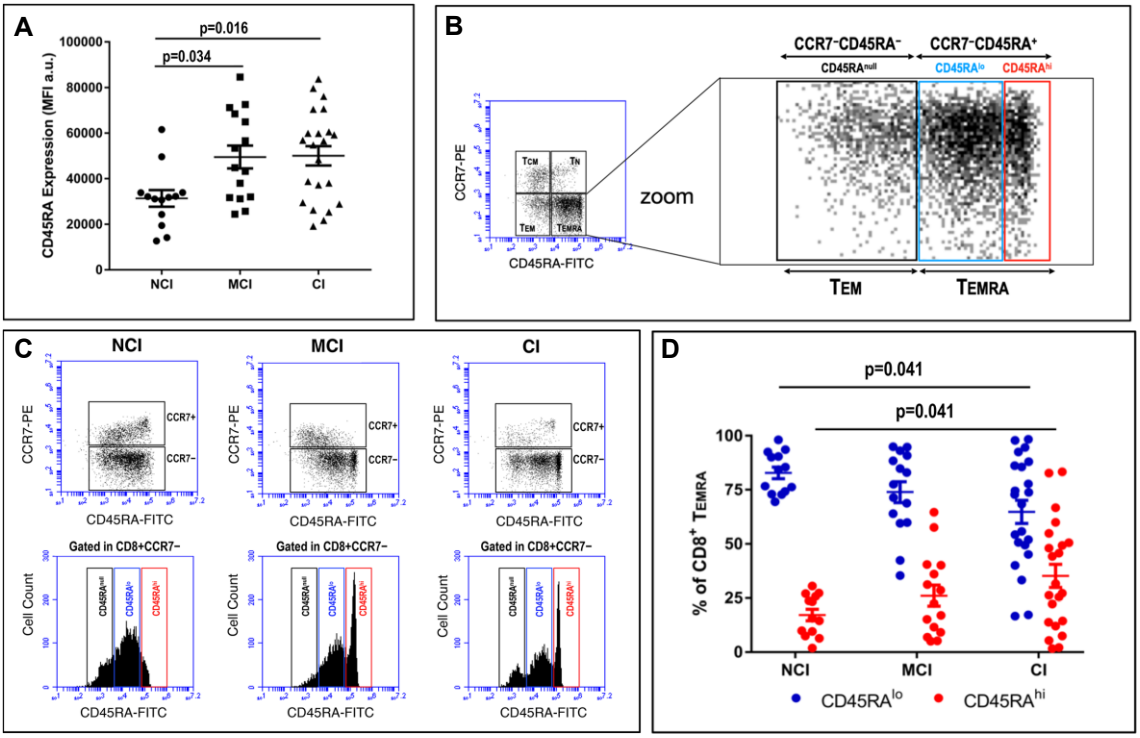


**Figure 3.2.** PBMC were isolated, stained and acquired as described in the legend of **Figure 3.1**. **(A)** Mean Fluorescence Intensity (MFI) values (a.u., mean ± SEM) of the expression of CD45RA after gating on CD3+CD8+CD45RA+ T cells in the three groups of elderly volunteers. Statistically significant differences between groups are indicated (ANOVA with Bonferroni's correction). (**B**, left) Dot-plot of CCR7 vs. CD45RA expression in CD3+CD8+ gated T cells showing naïve (TN), central-memory (TCM), effector-memory (TEM) and effector-memory CD45RA+ (TEMRA) cells. (**B**, right) Zoom of dot-plot lower quadrants (TEM + TEMRA) showing the existence of distinct subpopulations according to the level of expression of CD45RA: $CD45RA^{null}$, $CD45RA^{lo}$, and $CD45RA^{hi}$. **(C)** Plots of CCR7 vs. CD45RA expression in CD3+CD8+ gated T cells (upper row dot-plots) and CD45 expression (lower row histograms) in three representative volunteers. Histograms show CD45RA expression in CD3+CD8+CCR7− gated $T_{EM}$+$T_{EMRA}$ cells. Three distinct CD8+CD45RA T cell populations differing in their MFI values can be distinguished: $CD8+CD45RA^{null}$, $CD8+CD45RA^{lo}$ and $CD8+CD45RA^{hi}$. **(D)** Graph showing the relative percentage of $CD45RA^{lo}$ (blue circles) and $CD45RA^{hi}$ (red circles) in CD8+CCR7−CD45RA+ gated T cells in the three volunteer groups (mean ± SEM). Statistically significant differences between groups are indicated (ANOVA with Bonferroni's correction). a.u., arbitrary units.

On the other hand, the large majority of studies characterizing phenotypically and functionally CD8+ T cell populations in humans have used antibodies that recognize the CD8α chain. Here, we show that the use of antibodies against the CD8β chain revealed the existence of two distinct CD8+ T cell subpopulations based on the level of expression of the CD8β chain: $CD3+CD8\beta^{lo}$ T cells and $CD3+CD8\beta^{hi}$ T cells (**Figure 3.3A**). Determination of the percentages of these two CD8β+ T cell populations showed that NCI volunteers have higher percentages of $CD3+CD8\beta^{lo}$ T cells than CI volunteers (51.7 ± 4.8 vs. 33.5 ± 4.1, mean ± SEM, p=0.016), while presenting lower percentages of $CD3+CD8\beta^{hi}$ T cells (26.3 ± 3.3 vs. 46.0 ± 3.6, mean ± SEM, p=0.002) (**Figure 3.3B**). Given the resemblances between the expression of CD45RA and CD8β in cognitively unimpaired and cognitively impaired elderly, we decided to ascertain whether they were related populations. The existence of a robust positive correlation between CD8+ $T_{EMRA}^{lo}$ cells and $CD8\beta^{lo}$ T cells (**Figure 3.3C**, r=0.549, p<0.001) is a strong indication that they are similar CD8+ T cell populations. Finally, in order to better visualize the distribution of the aforementioned CD8+ T cell populations among the different cognitive groups, a heatmap representation was generated. This analysis revealed the existence of two clusters whereby the NCI group was enriched for CD8+ $TEMRA^{lo}$ and $CD8\beta^{lo}$ T cells, while the CI group was enriched for CD8+ $TEMRA^{hi}$ and $CD8\beta^{hi}$ T cells (**Figure 3.5**).

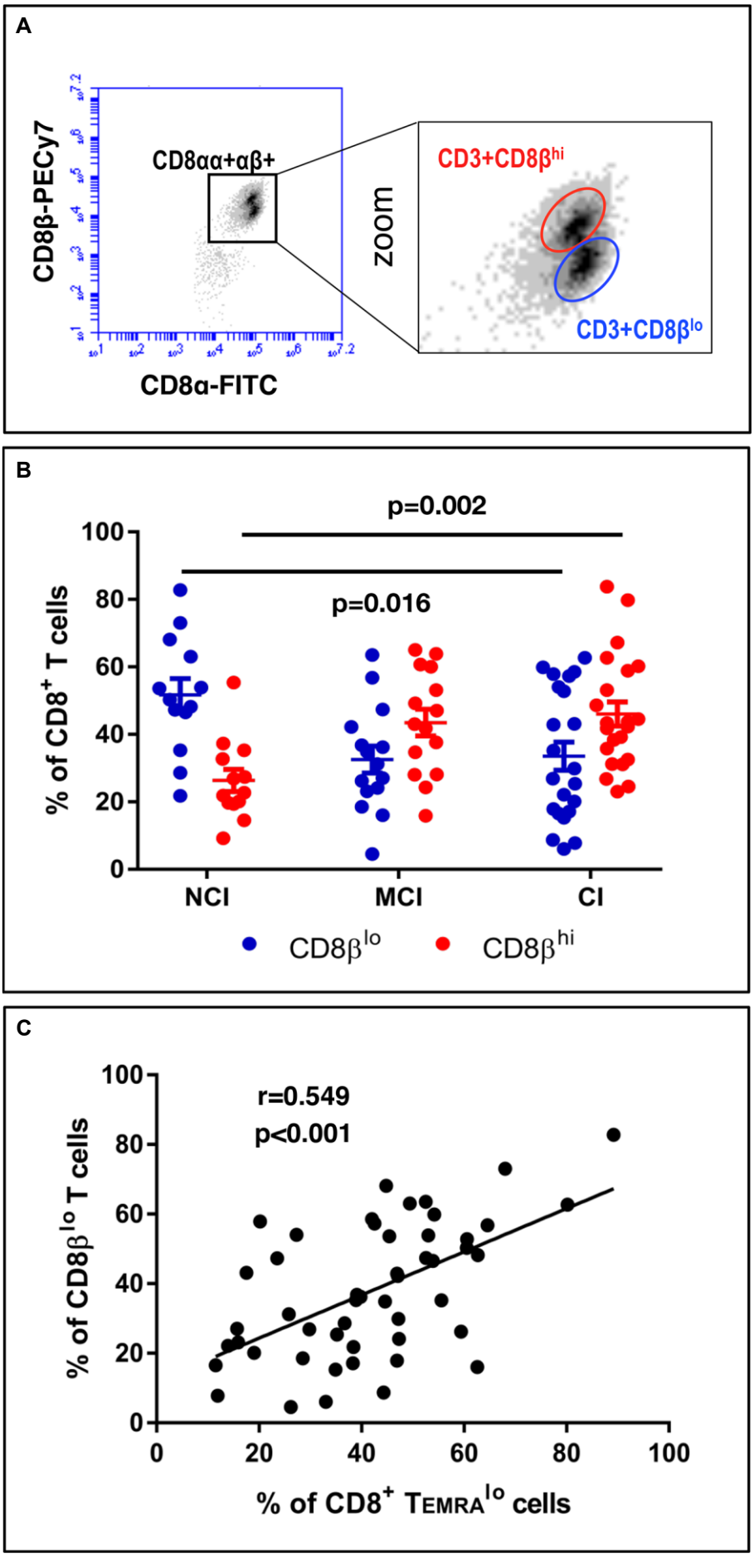

A
CD8β-PECy7
CD8αα+αβ+
CD8α-FITC
zoom
CD3+CD8β^hi
CD3+CD8β^lo
B
p=0.002
p=0.016
% of CD8+ T cells
100
80
60
40
20
0
NCI
MCI
CI
CD8β^lo
CD8β^hi
C
r=0.549
p<0.001
% of CD8β^lo T cells
% of CD8+ TEMRA^lo cells
0
20
40
60
80
100

**Figure 3.3.** PBMC were isolated, stained and acquired as described in the legend of **Figure 3.1.** (**A**, left) Dot-plot of CD8β vs. CD8α expression in CD3+CD8+ gated T cells. (**A**, right) Zoom of CD3+CD8αβ+ T cells showing the existence of two distinct populations according to the level of expression of CD8β: CD3+CD8β$^{hi}$ and CD3+CD8β$^{lo}$. **(B)** Graph showing the percentage of CD3+CD8β$^{lo}$ (blue circles) and CD3+CD8β$^{hi}$ (red circles) in CD3+CD8+ gated T cells in the three volunteer groups (mean ± SEM). Statistically significant differences between groups are indicated (ANOVA with Bonferroni's correction). **(C)** Scatter-plot showing a significant positive correlation between the percentages of CD8+ TEMRA$^{lo}$ and CD8β$^{lo}$ T cells (Pearson correlation, n=50). a.u., arbitrary units.

Comparison of the expression of CD28 by CD8β$^{lo}$ and CD8β$^{hi}$ T cells showed that the former are more enriched for CD28– cells (74.6 ± 2.3 vs. 45.5 ± 3.7, mean ± SEM, $p<0.001$). Interestingly, cross-correlation analysis revealed that the percentage of CCR7–CD45RA$^{lo}$, but not of CCR7–CD45RA$^{hi}$ CD8+ T cells, positively correlated with the percentage of CD8+CD28– T cells ($r=0.506$, $p<0.001$). These results indicate that CD8+CD45RA$^{lo}$ and CD3+CD8β$^{lo}$ T cells are predominantly CD28–, which is in accord with previous studies (37).

## Activated CD4+ T Cells From Cognitively Impaired Volunteers Produce Higher Levels of IFNγ Than Cognitively Unimpaired Volunteers

In order to evaluate production of IFNγ by activated T cells, a measure of the effector/regulatory potential of T cells, among the elderly groups, we stimulated PBMC with a combination of PMA and Ionomycin (Iono), followed by flow cytometry studies using combinations of anti-CD3, anti-CD28, anti-CD4, anti-CD8, and anti-IFNγ antibodies. Since PMA+Iono is known to induce a marked down-regulation of the CD4 receptor, we studied the production of IFNγ by CD4+ T cells by analyzing CD3+CD4+ T cells as well as CD3+CD8– T cells. As shown in **Figure 3.4A**, the percentage of CD4+IFNγ+ T cells (dot-plots) as well as the level of IFNγ produced by CD4+ T cells (histograms) were almost identical in either analysis. Likewise, we observed that the percentage of CD8+IFNγ+ T cells in response to PMA+Iono was about two-fold higher than the percentage of CD4+IFNγ+ T cells (79.5 ± 2.6 vs. 35.4 ± 2.9, respectively, mean ± SEM, $p<0.001$). This two-fold increase in the percentage of IFNγ-producing CD8+ T cells was observed regardless of the cognitive status of the volunteers (data not shown). However, when we analyzed the actual level of expression of intracellular IFNγ by activated CD4+ and CD8+ T cells, by determining the mean fluorescence intensity (MFI) values, we observed higher levels of IFNγ in activated CD4+ T cells from CI volunteers when compared to the NCI volunteers, with the MCI group displaying intermediate values (**Figure 3.4B**). As a result, the levels of expression of intracellular IFNγ in activated CD4+ T cells observed in the CI group were statistically significantly higher

than the levels of expression observed in the NCI group (99124 ± 3821 vs. 78069 ± 6236, mean ± SEM, p=0.038) (**Figure 3.4C**). Importantly, cross-correlation studies revealed that the values of MFI for IFNγ in activated CD4+ T cells correlated positively with cognition scores (ρ=0.439, p=0.022). In line with the heatmap representation generated for the CD8+ T cell populations, the CI group was enriched for CD4+ T cells producing high levels of IFNγ, contrasting with the NCI group, where the expression was lower (**Figure 3.5**).

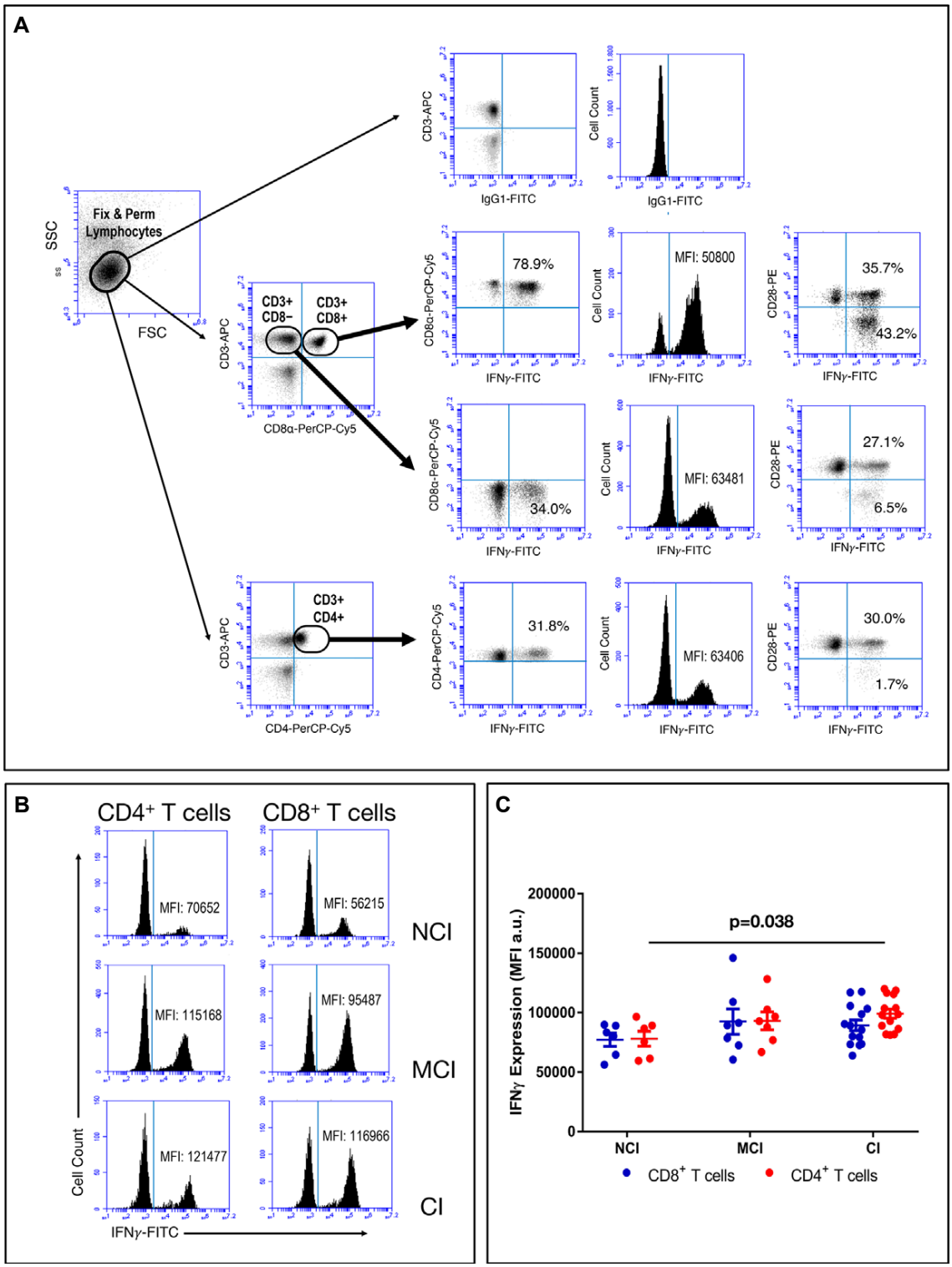



**Figure 3.4.** PBMC were isolated, activated with PMA+Iono for 4 h, washed and stained for CD3, CD4, CD8 and CD28, then fixed & permeabilized and stained again for IFNγ, and acquired in an Accuri C6 flow cytometer, as described in the Material and Methods section. **(A)** Graph illustrating the gating strategy to analyze IFNγ expression by activated T cells. Results show a representative experiment of the percentage of CD4+ and CD8+ T cells expressing intracellular IFNγ. Upper graphs show background staining using an irrelevant IgG isotype. Percentages of CD3+CD4+IFNγ+ T cells and CD3+CD8+IFNγ+ T cells, as well as IFNγ MFI values are shown. **(B)** Illustrative results of IFNγ MFI values in activated CD4+ (left histograms)

and CD8+ (right histograms) T cells from representative volunteers. **(C)** Graph showing MFI values of intracellular IFNγ (mean ± SEM) in gated CD4+ (red circles) and CD8+ (blue circles) T cells. Statistically significant differences between groups are indicated (ANOVA with Bonferroni's correction).

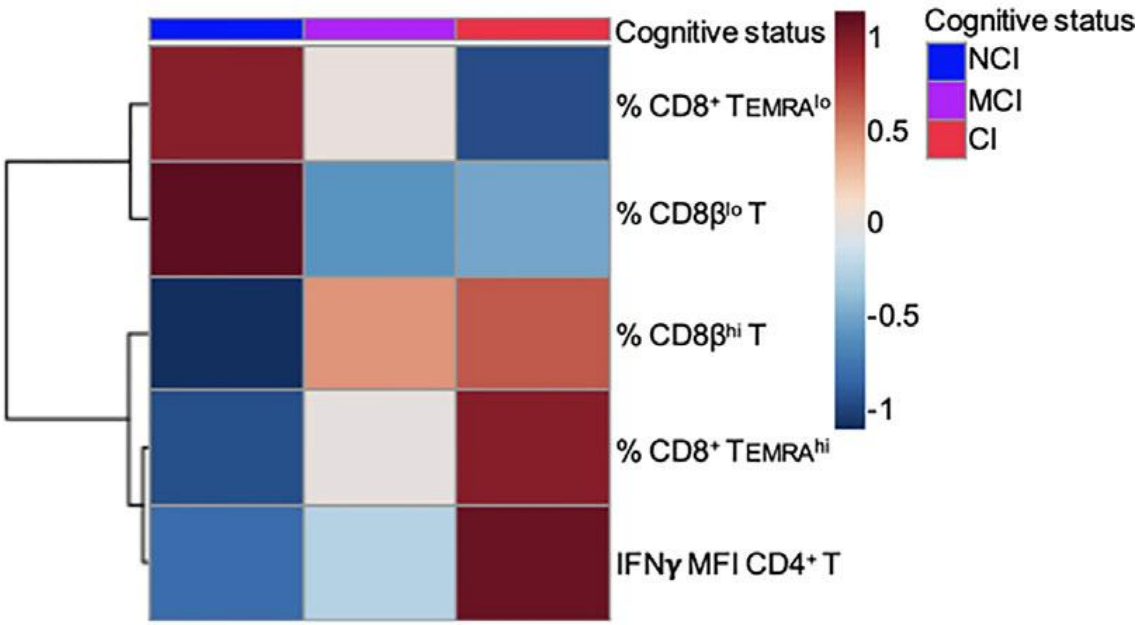
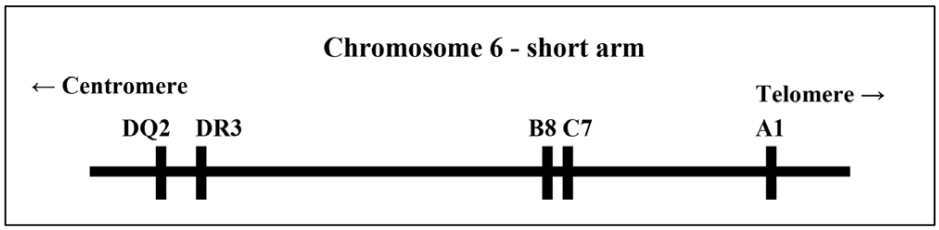



**Figure 3.5.** Heatmap showing two clusters of variables, Cluster 1 (% CD8+ TEMRA$^{lo}$ and % CD8β$^{lo}$) and Cluster 2 (% CD8+ TEMRA$^{hi}$, % CD8β$^{hi}$ and IFNγ MFI CD4+ T), associated with no cognitively impaired (NCI) and cognitively impaired (CI) volunteers, respectively.

## Higher Prevalence of the HLA-B8 Serotype in Cognitively Impaired Elderly

All the volunteers that were enrolled in this study were HLA typed for HLA-A, -B, -C, -DRB1, -DQA1 and -DQB1 loci and data presented at serological equivalent or at antigen allele level when there was no serological equivalent. The results of this analysis revealed that one-sixth of chromosomes of the CI volunteers expressed the HLA-B8 in heterozygosity, which contrasted with its complete absence in the NCI volunteers (17.4 vs. 0.0%, respectively, p=0.034). In other words, one-third of the CI volunteers were HLA-B8. Analysis of other HLA alleles revealed that a large percentage of the HLA-B8+ volunteers presented HLA alleles of the so-called ancestral haplotype, i.e., HLA-DQ2, HLADR3, HLA-B8, HLA-Cw7, and HLA-A1. Further analysis revealed that that the HLA-B8 and HLA-Cw7 alleles were overrepresented among the CI group (**Figure 3.6**). No other significant differences were observed in the expression of other HLA serotypes between NCI and CI volunteers (**Figure 3.6**). The only finding was an increase in the frequency of MCI volunteers expressing the HLA-A3, HLAC12 and HLA-DQB1_6 serotypes when compared to CI volunteers (see **Table 3.1**). Interestingly, analysis of the prevalence of HLA-B8 among the volunteers revealed that the percentage of CD3+CD8β$^{hi}$ T cells, but not the percentage of CD8+CD45RA$^{hi}$ T cells, is statistically

significantly increased in volunteers displaying the HLA-B8 serotype (53.6 ± 5.2 vs. 37.2 ± 2.5, mean ± SEM, p=0.008).




**Percentage of volunteers displaying the ancestral B8 haplotype alleles (DQ2, DR3, B8, Cw7, A1)**

| Ancestral Haplotype Alleles | DQ2 | DR3* | B8 | Cw7 | A1 | NCI (n=14) n (%) | MCI (n=15) n (%) | CI (n=23) n (%) | *P*-value# |
|---|---|---|---|---|---|---|---|---|---|
| Full Haplotype (1) | ■ | ■ | ■ | ■ | ■ | 0 (0.0) | 1 (6.7) | 3 (13.0) | 0.563 |
| Partial Haplotype I (2) | ■ | ■ | ■ | ■ | | 0 (0.0) | 0 (0.0) | 3 (13.0) | 0.245 |
| Partial Haplotype II (3) | | | ■ | ■ | ■ | 0 (0.0) | 0 (0.0) | 2 (8.7) | 0.497 |
| **Shared Ancestral Haplotype Alleles** | | | | | | | | | |
| (1) and (2) | ■ | ■ | ■ | ■ | | 0 (0.0) | 1 (6.7) | 6 (26.1) | 0.059 |
| (1) and (3) | | | ■ | ■ | ■ | 0 (0.0) | 1 (6.7) | 5 (21.7) | 0.150 |
| (1) and (2) and (3) | | | ■ | ■ | | 0 (0.0) | 1 (6.7) | 8 (34.5) | **0.012**** |

**Figure 3.6.** Upper figure represents part of the major histocompatibility complex (MHC) region, also known as the human leukocyte antigen (HLA) region on the short arm of chromosome 6. The table beneath shows the ancestral B8 haplotype alleles or its partial fragments (in red). The haplotypes were reconstructed by imputation of the allele markers and are putative, because it was not possible to strictly prove their segregation from family studies. The number of volunteers and the percentage within the cognitive status groups are shown. The shared B8 ancestral haplotypes are also shown (in green). *DR17 serotype; NCI, No-Cognitive Impairment; MCI, Mild Cognitive Impairment; CI, Cognitive Impairment. #P-values were calculated using Fisher's Exact Test with *post hoc* z-test to compare column proportions with p-values adjusted using Bonferroni's method. **Statistically significantly different between NCI and CI groups.

## Discussion

During the last years there has been a change of dogma whereby T cells are also endowed with properties that promote behavioral improvement, brain plasticity and cognition (4, 38–41). Indeed, recent studies in clinical and experimental neurodegenerative settings have provided important insights into the neuroprotective role of T cells (42–44). Even though the molecular cues behind these associations are presently uncertain, these studies suggest that certain effector-memory T cell populations may favor a healthy aging, while others may be detrimental (18).

In order to gain further insights into the relationship between T cell populations in peripheral blood and healthy aging, we undertook a comprehensive immunological analysis of lymphocyte and monocyte populations as well as of HLA molecules expression in an aged-matched cohort of elderly volunteers differing in their cognitive status. Our results show for the first time that a subpopulation of CD3+CD8+ T cells characterized by the expression at the plasma membrane of high levels CD45RA (CD8+ TEMRA$^{hi}$) and high levels of the CD8β chain (CD3+CD8β$^{hi}$) is overrepresented in elderly people with impaired cognitive status. On the contrary, elderly people with unimpaired cognitive status have CD3+CD8+ T cells characterized by the expression of low levels of cell surface CD45RA (CD8+ TEMRA$^{lo}$) and CD8β (CD3+CD8β$^{lo}$). Likewise, we show that CD4+ T cells from elderly people with impaired brain cognitive status activated *in vitro* with PMA/Ionomycin produce about two-fold more IFNγ than elderly people with unimpaired brain cognitive status. Finally, we report that the HLA-B8 serotype is absent in the group of elderly people with unimpaired brain cognitive status but present in 34% of elderly people with impaired brain cognitive status. More precisely one-sixth of chromosomes of the CI volunteers (17.4%) expressed the HLA-B8 in heterozygosity. On the one side, these results can be interpreted as meaning that CD8+ TEMRA$^{hi}$ and CD8β$^{hi}$ T cells, together with high IFNγ-producing CD4+ T cells and the presence of HLA-B8, are immunological biomarkers associated with unhealthy aging (i.e., impaired brain cognitive function). On the other side, the same results can be interpreted to mean that CD8+ TEMRA$^{lo}$ and CD8β$^{lo}$ T cells are immunological biomarkers associated with healthy aging (i.e., unimpaired brain cognitive function). In any case, these are novel and insightful data that point to selected subsets of highly differentiated CD8+ T cells as potential protectors of normal cognitive functions, as proposed by others (18, 40), and substantiate the double-edged role of IFNγ on cells of the CNS (45). Moreover, the finding of a prevalence of the HLA-B8 serotype among cognitively impaired volunteers unveils a novel association between impaired cognition and an HLA class I allele that is worthwhile confirming on a larger scale. The existence of two populations of circulating human CD3+CD8+ T cells differing in the expression of the tyrosine phosphatase isoform CD45RA and the CD8β chain is not new and has been previously reported (46). However, to our knowledge, this is the first proof in humans that the levels of expression of CD45RA and CD8β by CD8+ T cells are associated with cognition in a cohort of elderly volunteers.

These results are highly relevant because they suggest that CD8+ TEMRA$^{lo}$ and CD8β$^{lo}$ T cells are related subsets that might play important roles in keeping brain's cognitive function. Thus, they are highly differentiated oligoclonal CD8+ T cells that descend directly from expanded CD8αβ T cells *in vivo*, after down-regulation of the CD8β chain

(37, 47) and re-expression of CD45RA (48, 49). Moreover, CD8αβ$^{lo}$ T cells and CD8+ TEMRA$^{lo}$ cells are majoritarily CD28−, express CD45RA, respond poorly to TCR-mediated signals, and secrete IFNγ, perforin and granzyme upon stimulation (37, 47, 50, and this study). Interestingly, the CD8+ TEMRA$^{lo}$ cells described in this study resemble the CD8+ TEMRA+ cells described by Romero et al. (51) but using a different combination of surface markers. In this respect, a recent study has shown that the percentages of CD8+ TEMRA cells in peripheral blood and cerebrospinal fluid (CSF) of a human cohort encompassing mild cognitive impairment (MCI) and Alzheimer's disease (AD) patients are negatively associated with cognition. The authors discuss the likelihood that the presence of CD8+ TEMRA cells in the CSF may promote neurodegeneration through their cytotoxic effector function (16). Our findings with a cohort of elderly people extend these results by showing that not all CD8+ TEMRA are necessarily negatively associated with cognition, but only those expressing high levels of CD45RA, i.e., CD8+ TEMRA$^{hi}$ cells. At the same time, we have unveiled an association between CD8+ TEMRA$^{lo}$ cells and preserved brain cognitive functions. Therefore, while CD8+ TEMRA$^{lo}$ cells and CD8αβ$^{lo}$ T cells might constitute potential pro-cognitive immunological biomarkers that favor brain's cognitive function, CD8+ TEMRA$^{hi}$ cells and CD8β$^{hi}$ T cells might constitute potential detrimental immunological biomarkers that disfavor brain's cognitive function and healthy aging. Although previous studies have shown an association between high percentages of CD8+ TEMRA cells and infection by CMV (20, 21), the fact that 49 out of 50 elderly volunteers were CMV seropositive argues against the possibility that the differences found between NCI and CI volunteers is due to CMV infection. Nevertheless, the possibility that the CD8+ TEMRA$^{hi}$ cells found in the CI volunteers is being driven in some unidentified way by HCMV or other viral antigens cannot be discarded completely.

Even though the environmental signals driving the increase in the levels of CD45RA and the downmodulation of the CD8β chain by circulating effector-memory CD8+ T cells are uncertain, TCR-mediated stimulation and cytokines such as IL-1, IL-2 and IL-6, but not IFNγ, are known to regulate their expression (37, 52–57). Of note, IL-1/IL-6 are increased in neurodegenerative disorders such as Alzheimer's and Parkinson's disease, as well as in cognitive dysfunction (58, 59). Moreover, in animal models, IL-1 was shown to influence cognitive function by affecting long-term potentiation and possibly neurogenesis, while IL-6 impacts cognitive function via effects on neurogenesis and synaptic plasticity (60). These findings warrant further investigations to elucidate what signals enhance CD45RA expression by circulating CD8+ TEMRA$^{lo}$ cells and, at the same time, slow down CD8β chain downmodulation by CD8β$^{hi}$ T cells during aging, and whether these changes are associated with the secretion of IL-1/IL-6 by CNS parenchymal cells. Regarding IFNγ, prior animal studies have shown that low doses can

stimulate neurogenesis and induce protective signaling pathways in microglia, oligodendrocytes and primary neurons, thus raising the possibility of playing a role in development and repair of the CNS (61, 62). However, when present at high levels it induces disease worsening effects in both glial cell types and has detrimental effects on cognitive function, perhaps by inhibiting neural stem/progenitor cell proliferation (63, 64). These, apparently, contradictory biological effects of IFNγ are in accord with the opposing roles of this cytokine on cells of the CNS (45). In this respect, Gate et al. reported that production of IFNγ by activated CD8+ TEMRA cells was increased in the cohort of MCI and AD patients, suggesting that it may play a harmful role (16). In this scenario, the higher levels of IFNγ produced by CD4+ T cells upon activation found in the CI volunteers constitute a further detrimental immunological biomarker in the elderly. Although in our study, IFNγ production by activated CD8+ T cells was also increased in the CI group in comparison to the NCI group, this difference did not reach statistical significance.

Finally, the prevalence of the HLA-B8 serotype in volunteers with impaired cognition adds to the body of knowledge linking HLA-class I molecules with disease, a finding worthwhile confirming on a larger scale. If it proves true in a large cohort study, the role of HLA-B8 might give clues as where to go next in elucidating cognitive impairment in the elderly. In this regard, it is worth mentioning that HLA-B8 is part of the so-called "autoimmune" ancestral haplotype (HLA-DQ2, HLA-DR3, HLA-B8, HLA-Cw7, and HLA-A1) which is carried by most Caucasians and known to be positively associated with autoimmune disorders and by elevated circulating levels of inflammatory cytokines, such as IL-1 and TNFα (65). Thus, to our knowledge, this is the first report showing and association between cognitive impairment and the presence of HLA-B8, or alleles of the ancestral haplotype. Given the special features of HLA-class I molecules, we propose that the association found between the presence of alleles of the ancestral haplotype, namely HLA-B8 and HLA-Cw7, and cognitive impairment in the elderly cohort could result from three circumstances. First, as a result of the role of HLA-class I molecules as peptide presenting molecules that drive CD8+ T cell expansion and differentiation (66). In this regard, the study of Gate et al., is the first report showing that CD8+ T cells found in the CSF of AD patients carry a clonal TCR specific for an EBV-derived peptide presented by HLA-B8 (16). However, as pointed out by the authors, the EBV-specific clones detected in the study were not the most highly expanded ones and the data are not evidence of a causal link between EBV infection and AD (16). Second, as a result of the non-immunological functions of HLA-class I molecules (67), whereby these could modulate receptor-mediated signaling and endocytosis in CNS cells, and regulate brain function and plasticity (68, 69). In this context, it is worth mentioning recent studies in animal

models showing that interactions between MHC-class I molecules and the insulin receptor in the brain could regulate neuronal insulin sensitivity in the aging and diseased brain (70). Third, as a result of shedding of cell surface HLA-class I molecules into the extracellular media, where they can exert immunoregulatory functions on neighboring cells (71). In this respect, a potential role for shed MHC-I molecules as modulators of neurodevelopment and neurorepair responses has been reported (72).

Although the possibility that common factors might impact simultaneously immune system and cognitive impairment cannot be disregarded, the results of this study reinforce the view that cells of the adaptive immunological system, namely highly differentiated CD8+ T cells, might play a pro-cognitive role in humans. On the other hand, the possibility that changes in cognition could also alter behavior/lifestyle and that this may then alter immune composition is an interesting possibility that deserves further investigation. Therefore, whether the CD8+ TEMRA$^{hi}$ cells, CD8β$^{hi}$ T cells, and IFNγ-producing CD4+ T cells identified in this study as potential deleterious biomarkers of brain's cognitive function in the elderly, are truly detrimental needs further investigations. If these adaptive T cell subsets are confirmed to have negative effects in brain's cognitive function, they may be subjected to therapeutical manipulation. Similarly, if the CD8+ TEMRA$^{lo}$ and CD8β$^{lo}$ T cells are corroborated as pro-cognitive, further investigations must address the mechanisms involved and how to guarantee their maintenance in peripheral blood.

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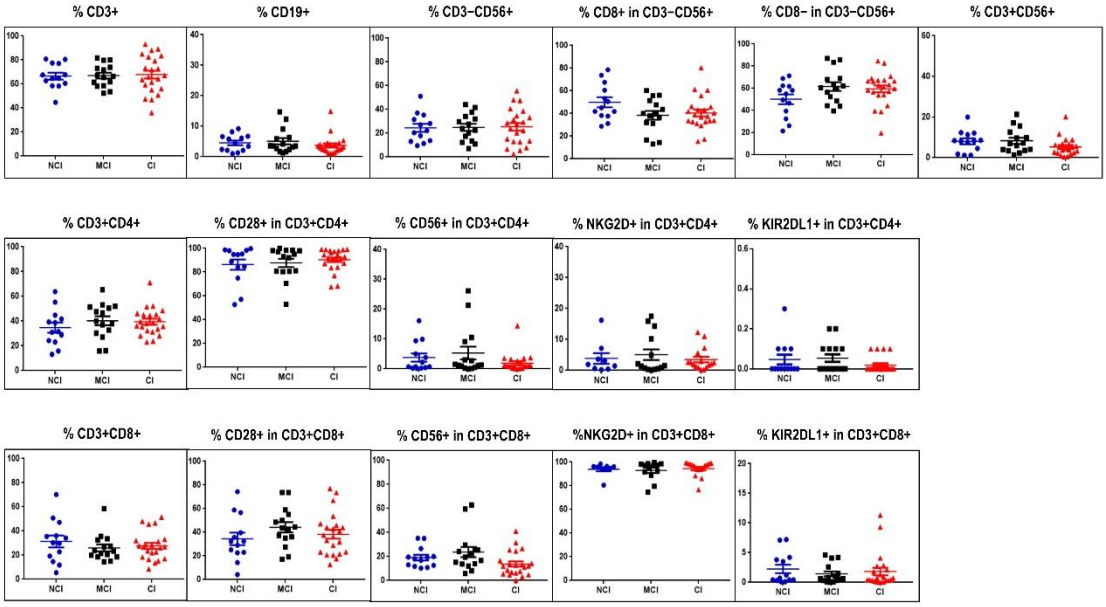
# Supplementary Material

**Supplemental Table 3.1.** List of Antibodies used in the study

| Antibody | Clone | Conjugate |
|---|---|---|
| CD3 | OKT3 | APC |
| CD4 | RPA-T4 | PerCP-Cy5.5 |
| CD8α | SK1 | FITC |
| CD8α | SK1 | PerCP-Cy5.5 |
| CD8β | SIDI8BEE | PE-Cy7 |
| CD14 | 63D3 | FITC |
| CD16 | 3G8 | PerCP |
| CD19 | HIB19 | FITC |
| CD28 | CD28.2 | PE |
| CD45RA | HI100 | FITC |
| CD56 | HCD56 | PE |
| CD202b (Tie2/Tek) | 33.1(Ab33) | Alexa Fluor 647 |
| CCR7 (CD197) | G043H7 | PE |
| NKG2D (CD314) | 1D11 | PE |
| KIR2DL1 (CD158a) | HP-MA4 | PE |
| IFNγ | 4S.B3 | FITC |
| Mouse IgG1 | MOPC-21 | FITC |

*All antibodies but CD8β (from eBioscience) were from Biolegend




**Supplementary Figure 3.1.** T cell populations in elderly volunteers. PBMC were isolated, stained and acquired as described in the legend of Figure 3.1. The distribution of the different T cell populations (percentage, mean ± SEM) in the three volunteer groups (NCI, MCI, CI) is shown in the different graphs.

# Chapter 4

## *In vitro* IL-15-activated human naïve CD8+ T cells down-modulate the CD8β chain and become CD8αα T cells

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# *In vitro* IL-15-activated human naïve CD8+ T cells down-modulate the CD8β chain and become CD8αα T cells


## Abstract

Antigen-driven human effector-memory CD8+ T cells expressing low levels of the CD8β chain have been previously described. However, little is known on a possible antigen-independent trigger. We have examined the impact that IL-15 has on the expression of CD8β on purified human naïve CD8+ T cells after CFSE labeling and culture with IL-15. As expected, IL-15 induced naïve CD8+ T cells to proliferate and differentiate. Remarkably, the process was associated with a cell-cycle dependent down-modulation of CD8β from the cell surface, leading to the generation of $CD8\alpha\beta^{low}$ and $CD8\alpha\beta^{-}$ (i.e., CD8αα) T cells. In contrast, expression of the CD8α chain remained steady or even increased. Neither IL-2 nor IL-7 reproduced the effect of IL-15. Determination of mRNA levels for CD8α and CD8β isoforms by qPCR revealed that IL-15 promoted a significant decrease in mRNA levels of the CD8β M-4 isoform, while levels of the M-1/M-2 isoforms and of CD8α increased. Noteworthy, CD8+ T cell blasts obtained after culture of CD8+ T cells with IL-15 showed a cell-cycle dependent increase in the level of the tyrosine kinase Lck, when compared to CD8+ T cells at day 0. This study has shown for the first time that IL-15 generates $CD8\alpha\alpha^{+}\alpha\beta^{low}$ and $CD8\alpha\alpha^{+}\alpha\beta^{-}$ T cells containing high levels of Lck, suggesting that they may be endowed with unique functional features.



## Keywords

IL-15, naïve CD8+ T cells, effector-memory CD8+ T cells, CD8β chain, downregulation, CD8α chain.


## Introduction

Human effector-memory CD8+ T cells (CD8+ TEM) have been thoroughly studied regarding their origin, phenotype and function. They are usually CD28–CCR7–CD45RA– but under certain conditions can re-express CD45RA, being designated as effector memory CD8+CD45RA+ T cells (CD8+ TEMRA) (1–3). An interesting study by Werwitzke et al., showed that about 80% of human peripheral blood CD8+ TEM cells re-express CD45RA and display low levels of the CD8αβ receptor, hence being $CD28^{-}CD45RA^{+}CD8\alpha\beta^{low}$ (4). Interestingly, the phenotypic similarities between

CD8+ TEMRA and effector-memory CD8αβ$^{low}$ T cells also include expression of a diverse array of activation and inhibitory receptors, and secretion of IFN-γ, perforin and granzymes upon activation (4–6), suggesting that they are similar CD8+ T cell populations. Noteworthy, these studies identified a population of CD8αα$^{+}$αβ$^{-}$ T cells (i.e., CD8αα) in the peripheral blood of healthy and HIV-infected humans and endowed with functional effector-memory features (4–7). Konno et al. suggested that the CD8αα T cells were descendants of clonally expanded CD8αβ$^{high}$ T cells *in vivo*, while Walker et al. suggested that the CD8αα T cells may represent circulating mucosal associated invariant T cells. By performing a TCRVβ CDR3-spectratyping analysis, the study of Konno et al. showed that CD8αβ$^{high}$ T cells were polyclonal, while CD8αβ$^{low}$ and CD8αα T cells were oligoclonal, suggesting that a fraction of CD8αβ$^{high}$ T cells clonally expanded, differentiated, and downregulated the CD8β chain (5).

Besides identifying functional populations of human CD8+ T cells, the β chain of the CD8αβ receptor plays an important role in assisting CD8αβ-mediated signaling and activation mediated by the TCR. Thus, the CD8β chain is thought to increase the efficiency of the *trans*-interaction between the TCR and MHC class I molecules, allow CD8αβ heterodimers to move into lipids rafts, and enhance the activation of the fraction of Lck molecules associated with the CD8α chain (8–12). The identification that the human CD8β gene encodes four alternatively spliced membrane-bound forms (M-1, M-2, M-3 and M-4) and two secreted forms (S-1 and S-2) (13, 14) introduced more complexity into this area. Indeed, the CD8β isoforms were shown to be differentially expressed by CD8+ T cell populations, with the M-1 isoform being mainly expressed in naïve CD8+ T cells (TN) and the M-4 isoform being predominantly expressed in CD8+ TEM (15). Moreover, mutational and functional studies with co-transduced human CD4+ T cells showed that expression of the M-4 isoform enhanced TCR-mediated recognition of antigen (16), reinforcing the importance of the CD8β chain in CD8+ T cell activation.

Despite the body of knowledge supporting the importance of the CD8β chain and the tyrosine kinase Lck in CD8+ T cell signaling and responses, studies examining the influence of factors other than peptide antigens on the expression of the CD8β chain and the tyrosine kinase Lck in human CD8+ T cells are lacking. Thus, while human CD8+ $T_{EM}$ cells that have reexpressed CD45RA (i.e., CD8+ TEMRA) have been shown to be generated by TCR-dependent and independent (i.e., cytokines) signals, such as IL-15 both *in vitro* (17–20) and *in vivo* (21, 22), human CD8αα T cells are only known to be generated by TCR-dependent signals (23). Whether IL-15 plays a role in the formation of human CD8αα T cells is not known. Prompted by our interest in characterizing molecular cues leading to the generation of CD8+ TEMRA cells and CD3+CD8αβ$^{low}$ T

cells, due to their association with chronic inflammatory disorders, including neurodegenerative disorders (3, 24), we used *ex vivo* human CD8+ T cells from the peripheral blood of regular healthy blood donors and cultured them *in vitro* in the presence of IL-15, and other members of the gamma common (γc) chain-dependent cytokines such as IL-2 and IL-7. The results demonstrated that IL-15, but not IL-2 or IL-7, induces a cell-cycle dependent downregulation of the CD8β chain, generating pools of CD8αβ$^{low}$ and CD8αα T cells, and a marked increase in the amount of total Lck.

# Material and Methods

## Ethics statement

Human peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of anonymous healthy regular blood donors kindly provided by the Centro do Sangue e da Transplantação de Coimbra (CST-C, Portugal) under a protocol approved by the Portuguese Institute of Blood and Transplantation (IPST, IP, Lisbon), the University of Beira Interior (UBI), and the Faculty of Health Sciences (FCS-UBI). The study protocol (INSIGHTHEM) was approved by the Ethics Committee of the University in accordance with the Declaration of Helsinki (Ref. Number CE-UBI-Pj-2017–012). PBMC were also obtained from the Blutspendezentrale at the University Hospital Düsseldorf under protocol accepted by the institutional review board at the University of Düsseldorf (study number 2019–383).

## Isolation of cells

PBMC were isolated from buffy coats after centrifugation over Lymphoprep (STEMCELL Technologies, France). Contaminating red blood cells were lysed in lysis solution (10 mM Tris, 155 mM $NH_4Cl$, pH 7.4) for 10 minutes at 37°C. Total and naïve CD8+ T cells were obtained from PBMC preparations by negative selection using MojoSort kits (BioLegend, USA). Purity was checked by flow cytometry using antibodies against CD3, CD4, CD8α, CD8β, CD45RA, CCR7, and was always higher than 96–97%.

## CFSE labeling and cell culture conditions

Freshly collected PBMC or isolated CD8+ T cells were immediately used for all studies. Cells were first labeled with CellTrace™ CFSE Cell Proliferation kit (Thermo-Fisher Scientific, USA) at a final concentration of 5μM for 5 minutes at room temperature (RT) in phosphate-buffered saline (PBS) with occasional mixing, followed by three washes with RPMI-1640 medium (Thermo-Fisher Scientific) containing 10% heat-inactivated fetal bovine serum (FBS). Then, CFSE-labeled PBMC ($1.0x10^6$/mL), total CD8+ T cells ($1.0x10^6$/mL) and naïve CD8+ T cells ($1.5x10^6$/mL) were cultured in 24-well plates

(Greiner Bio-One, Austria) in RPMI-1640 GlutaMAX medium (Thermo-Fisher Scientific) supplemented with 5% human serum (Sigma-Aldrich, USA) and 1% antibiotic-antimycotic solution (Sigma-Aldrich) at 37°C, 5% $CO_2$, and 95% humidity for 12 days. PBMC and total CD8+ T cells were cultured in the presence of IL-15 (R&D Systems, USA), whereas naïve CD8+ T cells were cultured in the presence IL- 15, IL-2 (Clinigen, Germany), IL-7 (R&D Systems), and combinations of IL-15+IL-2 and IL-15+IL-7. All cytokines were added at the beginning and at the sixth day of culture at a final concentration of 10 ng/mL.

## Flow cytometry studies

For cell surface staining, approximately $0.5x10^6$ cells were incubated in 96-well round-bottom plates or 5mL round-bottom tubes at 4°C in the dark for 30 minutes with combinations of different fluorochrome-conjugated antibodies in staining solution (PBS, 0.2% BSA, 0.1% $NaN_3$). **Supplementary Table 4.1** summarizes the antibodies used in this study. After staining, cells were washed, and a minimum of 20,000 events were acquired in a BD Accuri C6 (BD Biosciences, USA) or in a CytoFLEX flow cytometer (Beckman Coulter, USA). For intracellular staining, cells were first incubated with antibodies against cell surface receptors, as described. Then, cells were fixed for 30 minutes and permeabilized using the eBioscience™ Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific). After washing, cell aliquots were stained separately with irrelevant or anti-Lck antibody (see **Supplementary Table 4.1**) for 30 minutes at room temperature. After the washing steps, cells were resuspended in PBS and a minimum of 20,000 events were acquired in a BD Accuri C6 (BD Biosciences). For all acquisitions, doublets were excluded by FSC-A vs FSC-H. Results for extracellular and intracellular stainings were analyzed using BD Accuri C6 software (BD Biosciences), FlowJo software (FlowJo, LLC) or Kaluza Analysis Software (Beckman Coulter).

## Quantification of cell divisions

Cell divisions were determined by sequential halving of CFSE fluorescence intensity after the period of culture. In all the experiments performed, CFSE halving allowed to distinguish the different cell division cycles undergone by the CD8+ T cells. To quantitate changes in the expression of selected cell surface receptors (i.e., CD8α, CD8β, CCR7 and CD45RA) during the proliferation process electronic regions were created around positive cells in each cycle of cell division and mean fluorescence intensity (MFI) values were obtained (see **Supplementary Figure 4.1**). MFI values of cells that did not divide were used to normalize the MFI values of the dividing cells as follows: (MFI dividing

cells/MFI non-dividing cells) x 100. For each receptor, MFI was measured on positive cells, gated within each cell division (from 0 to 5, or more).

## RNA isolation, cDNA synthesis and qPCR

Total RNA was isolated from CD8+ T naïve cells prior and after culture with IL-15 using the RNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. All samples were treated with DNase I stock solution (Qiagen) as recommended by the manufacture. RNA quantification was performed using UV/Vis Nanophotometer spectrophotometer (Implen GmbH, Germany) and the purity was assessed using A260/280 ratio. The integrity of total RNA was based on visualization of 28S and 18S ribosomal RNA subunits under 1% agarose. Band intensity was assessed using gel documentation system 2000 (Bio-Rad, Germany). cDNA was synthesized from 0.5 or 1µg total RNA, in a total reaction volume of 20µL, using the Xpert cDNA Synthesis Kit (Ref. GK80.0100, GRiSP Research Solutions, Portugal) with Oligo(dT)20 primer according to the manufacturer's instructions. cDNA was either immediately used as template in qPCR or stored at -20°C prior to qPCR. Regarding qPCR, for each assay, 100 ng of cDNA (2µL or 1µL, depending on if cDNA was prepared with 0.5 or 1µg, respectively) was mixed in 20 µl final reaction mixed of a solution containing 10 µl of NZYSupreme qPCR Green Master Mix (2x), ROX plus (Ref. MB44002 NZYTech, Portugal), 0.4µM of each primer (**Supplementary Table 4.2**) and sterile water. All primers were evaluated and/or designed with NCBI Primer Blast and synthesized by STAB VIDA (Portugal). Real-time PCR reactions were settled with an initial denaturation and polymerase activation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds denaturation and 60°C for 1 minute annealing/extension. All reactions were run in duplicates in 0.2mL non-skirted 96-well PCR plates (Ref. AB0600, Thermo Fisher Scientific) with adhesive PCR plate seals (Ref. AB0558, Thermo Fisher Scientific). Negative controls without any template were processed in parallel and did not result in any qPCR signal. Specificity of primer pairs was verified by electrophoresis on a 2% (w/v) agarose gel in the presence of 0.05% Xpert Green DNA Stain (Ref. GS01.0001, GRiSP Research Solutions). The expected product size was confirmed by using the GRS Low Range Ladder Ref. GL011.0050, GRiSP Research Solutions). Standard curves were generated based on a five-fold dilution replicates series (without dilution, 1:5, 1:25 and 1:125) of a pool of different cDNAs (control and activated) analyzed in this work. Amplification efficiencies and correlation coefficients for each primer pair were calculated from the slopes of the standard curves using Excel. The relative mRNA expression of CD8α and CD8β were normalized using the measured expression level of two reference genes (GAPDH and RPS18), that were not regulated in the biological

system, using the ΔCq method: relative quantity reference/target = $2^{(Cq(\text{geometric mean of reference genes})-Cq(\text{gene of interest}))}$. The reference stability values for the reference genes were calculated using the CFX Maestro Software version 2.2 (Bio-Rad), according to the geNorm algorithm (25).

### Statistical Analysis

For flow cytometry data, statistical analysis was performed using SPSS 28 software (IBM, USA). Continuous variables were expressed as the Mean ± Standard Error of the Mean (SEM). Differences between the means of two continuous variables were analyzed using the paired samples T-test, whereas differences among the means of three or more continuous variables were analyzed using the one-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's *post hoc* tests. All data were checked for normality. Statistical significance was defined as $p<0.05$. For the qPCR data, comparison of relative quantity between control and activated cells was performed using Mann-Whitney U test, and the differences were regarded as significant when $p<0.05$. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad software Inc., USA).

## Results

### IL-15 induces CD8β chain down-modulation

In agreement with our previous results, long-term culture of human PBMC with IL-15 induced several cycles of CD8+ T cell division, as determined by CFSE halving. Noteworthy, analysis of the expression of CD8β after gating on the pool of CD3+CD8α$^+$ T cell blasts (**Figure 4.1A**, upper right quadrant), showed an evident down-modulation of the CD8β chain after several cycles of cell division, which led to the generation of a population of CD3+CD8αβ$^-$ (i.e., CD8αα) T cells in the most dividing CD8+ T cells (**Figure 4.1B**). Analysis of the percentage of dividing CD8αβ$^-$ versus dividing CD8αβ$^+$ T cells revealed that approximately 20% of the CD3+CD8αβ$^+$ T cells that expanded lost expression of CD8β, becoming CD8αα T cells (**Figure 4.1C**). However, as expected, it was the pool of CD3–CD8+ blast cells (**Figure 4.1A**, lower right quadrant) that took most advantage of the presence of IL-15 in culture. Thus, as shown in **Figure 4.1D**, the proliferating lymphocytes were almost completely negative for CD8β expression and were largely comprised on NK cells, as demonstrated by CD56 expression (**Figure 4.1E**). Next, we wanted to analyze the expression of CD8α vs CD8β in CD8+ TN, TCM, TEM or TEMRA at baseline levels in order to understand the relative expression of the CD8α and CD8β chains after culture with IL-15. To that, we labeled fresh collected PBMC with antibodies against CCR7, CD45RA, CD8α and CD8β. As shown in **Figure 4.1F**, while CD8+ TN cells are mainly constituted by CD8αβ$^{high}$ T cells, CD8+ TCM cells already

showed an inversion of the proportions of CD8αβ$^{high}$ vs. CD8αβ$^{low}$ T cells. In contrast, in the CD8+ TEM and TEMRA pools, the percentage of CD8αβ$^{low}$ T cells was predominant.

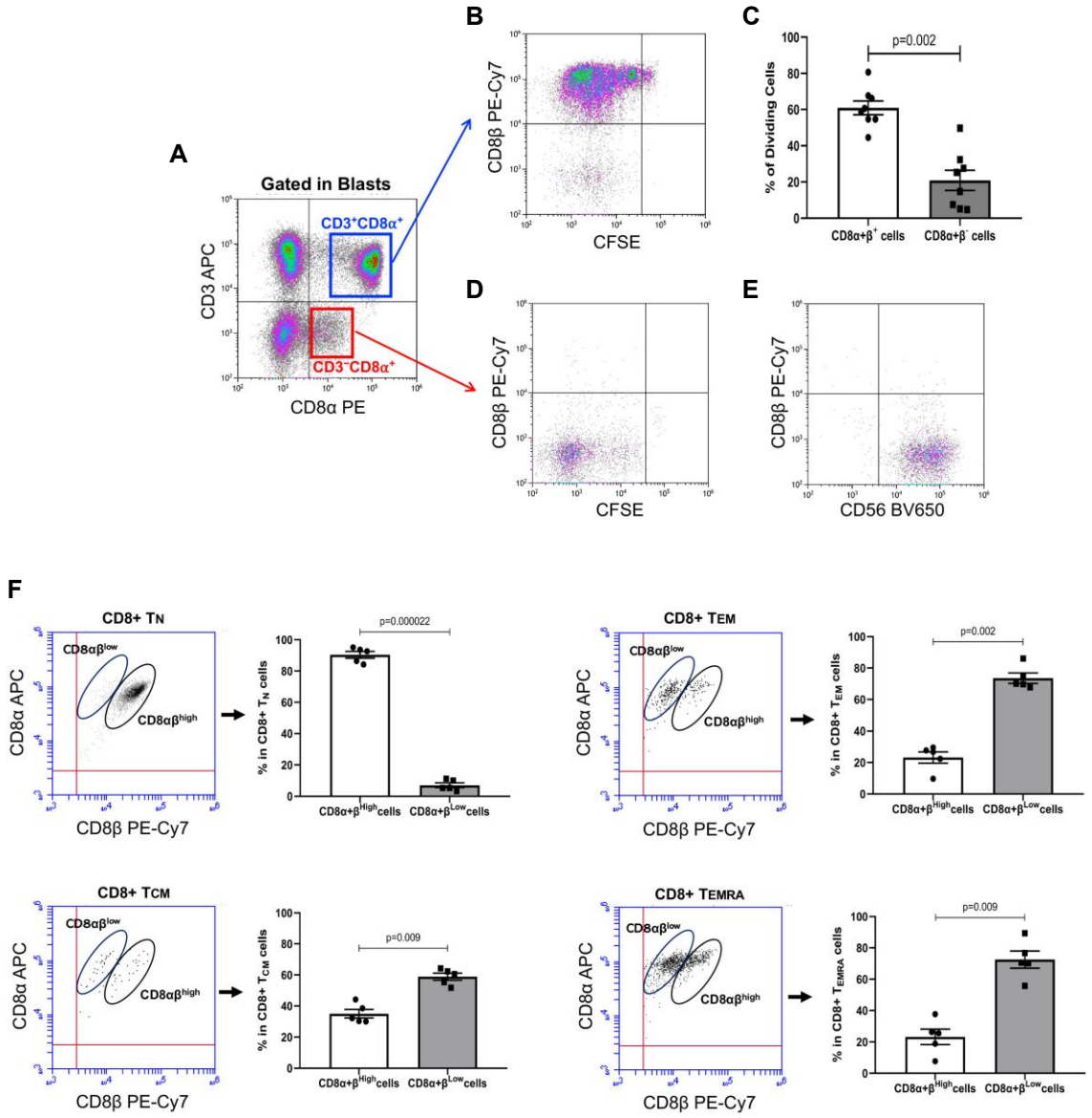


**Figure 4.1.** IL-15 induces down-modulation of CD8β in CD8+ T cells present in PBMC samples. PBMC were isolated from buffy coats from regular healthy donors by centrifugation over Lymphoprep, labeled with CFSE and cultured with IL-15 (10 ng/mL) for 12 days. At the end of the culture, cells were harvested, washed, and approximately $0.5\times10^6$ cells stained with fluorochrome-conjugated antibodies against CD3, CD8α, and CD8β (Accuri C6) or CD3, CD8β, and CD8α, CCR7, CD45RA and CD56 (CytoFLEX) and acquired. An electronic region was created around blast cells based on FSC and SSC, which were subsequently analyzed for CFSE fluorescence halving and cell surface expression of CD3, CD8α, CD8β, CD45RA, CCR7 and CD56. **(A)** Representative dot-plot showing CD3 vs. CD8α expression on blast cells after the 12-day culture period with IL-15. Electronic gates were created around CD3+CD8α$^+$ and CD3−CD8α$^+$ cell blasts. **(B)** Dot-plot shows CFSE halving vs. CD8β expression after gating in CD3+CD8α+ T cell blasts (upper right quadrant in Panel **(A)**. This gives us an unambiguous fluorescence emission of CD8β along the cycles of cell division. **(C)** Graph showing the percentage of dividing CD8αβ$^+$ and CD8αβ$^-$ T cells after 12-day culture with IL-15 (mean ± SEM, n=8). **(D)** Dot-plot shows CFSE halving vs. CD8β expression after gating in CD3−CD8α$^+$ T cell blasts

(lower right quadrant in Panel **(A)**. **(E)** Dot-plot shows CD8β vs. CD56 expression after gating in CD3−CD8α+ T cell blasts (lower right quadrant in Panel **(A)**. **(F)** Figure shows dot-plots of CD8β vs. CD8α expression in CD8+ TN, TCM, TEM and TEMRA in gated CD45RA+CCR7+, CD45RA−CCR7+, CD45RA−CCR7− and CD45RA+CCR7−, respectively, after labeling of fresh PBMC preparations. CD8β$^{high}$ and CD8β$^{low}$ populations in each subset are circled. Next to each dot-plot is a graph displaying the percentage of CD8β$^{high}$ and CD8β$^{low}$ T cells in CD8+ TN, TCM, TEM or TEMRA cells (mean ± SEM, n=5). P-values are indicated.

To avoid the possible confounding effect of the presence of NK cells on the PBMC preparations and to ascertain whether the CD8β chain downmodulation could also be seen in CD8+ TN cells, these were purified from PBMC preparations using negative isolation kits. The isolated CD8+ $T_N$ cells were CD45RA+CCR7+ and purity was usually higher than 97% (**Figure 4.2A**). These experiments showed that IL-15 also induced a cell-cycle dependent downmodulation of the CD8β chain in CD8+ TN cells, leading to the generation of a pool of CD8αβ$^{low}$ T cells and a pool of CD8β$^{-}$ T cells, namely in cells that divided ≥5 times (**Figure 4.2B**, left dot-plot). In marked contrast, expression of the CD8α chain remained constant or increased slightly with each cycle of cell division (**Figure 4.2B**, right dot-plot). Similar results were obtained with total bulk CD8+ T cells (see **Supplementary Figure 4.2**). The percentage of the four CD8+ T cell subsets framed on **Figure 4.2B** are illustrated in **Figure 4.2C**. Simultaneous analysis of CCR7 and CD45RA expression demonstrated that the CD8β downmodulation seen in the most dividing IL-15-activated CD8+ T cells was paralleled by a similar down-modulation of the naïve chemokine receptor CCR7 and the generation of CD8+CCR7− T cells. In contrast, although the CD45RA receptor was also downmodulated by IL-15, no CD8+CD45RA− were observed (**Figure 4.2D**), which is in accord with the fact that IL-15 stimulation drives CD8+ TN cells toward the acquisition of a highly differentiated phenotype (19, 20). **Figure 4.2E** summarizes the results of the expression of CD8α, CD8β, CCR7 and CD45RA in CD8+ TN samples after culture with IL-15. The normalized MFI values (see Material & Methods) for the expression of CD8β and CCR7 paralleled each other and started to decrease significantly after the 4$^{th}$ cycle of cell division ($p<0.001$). Regarding the normalized MFI values for CD45RA expression, an initial increase in the 1$^{st}$ to 3$^{rd}$ division cycles was followed by a slight downmodulation after 5$^{th}$ cycle of cell division ($p<0.01$). In contrast, the normalized MFI values for the expression of CD8α increased, being statistically significantly higher at cell division cycles 2, 3, 4 and ≥5 ($p<0.05$).

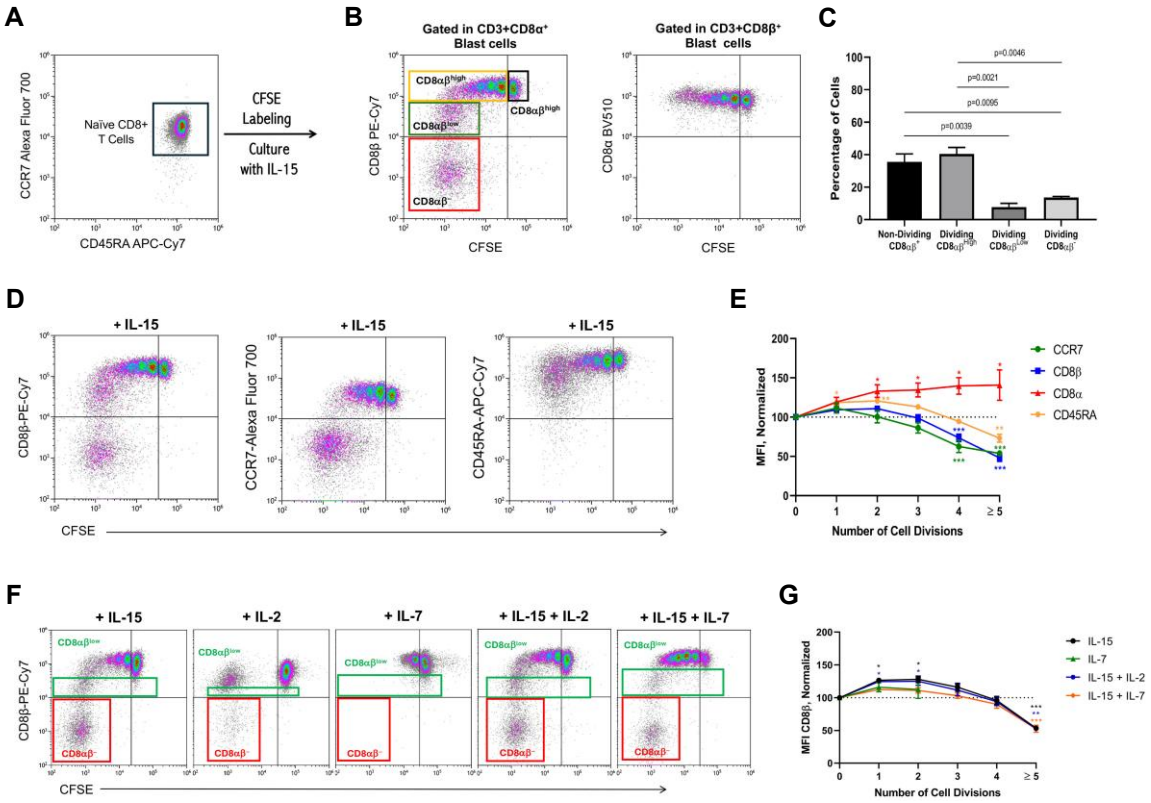


**Figure 4.2.** IL-15 induces down-modulation of CD8β in naïve CD8+ T cells. PBMC were isolated as described in the legend of **Figure 4.1**. Naïve CD8+ T cells were purified by using negative selection kits, labeled with CFSE and cultured with IL-15, IL-2, IL-7 or combinations of them (10 ng/mL) for 12 days. At the end of the culture, cells were harvested, washed, and approximately $0.5\times10^6$ cells stained using fluorochrome-conjugated antibodies against CD3, CD8α, CD8β, CD45RA, CCR7 and CD56, and acquired in an Accuri C6 or a CytoFLEX flow cytometers. **(A)** Representative flow graph showing CD45RA vs. CCR7 expression on the negatively selected naïve CD8+ T cells (purity was usually higher than 97%) before CFSE-labeling and culture with IL-15. (**(B)** left dot-plot) Flow graph shows CFSE vs. CD8β expression after the 12-day culture period after gating on $CD3^+CD8\alpha^+$ T cell blasts in order not to interfere with the analysis of the CD8β chain. Four CD8+ T cell populations can be distinguished: non-dividing $CD8\alpha\beta^{high}$ (black rectangle), dividing $CD8\alpha\beta^{high}$ (orange rectangle), dividing $CD8\alpha\beta^{low}$ (green rectangle), and dividing $CD8\alpha\beta^-$ (red rectangle). (**(B)**, right dot-plot). Flow graph shows CFSE vs. CD8α expression after the 12-day culture period after gating on $CD3^+CD8\beta^+$ T cell blasts in order not to interfere with the analysis of the CD8α chain. **(C)** Graph shows the percentage of the four different subsets framed in **Figure 4.2B** (left dot-plot, mean ± SEM, n=7). P values are indicated. **(D)** Dot-plots show CFSE halving vs. CD8β (left dot-plot), CCR7 (middle dot-plot) and CD45RA (right dot-plot) in CD8+ TN cells after 12-day culture with IL-15. **(E)** Graph shows the normalized mean fluorescence intensity (MFI) values for the CD8α, CD8β, CCR7 and CD45RA receptors, calculated as indicated in the Material & Methods, from purified naïve CD8+ T cells that undergone none, one to 4, or >5 divisions, after culture with IL-15 for 12 days. Data are presented as the mean±SEM; n=6 for zero to 4 divisions, and n=3 for ≥ 5 divisions (for CD8α CD8β and CCR7) and n=2 for CD45RA. *p<0.05; ***p<0.001, as determined by one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test, comparing each cell division with cells that did not divide (0 divisions). **(F)** Dot-plots show CFSE halving vs. CD8β expression in CD8+ TN cells after 12-day cultures with the indicated cytokines. Dividing $CD8\alpha\beta^{low}$ (green rectangle) and dividing $CD8\alpha\beta^-$ (red rectangle) are indicated. **(G)** Graph shows the normalized mean fluorescence intensity (MFI) values for CD8β, calculated as indicated in the Material & Methods, from

purified naïve CD8+ T cells that undergone none, one to 4, or >5 divisions, after culture with IL-15, IL-7, IL15+IL-2 and IL-15+IL-7 for 12 days. Data are presented as the mean±SEM; n=2, *p<0.05; **p<0.01, ***p<0.001, as determined by one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test, comparing each cell division with cells that did not divide (0 divisions).

Next, we wanted to ascertain whether the effect of IL-15 can also be seen with other γc cytokines, such as IL-2 and IL-7, which have been used for expanding CD8+ T cells for experimental and clinical therapeutic purposes. As shown in **Figure 4.2F**, IL-2 neither induced CD8αβ$^{low}$ T cells nor a significant pool of CD8αα T cells when compared to IL-15 (3.8 ± 0.5 vs. 14.6 ± 0.7, mean ± SEM, p=0.0027). Indeed, the number of CD8+ T cells entering successive cell divisions was minimal when compared to IL-15 or the combination of IL-15+IL-2 and IL-15+IL-7 (see **Supplementary Table 4.3**). Of note, IL-7 was only capable to drive naïve CD8+ T cells into two cycles of cell division and, consequently, no CD8β downmodulation was observed (see **Supplementary Table 4.3**). Finally, the combined use of these cytokines showed that IL-15+IL-2 did not significantly affect the percentage of CD8αα T cells when compared to IL-15 alone (16.1 ± 2.2 vs. 14.6 ± 0.7, p=0.7578). Although the use of IL-15+IL-7 slightly inhibited the formation of CD8αα T cells when compared to IL-15 alone, the difference was not statistically significant (9.7 ± 0.4 vs. 14.6 ± 0.7, p=0.0704). **Figure 4.2G** summarizes the results of the expression of CD8β in CD8+ TN samples after culture with IL-15.

## IL-15 preferentially down-modulates the CD8β M-4 isoform

Next, we wanted to ascertain whether the results of CD8β down-modulation were related, or not, with the expression levels of any of the CD8β mRNA isoforms described. For that, we performed qPCR using specific primers designed to amplify the six described CD8β mRNAs isoforms (**Figure 4.3**; **Supplementary Table 4.2**): four membrane-anchored (M-1, M-2, M-3 and M-4) and two secreted (S-1 and S-2) in CD8+ TN cells before and after activation with IL- 15 (see **Figure 4.3A** for exons' nomenclature and for visualization of primers location). As summarized in **Figure 4.3B**, the mRNA levels of the isoforms M-1 and M-2 were significantly increased in IL-15- activated CD8+ T cells (p<0.05). In marked contrast, the mRNA level for the M-4 isoform was significantly decreased (p<0.001). Although the mRNA for the M-3 isoform was also decreased, it did not reach statistical significance. Interestingly, we detected the two soluble isoforms described earlier (S-1 and S-2), with the mRNA of the former being significantly decreased (p<0.05). Also illustrated in **Figure 4.3** are the mRNA levels for all CD8β isoforms, which remained constant, and the mRNA levels for the CD8α chain, which was significantly increased in IL-15 activated CD8+ TN cells (p<0.05), in accordance with the

results of cell surface CD8α expression (see **Supplementary Figure 4.3** for a representative gel).

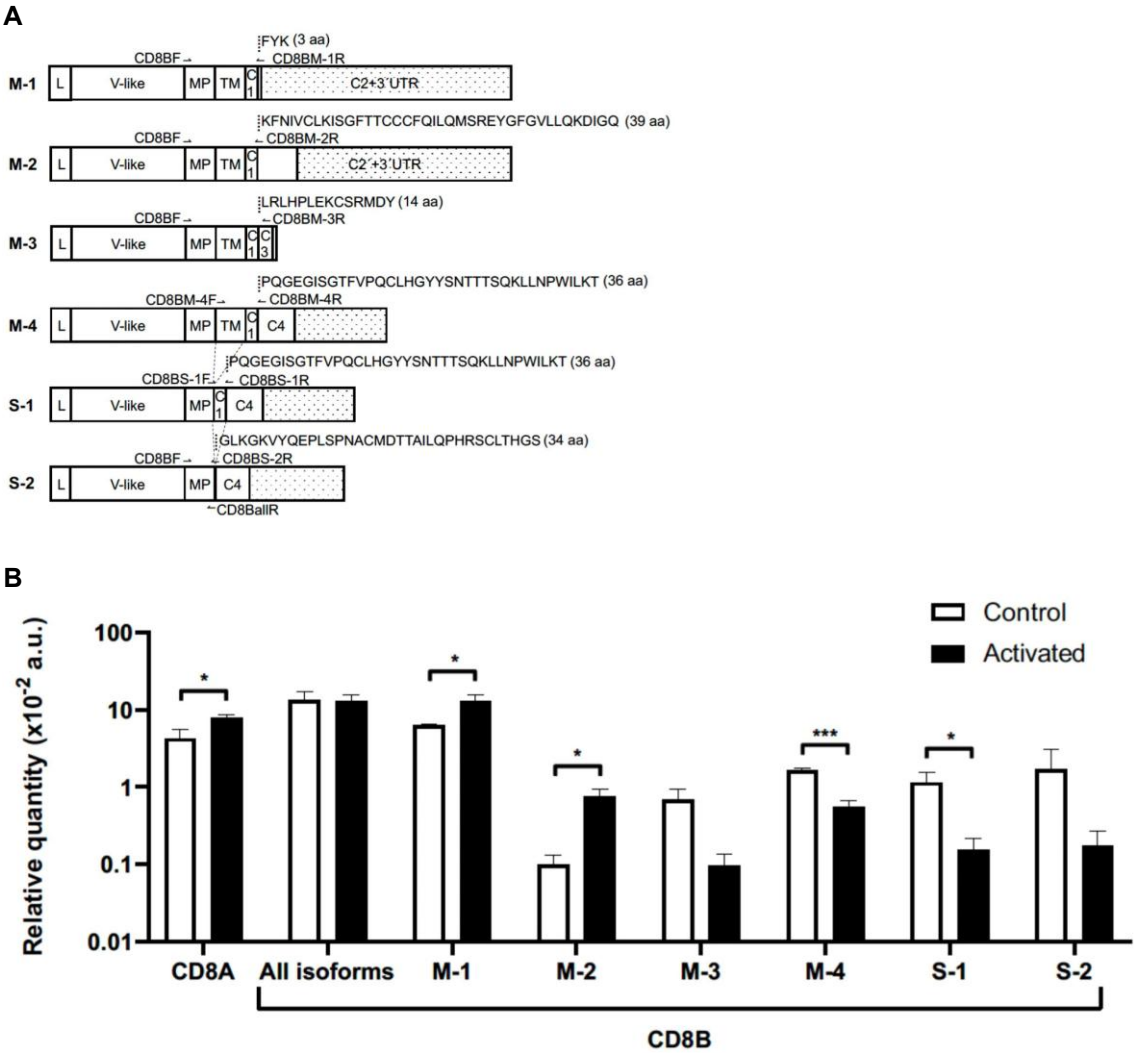


**Figure 4.3.** Expression of *CD8A* and *CD8B* genes in naïve CD8+ T cells before and after culture with IL-15. **(A)** Schematic representation of *CD8B* studied isoforms, the location of primers used for qPCR, and respective unique C-terminal amino acid sequences. White squares and doted squares represent exons and 3'UTR, respectively. For more information about *CD8B* isoforms see **Supplementary Table 4.2**; **Figure 4.3**. **(B)** Relative mRNA expression levels normalized to the geometric mean of two reference genes (*GAPDH* and *RPS18S*) multiplied by 100. Graphed qPCR data represents the mean relative quantity of four experiments ± SEM in naïve CD8+ T cells before (Control) and after (Activated) culture with IL-15 for 12 days. *p<0.05; ***p<0.001, Mann-Whitney U test. aa, amino acids; a.u., arbitrary units; C, Cytoplasmic; IgV-like, Immunoglobulin V-like; L, Leader peptide; MP, membrane proximal; TM, transmembrane; UTR, untranslated region.

## IL-15 increases the amount of Lck in IL-15- activated CD8+ T cells

Considering the importance of the tyrosine kinase Lck in the transmission of intracellular activation signals in human CD8+ T cells, we wanted to ascertain if the levels of total Lck changed between freshly isolated CD8+ T cells and differentiated CD8+ T cells after 12

days in culture with IL-15. By using total CD8+ T cells, an increase in the amount of Lck, as determined by the mean fluorescence intensity (MFI), was consistently observed between resting CD8+ T cells at day 0 and CD8+ T cell blasts at day 12 in all the experiments performed (compare **Figures 4.4A, B**). As shown in **Figure 4.4B**, on average, the MFI values of Lck observed between CD8+ T cells at day 0 and CD8+ T cell blasts at day 12 increased by 4.5-fold (10728 ± 996 vs. 45739 ± 3778, mean ± SEM, p=0.001). Kinetic studies at day 0, 6 and 12, showed a time-dependent increase in Lck expression, as determined by MFI values, from day 0, to day 6, to day 12 (13078 vs. 16846 vs. 47133, respectively). Next, we performed an analysis of Lck expression in CD8+ T cells according to their dividing status, that is CD8+ T cells at day 0 (resting cells, RC), CD8+ T cells that survived the 12-day culture with IL-15 but did not divide (non-dividing cells, NDC), and CD8+ T cells that entered successive cycles of cell division (dividing cells, DC). As shown in **Figure 4.4C**, the analysis showed that there was a gradual increase in the amount of Lck, as determined by the MFI values, from RC to NDC (10708 vs. 33299, p=0.013, n=5) and from NDC to DC (33299 vs. 45739, p=0.002, n=5).

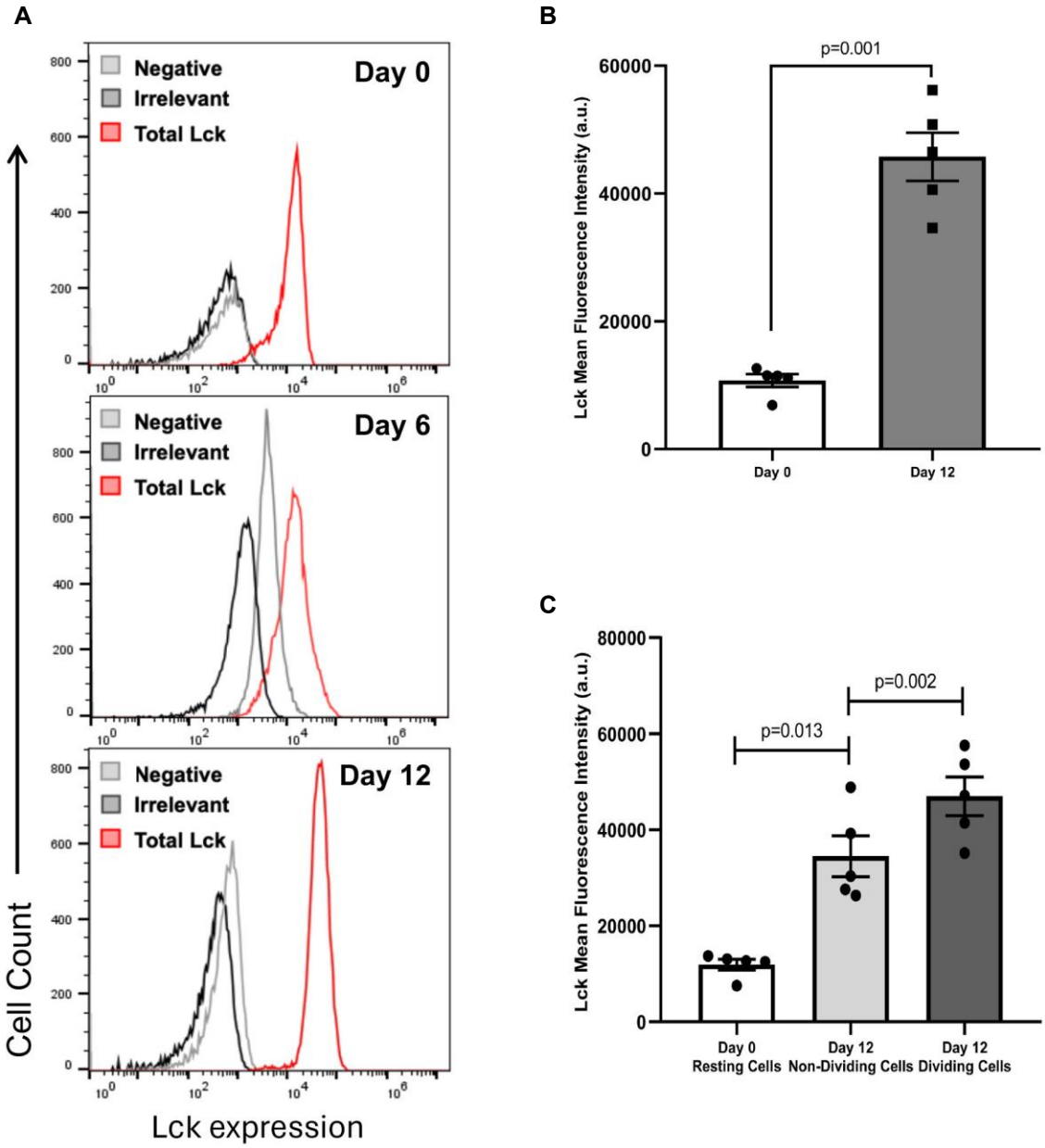


**Figure 4.4.** IL-15 increases the amount of total Lck in CD8+ T cells. PBMC were isolated as described in the legend of **Figure 4.1**. Total CD8+ T cells were purified using negative selection kits, labeled with CFSE and cultured with IL-15 (10 ng/mL) for 12 days. Approximately $0.5\times10^6$ CD8+ T cells at day 0 and at day 12 were stained using fluorochrome-conjugated antibodies against CD3 and CD8α, or CD3 and CD8β. Then, labeled CD8+ T cells were fixed, permeabilized and incubated with fluorochrome-conjugated antibodies against total Lck and irrelevant IgG, as indicated in Materials and Methods, and acquired in a BD Accuri C6 flow cytometer. **(A)** Representative histograms showing the fluorescence emission in freshly isolated CD8+ T cells after incubation with anti-Lck, irrelevant IgG, or none at day 0, 6 and 12. **(B)** Graph showing the amount of total Lck, as determined by the MFI values obtained after subtracting background fluorescence obtained with the irrelevant IgG, at day 0, 6 and 12. Data are presented as the mean±SEM; n=5. Paired samples T-test was used to assess the significance of the differences in MFI values between days 0 and 12. **(C)** Graph showing the amount of total Lck, as determined by the MFI values obtained after subtracting background fluorescence obtained with the irrelevant IgG, at days 0 (Resting Cells), 12 (Non-Dividing Cells) and 12 (Dividing Cells). Data are presented as the mean±SEM; n=5. Paired samples T-test was used to assess the significance of the differences in MFI values between days 0 and 12.

## Discussion

Apart from initial reports describing the expression of CD8β in human T cells (13, 14, 26–30), studies examining factors and molecular mechanisms regulating the expression of the CD8αβ receptor in humans are scarce. Thus, with the exception of one study using retrovirally transduced peripheral blood cells and cell lines that showed a Nef-mediated down-modulation of the CD8β chain (31), most other studies in humans have been performed with *ex vivo* blood lymphocytes from healthy blood donors (4, 5, 32), diseased patients (33–35), and virus-infected patients (6, 7). More recently, we have shown that the frequency of CD3+CD8β$^{low}$ T cells in peripheral blood of elderly people with cognitive impairment is decreased when compared to age-matched people without cognitive impairment, suggesting a possible protective role for CD3+CD8β$^{low}$ T cells against cognitive decline and neurodegeneration (24). Importantly, some of these studies showed that down-modulation or absence of the CD8β chain was associated with the generation of CD8+ T cells with an effector-memory phenotype, and defined by lack of CD28, and expression of CD8αα homodimers, perforin, granzymes, and IFN-γ (4–6, 32).

Our work has extended these studies allowing us to conclude that *in vitro*, IL-15 is a novel factor that contributes to the formation of a pool of conventional CD8αα T cells. Intriguingly, the decrease in the expression of total CD8β protein on the surface and that results in the generation of a pool of CD8αα T cells was not paralleled by a decrease in total mRNA (all isoforms). Therefore, in principle, the cell surface CD8β decrease cannot be explained solely by the IL-15-induced decrease of the M-4 isoform, unless there is some sort of mechanism where one of the mRNA forms is less stable or is less efficiently translated into protein. In that regard, the M-2 protein isoform has been shown to be ubiquitinylated, which directs the CD8β protein to the lysosomal compartment where it can be degraded (15). Thus, it is a likely possibility that the marked increase in mRNA for the M-2 isoform does not result in a net increase in CD8β protein at the plasma membrane. Nevertheless, these results show for the first time that IL-15 is a key cytokine that, *in vitro*, prompts the generation of conventional human CD8αβ$^{low}$ and CD8αα T cells previously reported, like CD8+ TEMRA cells, to exist *in vivo* (5, 6, 21). These results also reinforce the notion that IL-15 is a key cytokine that is capable of pushing naïve CD8+ T cells toward a more differentiated functional phenotype, characterized by loss of naïve cell surface receptors and *de novo* expression of NK-like receptors, as demonstrated by several groups, including ours (19, 36, 37). Interestingly, in the latter work, a significant decrease in the expression of the CD8β gene in CD8+ TEMRA cells when compared to CD8+ TN (-2,45-fold, p=0.000024, ANOVA) was found, which may

be an indication that a pool of CD8+ TEMRA cells found in peripheral blood may actually contain CD8αα+αβ$^{low}$ and/or CD8αα+αβ$^{-}$ (37).

Importantly, our results have shown that even the CFSE halving between CD8αβ$^{low}$ and CD8αα is comparable, their CD8β expression are different, i.e., the latter population has completely lost CD8β expression. In our view, *in vitro* IL-15-activated naïve CD8+ T cells enter successive cycles of cell division and, at some point around the 5$^{th}$ to 6$^{th}$ cycle the dividing CD8+ T cells start to diminish CD8β chain, with a sizeable fraction of these CD8+ T cells losing completely expression. One possibility explaining this novel result is that a subset of the most dividing CD8+ T cells is more susceptible to the epigenetic modifications induced by IL-15 signaling and result in the silencing the gene(s) coding for the CD8β chain(s). This view is not unlikely since a recent study has shown that IL-15, via STAT3 and STAT5 signaling, mediated silencing of Epstein-Barr Nuclear Antigens (EBNA) via epigenetic effects (38).

The novel findings reported in this study are important because recent studies have identified innate-like CD8αα T cells endowed with Treg functions and capable of controlling effector T cell responses in human peripheral blood (39), suggesting that expression of CD8αα homodimers, besides being a feature of expanded effector-memory CD8+ TEMRA cells, endows these CD8+ T cells with regulatory functions. Thus, in mice models, CD8αα receptors induce the expression of pro-survival molecules such as IL-7Rα and Bcl-2 (40). CD8αα receptors have also been proposed as repressors that negatively regulate CD8+ T cell activation (41), which is compatible with their pro-survival function. Moreover, a predominant expression of CD8αα homodimers by IL-15-induced CD8αβ$^{-}$ T cells may favor preferential *cis*-interactions with other receptors expressed by effector-memory CD8+ T cells, including KIR3DL1 (42), MHC class I molecules (43) and β2m-free heavy MHC class I chains, also known as open MHC-I conformers (44–46). In either case, the *cis*-interactions would regulate the CD8+ T cell responses.

Importantly, we have also shown that IL-15-activated CD8+ T cells markedly augmented the amount of the total amount of the tyrosine kinase Lck. In view of recent data by the Gascoigne group showing that free Lck is more active than the fraction of CD8α-bound Lck (47), these results may have implications at the signaling and responsive levels of the differentiated CD8αα T cells. Based on our results and the existing experimental data, it can be proposed a scenario where the CD8αα$^{+}$αβ$^{low}$ and CD8αα$^{+}$αβ$^{-}$ T cells would be, like CD8+ TEMRA cells, less responsive to TCR-mediated signals, an issue that warrants

further investigations. In any case, this work highlights the fact that the capacity of IL-15 to induce CD8+CD28– TEMRA cells *in vitro* and *in vivo* cannot be dissociated from the loss of the CD8β chain. In summary, these are novel findings that may have physiological relevance in settings where the plasma and tissue levels of IL-15 are increased. These settings include hematologic and solid tumors, autoimmune diseases, HIV infection, neurodegenerative disorders, and also exercise training in healthy subjects (48–53). In the latter situation, an inverse relationship between IL-15 and adipose tissue mass indexes was observed, suggesting that IL-15 has beneficial metabolic activities in obesity and type 2 diabetes. Whether these beneficial effects are mediated by CD8+ TEMRA and/or CD8αα T cells are issues that warrant further investigations.

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# Supplementary Material

**Supplemental Table 4.1.** List of antibodies used.

| Antibody | Conjugate | Clone | Isotype | Company |
|---|---|---|---|---|
| CD3 | APC | OKT3 | IgG2a | BioLegend |
| CD4 | PerCP-Cy5.5 | RPA-T4 | IgG1 | BioLegend |
| CD8α | FITC | SK1 | IgG1 | BioLegend |
| CD8α | PE | RPA-T8 | IgG1 | BioLegend |
| CD8α | PE | OKT8 | IgG2a | Thermo Fisher Scientific |
| CD8α | PerCP-Cy5.5 | SK1 | IgG1 | BioLegend |
| CD8α | APC | SK1 | IgG1 | BioLegend |
| CD8β | PE-Cy7 | SIDI8BEE | IgG1 | Thermo Fisher Scientific |
| CD28 | PE | CD28.2 | IgG1 | BioLegend |
| CD45RA | FITC | HI100 | IgG2b | BioLegend |
| CD56 | PE | HCD56 | IgG1 | BioLegend |
| CD197 (CCR7) | PE | G043H7 | IgG2a | BioLegend |
| Mouse IgG | Alexa Fuor®647 | MOPC-21 | IgG1 | BioLegend |
| Lck | Alexa Fuor®647 | LCK-01 | IgG1 | BioLegend |

**Supplemental Table 4.2.** Set of primers used in qPCR.

| Gene | Name in this paper | Name and/or transcript variants | SeqRef Accession number | Primer sequence (5'→3') | Amplicon length (bp) | Location# |
|---|---|---|---|---|---|---|
| *CD8A* | CD8A | CD8α molecule; all mRNA transcript variants* | See footnote | F: TGAGCAACTCCATCATGTACTTCAG<br>R: GGCGCCGGTGTTGGT | 97 | Exon IgV-like<br>Exon MP |
| *CD8B* | All isoforms | CD8β molecule; transcript variants X1, 2, 3, 4, 5 & 6** | See variants below | F: GTGTGGTTGATTTCCTTCCCA<br>R: TTCTGGGTCTCTGGCCTGG | 94 | Spanning exons IgV-like & MP<br>MP exon |
| | M-1 | CD8β molecule/transcript variant 5 | NM_004931.5 | F: GTGTGGTTGATTTCCTTCCCA<br>R: TTCTCTGCTCATTTGTAAAATTGTTTC | 244 | Spanning exons IgV-like & MP<br>Spanning exons C1 and C2 |
| | M-2 | CD8β molecule/ transcript variant X1 | XM_011533164.3 | F: GTGTGGTTGATTTCCTTCCCA<br>R: GGCAAACGATATTGAATTTCTGTTTC | 243 | Spanning exons IgV-like & MP<br>Spanning exons C1 and C2' |
| | M-3 | CD8β molecule/ transcript variant 3 | NM_172101.5 | F: GTGTGGTTGATTTCCTTCCCA<br>R: TTCTGGAACATTTCTCCAGTGG | 258 | Spanning exons IgV-like & MP<br>C3 exon |
| | M-4 | CD8β molecule/ transcript variant 2 | NM_172213.5 | F: CCCACTTTGTAGCCCCATCA<br>R: ATACCTTCCCCTTGAGGCTGTT | 144 | TM exon<br>Spanning exons C1 and C4 |
| | S-1 | CD8β molecule/ transcript variant 4 | NM_172102.5 | F: CAGAGACCCAGAAGGGCCGG<br>R: ATACCTTCCCCTTGAGGCTGTT | 70 | Spanning exons MP & C1<br>Spanning exons C1 & C4 |
| | S-2 | CD8β molecule/ transcript variant 6 | NM_001178100.2 | F: GTGTGGTTGATTTCCTTCCCA<br>R: ATACCTTCCCCTTGAGGCCCTT | 114 | Spanning exons IgV-like & MP<br>Spanning exons MP & C4 |
| *GAPDH* | GAPDH | glyceraldehyde-3-phosphate dehydrogenase*** | See footnote | F: CGCCAGCCGAGCCACATC<br>R: CGCCCAATACGACCAAATCCG | 76 | Spanning exons 1 & 2<br>Spanning exons 2 & 3 |
| *RPS18* | RPS18 | Homo sapiens ribosomal protein S18 (RPS18) | NM_022551.3 | F: CAGAATCCACGCCAGTACAAG<br>R: GCTTGTTGTCCAGACCATTG | 106 | Exon 4<br>Exon 5 |

F, forward; R, reverse

*Transcript variants 1, 2, 3 & 5 (NM_001768.7; NM_171827.4; NM_001145873.1; NM_001382698.1)

**Also, would amplify *CD8B2*. However, this *CD8B* paralog has been shown to lose expression specifically in T cells and to gain expression in brain tissues (cortex) (Doughert et al., 2018).

***Transcript variants 1, 3, 4 & 7 (NM_002046.7; NM_001289745.3; NM_001289746.2; NM_001357943.2)

#See Figure 4.3A for exons' nomenclature and for visualization of primers location. C2' results from an alternative splicing acceptor site of exon C2, originating the predicted CD8β molecule (CD8B), transcript variant X.

**Supplemental Table 4.3.** Events (CD8αβ+ T cells) gated in every cell cycle of cell division after culture with the different cytokines.

| **IL-15** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 Div.** | **1 Div.** | **2 Div.** | **3 Div.** | **4 Div.** | **≥5 Div.** | **TOTAL** |
| **Exp#1** | 6494 | 3957 | 1934 | 1521 | 889 | 2518 | 17313 |
| **Exp#2** | 9407 | 4618 | 2003 | 895 | 493 | 1905 | 19321 |
| **Mean** | **7951** | **4288** | **1969** | **1208** | **691** | **2212** | **18317** |

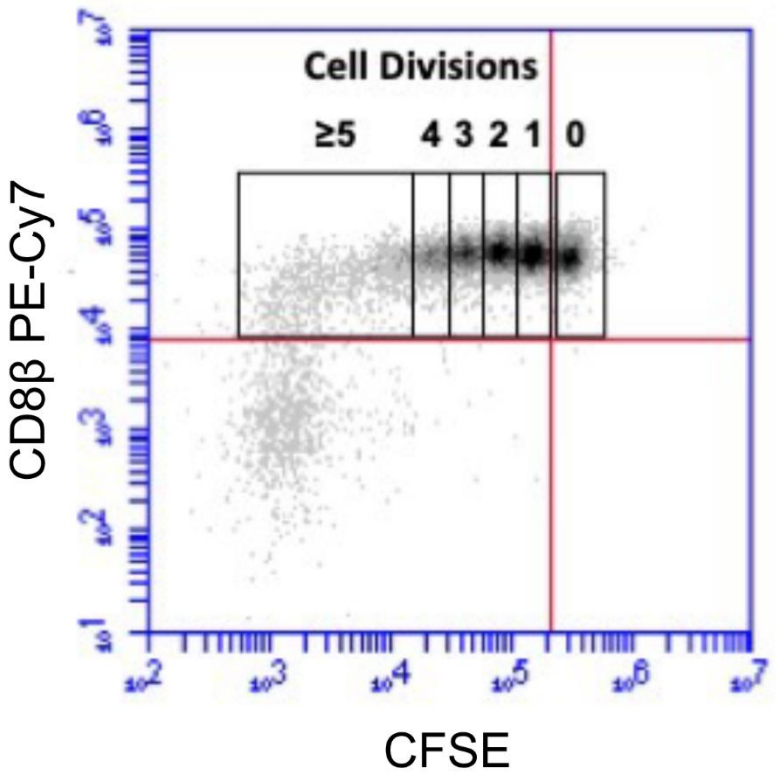
| **IL-2** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 Div.** | **1 Div.** | **2 Div.** | **3 Div.** | **4 Div.** | **≥5 Div.** | **TOTAL** |
| **Exp#1** | 16120 | 196 | 25 | 37 | 82 | 3067 | 19527 |
| **Exp#2** | 18394 | 160 | 24 | 44 | 74 | 291 | 18987 |
| **Mean** | **17257** | **178** | **25** | **41** | **78** | **1679** | **19257** |

| **IL-7** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 Div.** | **1 Div.** | **2 Div.** | **3 Div.** | **4 Div.** | **≥5 Div.** | **TOTAL** |
| **Exp#1** | 10686 | 7311 | 1432 | ND | ND | ND | 19429 |
| **Exp#2** | 18721 | 1506 | 70 | ND | ND | ND | 20297 |
| **Mean** | **14704** | **4409** | **751** | **ND** | **ND** | **ND** | **19863** |

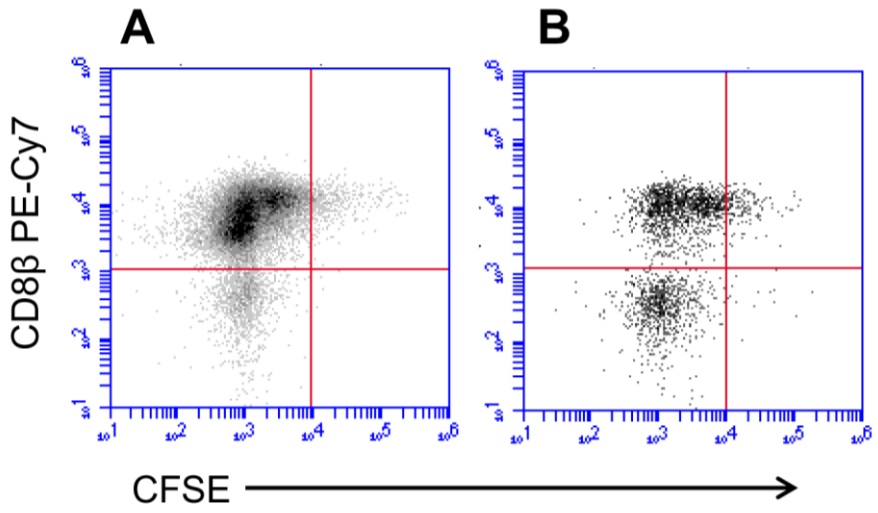
| **IL-15+ IL-2** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 Div.** | **1 Div.** | **2 Div.** | **3 Div.** | **4 Div.** | **≥5 Div.** | **TOTAL** |
| **Exp#1** | 7077 | 4321 | 2049 | 1458 | 906 | 2525 | 18336 |
| **Exp#2** | 7840 | 3933 | 1622 | 776 | 445 | 2408 | 17024 |
| **Mean** | **7459** | **4127** | **1836** | **1117** | **676** | **2467** | **17680** |

| **IL-15 + IL-7** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0 Div.** | **1 Div.** | **2 Div.** | **3 Div.** | **4 Div.** | **≥5 Div.** | **TOTAL** |
| **Exp#1** | 2007 | 3942 | 4603 | 4324 | 2947 | 1497 | 19320 |
| **Exp#2** | 2770 | 4861 | 4996 | 4166 | 1329 | 1397 | 19519 |
| **Mean** | **2389** | **4402** | **4800** | **4245** | **2138** | **1447** | **19420** |

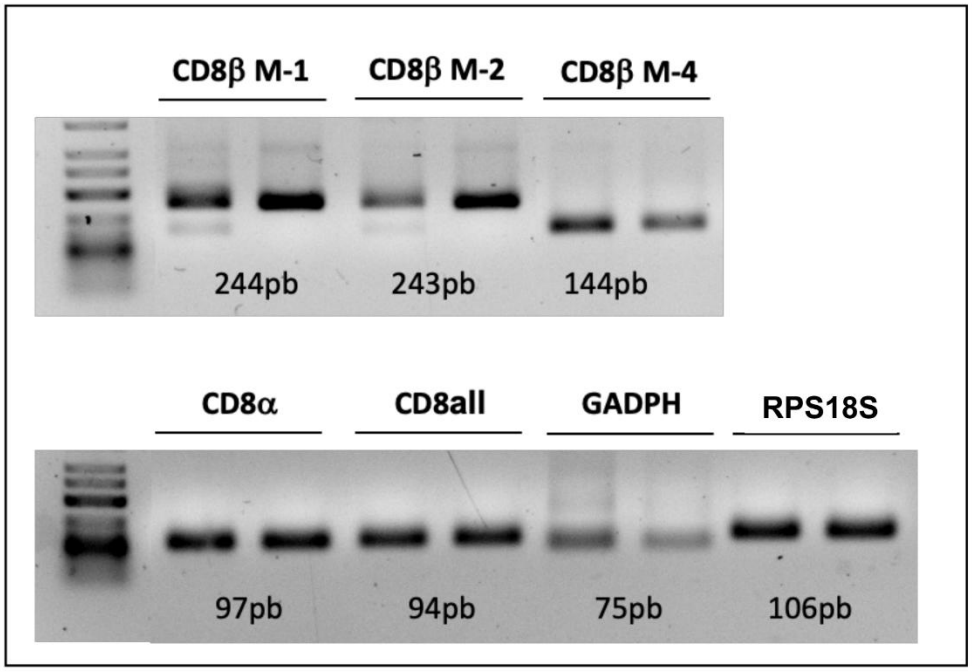
**ND, Not Detectable**




**Supplementary Figure 4.1.** Quantification of cell divisions. Representative dot-plot of CFSE fluorescence halving (X axis) vs. CD8β expression (Y axis) relative to one representative experiment and illustrating how the regions were created electronically to determine the mean fluorescence intensity (MFI) values in each cycle of cell division, as indicated in the Material and Methods.




**Supplementary Figure 4.2.** Effect of IL-15 on CD8β expression in bulk CD8+ T cells. Total CD8+ T cells were isolated by using negative isolation kits and cell cultured for 12 days in the presence of IL-15 as indicated in the legend of **Figure 4.2**. At the end of the culture, cells were harvested, washed, and approximately $0.5\times10^6$ cells were stained with fluorochrome-conjugated antibodies against CD3, CD8α, and CD8β and acquired in an Accuri C6 flow cytometer. An electronic region was created around $CD3^+CD8\alpha^+$ blast cells, which were subsequently analyzed for CFSE fluorescence halving and vs. CD8β expression. Results from two experiments **(A, B)** are shown.




**Supplementary Figure 4.3.** PCR products of selected gene transcripts. Photograph from a 2% (w/v) agarose gel from a representative sample of non-activated (CTR) and activated (ACT) naïve CD8+ T cells, showing the PCR bands of selected membrane CD8β isoforms (M-1, M-2, M-4), all CD8β, CD8α and the two reference genes (*GAPDH* and *RPS18*) used. On the left it is shown the molecular weight markers (MWM).

# Chapter 5

## Conclusions and Future perspectives

# Conclusions and Future perspectives

## General conclusions

During the last decades, studies both in human and murine models have provided important insights into the neuroprotective role of T cells. This, despite the fact that CD8+ T cells have predominantly been portrayed as a detrimental and harmful T cell subset in this context. Specifically, the expansion of CD8+ TEMRA cells in the peripheral blood and CSF of individuals with cognitive impairment and neurodegenerative diseases has been described as a factor contributing to the onset of disease. Remarkably, this view is starting to change, with studies demonstrating that certain subsets of CD8+ T cells may contribute to neuroprotection and impede the progression of neurodegenerative diseases. In order to gain further insight into the impact of CD8+ T cells, particularly CD8+ TEMRA cells, on the CNS, we have performed an extensive characterization of lymphocyte and monocyte populations, as well as of HLA class I molecules expression in a cohort of elderly volunteers differing on their cognitive status.

In this work, we did not observe a significant increase in the percentage of CD8+ TEMRA cells in the cognitively impaired elderly volunteers. However, a detailed analysis of the level of CD45RA allowed us to identify two discrete subsets of CD8+ TEMRA cells: CD8+ TEMRA$^{low}$ and CD8+ TEMRA$^{high}$, with the former being more prevalent in cognitively unimpaired elderly. Importantly, this population was characterized by the expression of low levels of the CD8β chain (CD8αβ$^{low}$ T cells). Taken together, this is the first demonstration that a population of CD8+ T cells characterized by cell surface expression of low levels of CD45RA and CD8β is associated with the cognitive status in the elderly and thus may play a role in maintaining good cognitive function. Our findings extend the current knowledge on CD8+ TEMRA cells by showing that this population is not necessarily harmful for human cognition, reflecting the heterogeneity and polyfunctionality of these highly differentiated CD8+ T cells. Notably, CD8αβ$^{low}$ T cells have been previously described as CD28- T cells that express CD45RA, and secrete IFN-γ, perforin and granzyme upon stimulation. Although previous studies have shown an association between high expansion of CD8+ TEMRA cells and CMV infection, our results do not corroborate this assumption. Indeed, all volunteers, except one, were CMV seropositive. We also show that CD4+ T cells from cognitively impaired elderly produce significantly increased levels of IFN-γ upon *in vitro* stimulation with PMA and Ionomycin, and a higher prevalence of the HLA-B8 serotype among elderly with cognitive impairment. In this sense, the production of IFN-γ by CD4+ T cells and the

presence of the HLA-B8 serotype can be considered as immunological biomarkers associated with cognitive impairment.

In this work we also demonstrate that IL-15 induces the *in vitro* down-modulation of the CD8β chain, leading to the generation of CD8αβ$^{low}$ and CD8αα T cells. This indicates that IL-15 is one of the factors associated with the generation of pro-cognitive CD8+ T cell populations. Although previous studies have shown that CD8β downregulation can be observed in antigen-expanded CD8+ T cells from healthy donors, diseased persons, and HIV-infected patients, this is the first work describing a TCR-independent stimuli capable of inducing the down-modulation of CD8β. Importantly, the observed decrease at the protein level was not paralleled by a decrease in total mRNA, suggesting the involvement of posttranscriptional modifications that should be further explored in the future. IL-15 has previously been shown to drive the differentiation of CD8+ TN cells into CD8+ TEMRA cells, expressing NKRs, and exhibiting redirected cytotoxicity, thereby leading CD8+ T cells to a highly differentiated phenotype. Expression of CD8αα homodimers has been proposed to be associated with the repression of CD8+ T cell activation and to favor the interaction with other molecules, such as NKRs and MHC class I molecules. Importantly, they also contribute to long-term survival of CD8+ T cells. Interestingly, intraepithelial lymphocytes (IELs) subsets expressing CD8αα homodimers are associated with regulatory and suppressive functions, which may also pertain to the cells described in the present work. Furthermore, IL-15 also increases the total amount of the tyrosine kinase Lck in CD8+ T cells, with functional implications for CD8+ T cell responses yet to be explored. The phosphorylation status and kinase activity of Lck, is a novel result that needs further investigations. Indeed, recent studies suggest that free Lck is in a more active state than the CD8α-associated Lck. Absence of CD8β has been shown to exclude CD8αα homodimers from lipid rafts, which has implications for signal transduction.

In conclusion, we have identified CD8+ TEMRA$^{low}$ and CD8αβ$^{low}$ T cells as subsets of highly differentiated CD8+ T cells associated with or apparently contributing to the maintenance of a good cognitive performance in a cohort of elderly people. This challenges the view that CD8+ TEMRA cells are detrimental to the CNS. On the other hand, IFN-γ-producing CD4+ T cells and HLA-B8 were identified as deleterious immunological biomarkers of human cognitive function. Moreover, we also identified IL-15 as a factor involved in the generation of CD8αβ$^{low}$ and CD8αα T cells that may constitute populations of suppressive and regulatory CD8+ T cells contributing to neuroprotection.

## Future perspectives

This thesis leaves open a number of questions, as it should be. First, can the results obtained with the volunteers of the EBIcohort be reproduced in a new different cohort? Second, what is the nature of the products/cytokines secreted by CD8+ TEMRA cells according to the level of expression of CD45RA and CD8β? Third, does the HLA phenotype indeed influence cognitive decline? How? Four, are the CD8αβ$^{high}$, CD8αβ$^{low}$, and CD8αα T cells generated *in vitro* functionally similar to the ones found *in vivo* in cognitively unimpaired elders? Five, what is the nature (i.e., protective vs. harmful) of the genes expressed by CD8aαβ$^{high}$, CD8aαβ$^{low}$, and CD8αα T cells generated *in vitro* and are they epigenetically regulated? Last, but not least, what are the phosphorylation status and activity of CD45RA, and other signaling molecules, such as Csk and ZAP-70?

The maintenance of a stable pool of circulating CD8+ TEMRA cells appears to be important for humans, namely during the aging process. Therefore, it is of crucial importance to gain new insights on how this pool is regulated and how it may exert protective functions.

To address all these questions, studies focusing on the comprehensive characterization of phenotypic and functional features of CD8+ TEMRA cells and the HLA allotype in a new and larger cohort of elderly volunteers are warranted. We are actually working on the assembly of such a cohort in the Guarda region under the umbrella of the EBI-Guardian project financed by the FCT in 2024. We will also perform studies delineating the expression and regulation of gene expression profiles of CD8αβ$^{high}$, CD8αβ$^{low}$, and CD8αα T cells by transcriptomics and epigenomics, respectively. These studies will allow to identify potentially protective and harmful genes and how their expression is regulated and linked to the CD8β downmodulation/silencing. The elucidation of their potential beneficial or detrimental role in cognition and neurodegeneration could be used to develop therapeutical interventions to slow-down or even revert cognitive decline. Finally, studies characterizing the phosphorylation status and activity of CD45, CD8-associated Lck, Csk and other signaling molecules hopefully will shed light on the signaling pathways active in CD8+ TEMRA cells.