The Immunosenescence of $\alpha\beta$ & $\gamma\delta$ T cells in human blood and skin

Xu, Weili

2019

Xu, W. (2019). The Immunosenescence of $\alpha\beta$ & $\gamma\delta$ T cells in human blood and skin. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/83643>

<https://doi.org/10.32657/10220/47613>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**THE IMMUNOSENESCENCE OF $\alpha\beta$ & $\gamma\delta$ T CELLS
IN HUMAN BLOOD AND SKIN**

XU WEILI

SCHOOL OF BIOLOGICAL SCIENCES

2019

**THE IMMUNOSENESCENCE OF $\alpha\beta$ & $\gamma\delta$ T CELLS
IN HUMAN BLOOD AND SKIN**

XU WEILI

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

30/01/2019

.....
Date

.....
XU WEILI

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it of sufficient grammatical clarity to be examined. To the best of my knowledge, the thesis is free of plagiarism and the research and writing are those of the candidate's except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

30/01/2019

.....
Date

.....
ANIS LARBI

Authorship Attribution Statement

This thesis contains material from [4] paper(s) published in the following peer-reviewed journal(s) where I was the first and/or corresponding author.

EBioMedicine: Weili Xu *et al.* Mapping of γ/δ T cells reveals V δ 2+ T cells resistance to senescence. *EBioMedicine*. 2018 Dec. doi.org/10.1016/j.ebiom.2018.11.053

The contributions of the co-authors are as follows:

W.X. designed, performed the experiments, analyzed, interpreted the data, prepared the figures and wrote the paper. G.M. designed the RNA-seq experiments, analyzed the multiplex data and prepared the figs. E.W.H., W.L.W.T., R.F. provided the expertise, performed and analyzed the epigenetics data set. Y.S., H.K., S.W.T, E.N. provided the expertise, performed and analyzed the CYTOF data set. W.Z.Y·H wrote the custom R scripts and did the analysis of the senescence genes data set. C.T.Y.T. processed the human samples. S.K.G., D.C. provided the expertise, performed and analyzed the microbiome data set. I.C.H.L., E.W.H.M., S.F., J.L. performed the sorting, luminex and rna-seq/nanostring experiments respectively. B.T.K.L. analyzed the gene-array data. T.P.N. organized the elderly cohort and provided the blood samples. A.N.A. provided the expertise for FLOW-FISH experiments and intellectual directions. H.L.T, W.P.T and T.F provided background discussions. L.A. provided overall directions and wrote the paper; all authors have read and approved the final version of this manuscript.

Experimental Gerontology: Xu W. and Larbi A. Immunity and inflammation: From Jekyll to Hyde. *Exp Gerontol*. 2017 Nov 26. pii: S0531-5565(17)30515-6. doi: 10.1016/j.exger.2017.11.018.

The contributions of the co-authors are as follows:

W.X.and L.A. contributed and wrote the manuscript.

International Journal of Molecular Science.: Xu W. and A. Larbi. Markers of T Cell Senescence in Humans. *Int.J. Mol. Sci.* 2017, 18(8), 1742; doi:10.3390/ijms18081742 W.

The contributions of the co-authors are as follows:

X.and L.A. contributed and wrote the manuscript.

Oncotarget: Tan CT, Wistuba-Hamprecht K, Xu W. Nyunt MS, Vasudev A, Lee BT, Pawelec G, Puan KJ, Rotzschke O, Ng TP, Larbi A. V δ 2+ and α/β T cells show divergent trajectories during human aging. *Oncotarget.* 2016 Jul 19;7(29):44906-44918. doi: 10.18632/oncotarget.10096.

The contributions of the co-authors are as follows:

C.T.Y.T., K.W-H., W.X., A.V., and K.J.P designed, executed the experiments and analysed the data. M.S.Z.N, O.R. and T.P.N provided the young and old cohort samples. B.T.K.L. provided input on the analysis. G.P. provided background scientific discussion. L.A. provided supervision and scientific discussion for the project.

[Input Date Here]

30/1 /2019
.....

Date

[Input Signature Here]

.....

[Input Name Here]

Xu Wei

Acknowledgements

Anis Larbi

First of all, I would like to express my gratitude towards my supervisor, Dr Anis Larbi, for his support and mentoring during the 4 years of my PhD studies. He gave me the freedom to explore the topic: "The Immunosenescence of $\alpha\beta$ & $\gamma\delta$ T cells in Human Blood and Skin" on my own, in search of the "holy grail of immortality" and with guidance only when I needed it. This approach has not only trained me to be more independent but has also empowered me to have the ability to think critically by myself and not be too risk-averse when comes to making important decisions (e.g. experimental design). These traits will definitely be essential and useful for me to overcome future challenges both in life and in my scientific career. Besides that, he is always constantly exposing me to new scientific methods (i.e. the latest technology and new analysis methods) in the immunology field. This has definitely added more skillsets to the list that I possessed prior to doing a PhD with him and has also allowed me to be at the forefront of immunological techniques that will be essential to discover new immunology knowledge.

Thesis Advisory Committee (TAC)

I will also like to take this opportunity to thank my Thesis Advisory Committee (TAC) as they have sacrificed some of their precious time and provided valuable feedback during our annual meetings. The feedbacks received during these meetings have trained me to view things from a different perspective and has help solved some of the scientific challenges that I have encountered during my PhD.

Agency for Singapore Technology and Research (A*STAR)

All these works would have been impossible without the infrastructure that exists in Singapore, both A*STAR and SIgN. No doubt Singapore is a small country, but this cohesiveness has allowed research to progress at a relative faster pace because the distance between hospital, research institutes and other research institutes are in close proximity, making the whole collaboration more fuss-free. With that, I would like to thank the people that I have worked closely with during these 4 years of my PhD and below are some of the individual organization, platforms and people that I will specially like to thank for their help in the advancement of science.

A*STAR Graduate Academy (AGA) for funding

Even though science is not a career that one should go for if a person is interested in earning big bucks, money is still essential to a certain extent for a living. For that, I would like to thank A*STAR Graduate Academy (AGA) for the funding during my PhD studies which has also allowed me to participate in many overseas conferences. These conferences has allowed me to meet, network with leading experts in my field and forged potential collaborative research ideas, which could be meaningful for the community in the pursuit of using immunology as a solution against present and future disease ailments.

Lab Members

I would also like to take this opportunity to thank the past and present members of Dr. Anis Larbi's lab. Not only did they provided scientific inputs during lab meetings and helped out during big experiments, they also made this PhD journey enjoyable with all the encouragement and laughter. This is important as good emotional well-being has been associated with reduced chronic stress which then helps to lessen chronic inflammation and mitigates the aging process.

Bioinformatics Platform (SIgN)

With the capabilities of technology these days, producing data is no longer a problem but analyzing them is. Therefore, a bioinformatician's ability to analyze these "big data" within days and uncover the meaningful observations is important. With that, I will like to take this opportunity to thank Dr Michael Poidinger, Dr Lee Teck Kwong, Bernett and their team for their help with all the analysis that they have done for me with the different datasets. Without their help and some of the tutoring in R programming, the PhD journey might have taken longer to complete as it will take many more hours or even weeks to complete the analysis, adding to the stress already present and accelerating the aging phenomenon.

Flow Core Platform (SIgN)

In order to understand the biology of a single population of immune cells, flow cytometry is a useful tool that allows scientists to interrogate them at the single cell level. With that, I will like to thank the Flow Core Facility in SIgN especially Miss Low Chay Huang, Ivy for keeping the flow analyzers in tip top conditions and also for helping me to sort and ensure the purity of the immune cell population of my interest for downstream studies.

Functional Genomics Platform (SIgN)

Transcriptomics is an important component of biology studies as transcription profile can determine to a certain extent what proteins a particular cell of interest is producing. However, it is not easy to execute these experiments properly as it requires high technical knowledge from the operator. Therefore, I will like to thank the Functional Genomics Platform in SIgN for providing all the inputs needed to ensure the transcriptomics experiment was ran successfully which definitely have saved me a lot of time and possibly resources.

Immunomonitoring Platform (SIgN)

ELISA (Enzyme Linked Immunosorbent Assay) is one of the favorite tools for an immunologist as it can assess the molecule of interest in the sample/solution. However, with the limited amount of patient sample we obtained and the vast amount of possible molecules that could differ between groups, this might not be possible with the conventional ELISA. With that, I will like to thank the Immunomonitoring platform for performing the Luminex experiments that had allowed me to interrogate more molecules with the limited of samples I have. This has helped in many new discoveries, which could be useful for future studies.

Genome Institute of Singapore (A*STAR)

Epigenetics is the modification of genetic material that either opens it up or condenses it. These epigenetic modifications could then determine whether gene transcription activity is highly active or repressed and these studies are important because it reveals another level of control in the expression of protein. However, to perform a whole genome epigenetic experiment and analyzing them requires lots of prior knowledge from the scientists. Therefore, I like to thank Dr Roger Foo, Dr Wong Huijin, Eleanor and Mr Tan Lek Wen, Wilson for helping with this set of experiment. Indeed, this approach has helped us in the understanding of V δ 2+ T cell biology with regards to cellular senescence.

National Skin Centre (NSC)

Collection of patient samples requires access, time and expertise. Therefore, I will like to thank Dr Tan Wee Ping, Melissa and Miss Mak Yan Qin for helping me to collect and coordinate the sample collection process, which has allowed me to focus on the actual study itself.

Family

Family support is always needed especially during a PhD journey, as it definitely will not be a smooth sailing one. Therefore, I will like to thank them for settling the daily household stuff and making the house a home that one will like to go after a day's work.

AGS Batchmates

I will also like to thank my group of AGS batchmates for going through this not-so-simple journey with me as we talk about scientific stuff, helped each other in troubleshooting the experiments and inspire each other to be the next great scientist in the world during our meetups.

Diving Friends

Diving has become an important part of my life as it does not only allow me to reconnect with nature and its wonders but also it is a moment whereby you get to enjoy the peace and tranquility in the ocean, away from the hustle and bustle of city life. This is important as it allows one to slow down, reflect and recollect the knowledge we have gained, in order to rethink the next step moving forward. With that, I will like to thank my diving friends, which has accompanied me on the dive trips in the past few years.

The PhD Journey in Aging

Doing a PhD in Aging has not only allowed me to understand some of the basic fundamentals of what happens when a cell reaches the senescence stage, the latest research and possible interventions to mitigate aging. It has also fueled my curiosity on how some human can live to 100+ with minimum life-threatening health complications (centenarians). Reading through the different books and scientific articles written by scientists on how to mitigate aging, it made me realized that lots of age-related diseases could be mitigated with good lifestyle choices and practicing these lifestyle choices has (I feel) helped improved my physical and mental well-being. This shows that we all have the power to have a control over the kind of life you want and age is just a number with the right and positive mindset.

Table of Contents

List of Figures

List of Tables

Abbreviations

Abstract

Introduction

CHAPTER 1: INTRODUCTION OF AGING IN HUMANS AND CELLULAR SENESCENCE

1.1 Aging in Human

- 1.1.1 Why is Aging Research important on the Global Scale?
- 1.1.2 Why is the population greying?
- 1.1.3 Human Aging and Centenarians

1.2 The Mechanisms involved in Cellular Senescence

- 1.2.1 The Mechanisms involved in Cellular Senescence, SASP and Chronic Inflammation

CHAPTER 2: INTRODUCTION OF THE IMMUNE SYSTEM AND IMMUNOSENESCENCE

2.1 Immune System and Vaccination

- 2.1.1 Immune System
- 2.1.2 Innate Immunity
- 2.1.3 Adaptive Immunity
- 2.1.4 Utilizing the Immune System against Infections and Diseases (Vaccination and Immunotherapy)

2.2 Immunosenescence and Vaccine Efficacy

- 2.2.1 Innate Immunity Immunosenescence
- 2.2.2 B cell immunosenescence
- 2.2.3 $\alpha\beta$ T cells
- 2.2.4 Aging, Persistent Infections and Immunosenescence of $\alpha\beta$ T cells
- 2.2.5 Innate T Cells

CHAPTER 3: FOCUS OF THE THESIS Part 1: HUMAN $\gamma\delta$ T CELLS IMMUNOSENESCENCE

3.1 Introduction

- 3.1.1 $\gamma\delta$ T Cells in Human
- 3.1.2 Human $\gamma\delta$ T cells Immunosenescence

CHAPTER 4: MATERIALS AND METHODS

4.1 Peripheral $\gamma\delta$ T cells study

- 4.1.1 Donors Information, Blood and Stool Collection
- 4.1.2 Cell Preparation
- 4.1.3 Cytokine response assay
- 4.1.4 Proliferation Assay
- 4.1.5 Flow Cytometry
- 4.1.6 CYTOF (Cytometry using Time of Flight)
- 4.1.7 FLOW-FISH (Fluorescent in-situ hybridization)
- 4.1.8 Soluble factor measurement by multiplex (DropArray)
- 4.1.9 Nanostring Experiments
- 4.1.10 CMV Serology
- 4.1.11 Epigenetic Methylation RRBS Data and Analysis
- 4.1.12 RNA-Seq
- 4.1.13 GSEA Analysis
- 4.1.14 DNA Damage Repair (DDR) Assay

- 4.1.15 16S Microbiome Sequencing
- 4.1.16 Data Analysis, Statistics and Visualization

4.2 Skin Aging of T cells Materials and Methods

- 4.2.1 Donor Information
- 4.2.2 Flow Cytometry
- 4.2.3 Data Analysis and Visualization
- 4.2.4 RNA-seq
- 4.2.5 Nanostring

Results

CHAPTER 5: CHAPTER 5: HUMAN $\gamma\delta$ T CELLS IMMUNOSENESCENCE

5.1: Human $\gamma\delta$ T Cells in Young and Old Peripheral Blood

- 5.1.1 Decrease in frequency of V δ 2+ T cells in peripheral blood of the elderly not associated to thymic involution
- 5.1.2 Peripheral $\gamma\delta$ T cells phenotypic alterations between the young and elderly
- 5.1.3 Peripheral $\gamma\delta$ T cells functional alterations between the young and elderly
- 5.1.4 CD27 delineate expression of molecules for V δ 2- but not V δ 2+
- 5.1.5 Peripheral $\gamma\delta$ T cells soluble factors secretion capacity between the young and elderly
- 5.1.6 Replicative senescence of $\gamma\delta$ T cells in young and elderly

5.2: Characterization of Human $\gamma\delta$ T Cells

- 5.2.1 Telomere length characterization of $\gamma\delta$ T cells
- 5.2.2 RRBS Methylation Epigenetic Profiles of CD4, CD8 and $\gamma\delta$ T cells

5.3: V δ 2+ Resistant to Cellular Senescence Mechanisms

- 5.3.1 Unique Epigenetic Signature of V δ 2+
- 5.3.2 Epi-Transcriptome analysis of $\gamma\delta$ T cells

- 5.3.3 DNA Damage repair response differs for V δ 2+ compared to CD8 and V δ 1+ with differentiation

CHAPTER 6: FOCUS OF THE THESIS Part 2: SKIN-RESIDENT T CELLS' IMMUNOSENESCENCE

Introduction

- 6.1.2 Tissue Resident T cells
- 6.1.2 Skin-Resident T cells Immunosenescence

6.2 Phenotypic and Transcriptomic analysis of elderly skin-resident and peripheral T cells

- 6.2.1 Skin-Resident and peripheral T cells adopt different phenotype in elderly individual.
- 6.2.2 CD4 and CD8 phenotype are associated in both skin and blood
- 6.2.3 Differential immunological gene expression in skin-resident and peripheral T cells in elderly individuals

6.3. Skin-resident T cells in young and elderly

- 6.3.1 PD-1 expression is decreased in skin-resident memory CD4 and V δ 1+ T cells with age.
- 6.3.2 No difference between young and old whole tissue and CD4, CD8 skin-resident T cells gene expression

CHAPTER 7: DISCUSSION

CHAPTER 8: CONCLUSION

References

Tables

Appendix A-F

- APPENDIX A. FACS Plot of Human $\gamma\delta$ T cell Staining with different fluorophore and clones
- APPENDIX B. PBMCs CD Marker Expression with and without overnight Collagenase Digestion
- APPENDIX C: Nanostring Panel (Senescence associated gene)
- APPENDIX D: FACSSymphony Configuration
 1. Blue Laser
 2. Ultraviolet Laser
 3. Red Laser
 4. Yellow Green Laser
 5. Violet Laser
 6. Violet Laser 2
- APPENDIX E: GENES IN SALMON MODULE FOR V δ 2+
- APPENDIX F: DEGS OF BLOOD AND SKIN IN ELDERLY

Author's Publications

Posters, Awards and Oral Presentations

List of Figures

5.1.1 FIGURE 1. Frequency of $\gamma\delta$ T cells subsets and CD4 RTE in the periphery of the young and elderly

5.1.2 FIGURE 2. Relationship between Microbiome profile and Periphery immune parameters in elderly.

5.1.3 FIGURE 3. Phenotypic Alterations of CD8, V δ 1+, V δ 2+ and DN $\gamma\delta$ + in the periphery of the young and elderly. (CD27, CD45RA)

5.1.4 FIGURE 4. Phenotypic Alterations of CD8, V δ 1+, V δ 2+ and DN $\gamma\delta$ + in the periphery of the young and elderly. (CD85j, CD244)

5.1.5 FIGURE 5. Phenotypic Alterations of CD8, V δ 1+, V δ 2+ and DN $\gamma\delta$ + in the periphery of the young and elderly. (CD56, CD16, KLRG1)

5.1.6 FIGURE 6. Functional Capacity of CD8, V δ 1+ and V δ 2+ in the periphery of the young and elderly.

5.1.7 FIGURE 7. Functional Capacity and correlation with phenotype of CD8, V δ 1+ and V δ 2+ in the young and elderly.

5.1.8 FIGURE 8. CYTOF analysis on CMV + Young $\gamma\delta$ T cells

5.1.9 FIGURE 9. Soluble Factors and Inflammation Gene Expression Analysis of CD8, V δ 1+ and V δ 2+ in the young and elderly

5.1.10 FIGURE 10. Frequency of CD57+ Pan $\gamma\delta$ -, V δ 1+, V δ 2+ and DN $\gamma\delta$ + in young and elderly and proliferation index of CD57-, CD57+ CD4, CD8 V δ 1+ and V δ 2+

5.2.1 FIGURE 11. Telomere Length of CD27, CD45RA and CD57 subsets of CD4, CD8 and $\gamma\delta$ subsets.

5.2.2 FIGURE 12: Epigenetic profiles of CD4, CD8 phenotypic subsets and the various $\gamma\delta$ subsets

5.3.1 FIGURE 13: Unique Epigenetic profiles of V δ 2+ T cells

5.3.2 FIGURE 14. DNA Damage repair response differs for V δ 2+ compared to CD8 and V δ 1+ with differentiation

6.2.1 FIGURE 15. Blood and Skin T Cells' CD27/CD45RA Phenotype in elderly individuals.

6.2.2 FIGURE 16. Blood and Skin T Cells' Senescent and Exhaustion Phenotype in elderly individuals.

6.2.3 FIGURE 17: Spearman correlation analysis in elderly individuals of CD8 respective phenotypes with CD4 and $\gamma\delta$ + phenotype.

6.2.4 FIGURE 18: DEGs and IPA analysis of Immunological genes of sorted CD4 and CD8 from elderly individuals' peripheral blood and skin

6.3.1 FIGURE 19: Frequency of $\gamma\delta$ T cells subsets in young and elderly.

6.3.2 FIGURE 20: Skin T Cells' Phenotype between young and old individuals

6.3.3 FIGURE 21. Gene expression analysis of young and old individuals in whole tissue and sorted CD4 and CD8 T cells isolated from skin

List of Tables

Table 1. Surface markers of T cells, B cells and Skin-Resident T cells Phenotype

Table 2. TABLE 2a. Donor Information

Table 3. TABLE 2b. Donor Information of blood samples for FIGURE 1f-1i

Table 4. TABLE 2c. Donor Information of blood and stool samples for FIGURE 2

Table 5. TABLE 3a Donor information of skin and blood samples for FIGURE 15 and 16

Table 6. TABLE 3b. Donor information of skin samples for FIGURE 19

Abbreviations

ADCC (Antibody dependent cell-mediated cytotoxicity)
APC (Antigen Presenting Cell)
B3GAT1 (Galactosylprotein 3- β -glucuronosyltransferase 1)
BD (Becton Dickinson)
BSA (Bovine Serum Albumin)
BV (Brilliant Violet)
BUV (Brilliant UltraViolet)
CCL (C-C motif Chemokine Ligand)
CCR (C-Chemokine Receptor)
CD (Cluster of Differentiation)
CM (Central Memory)
CMV (Cytomegalovirus)
CO₂ (Carbon Dioxide)
CPT (Cell Processing Tube)
CRP (C-Reactive Protein)
CXCL (C-X-C Ligand)
CYTOF (Cytometry by Time of Flight)
DA (DropArray)
DAMPs (Damage Associated Molecular Patterns)
DC (Dendritic Cell)
DDR (DNA Damage Repair)
DEG (Differential Expression Genes)
DMSO (Dimethyl Sulfoxide)
DN (Double Negative)
DNA (Deoxyribonucleic Acid)
EDTA (Ethylenediaminetetraacetic Acid)
EM (Effector Memory)
ELISA (Enzyme Linked Immunosorbent Assay)
EPCR (Endothelial protein C receptor)
FACS (Fluorescence-Activated Cell Sorting)
FBS (Fetal Bovine Serum)
FISH (Fluorescent in-situ Hybridisation)
FITC (Fluorescein isothiocyanate)

FOXP3 (Forkhead Box P3)

GE (General Electric)

GM-CSF (Granulocyte Macrophage- Colony Stimulating Factor)

GREAT (Genomic Regions Enrichment of Annotations Tool)

GSEA (Gene Set Enrichment Analysis)

HCL (Hydrochloric Acid)

HDI (Human Development Index)

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid))

HIV (Human Immunodeficiency Virus)

HMBPP ((E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate)

HTERC (Human Telomerase RNA gene complex)

HSA (Health Science Authority Singapore)

L/D (Live/Dead)

IFN γ (Interferon Gamma)

Ig (Immunoglobulin)

IL (Interleukin)

iNKT (Invariant Natural Killer T)

IPA (Ingenuity Pathway Analysis)

IPP (Isopentenyl pyrophosphate)

IRB (Institutional Review Board)

ITIM (Immunoreceptor tyrosine-based inhibitory motif)

KLRG1 (Killer cell lectin-like receptor subfamily G member 1)

MAIT (Mucosal Associated Invariant T)

MCP (Monocyte Chemoattractant Protein)

MFI (Mean Fluorescent Intensity)

MICA (MHC-Class 1 chain related protein A)

MICB (MHC-Class 1 chain related protein B)

MIP (Macrophage Inflammatory Protein)

MMP (Metalloproteinase)

NaCl (Sodium Chloride)

NAD (Nicotinamide)

NHG (National Healthcare Group)

NK (Natural Killer)

NLR (Nod-like receptors)

NUS (National University Singapore)

OTU (Operational Taxonomic Unit)

PAMP (Pathogen Associated Molecular Patterns)

PB (Pacific Blue)

PBMC (Peripheral Blood Mononuclear Cells)

PBS (Phosphate Buffered Saline)

PCA (Principal Component Analysis)

PCR (Polymerase Chain Reaction)

PD (Programmed Cell Death)

PDGF (Platelet Derived Growth Factor)

PE (Phycoerythrin)

PFA (Paraformaldehyde)

PM (Percent Methylation)

PMA (Phorbol 12-Myristate 13-Acetate)

PNA (Peptide Nucleic Acid)

QIIME (Quantitative Insights Into Microbial Ecology)

RAGE (Receptor for advanced glycation end-products)

RIN (RNA Integrity Number)

RLR (Rig-I-like receptors)

RNA (Ribonucleic Acid)

RPMI (Roswell Park Memorial Institute Medium)

RRBS (Reduced Representation Bisulfite Sequencing)

RT (Room Temperature)

RTE (Recent Thymic Emigrant)

SLAS (Singapore Longitudinal Aging Study)

SASP (Senescent Associated Secretory Phenotype)

SDS (Sodium Dodecyl Sulfate)

SPICE (Simplified Presentation of Incredibly Complex Evaluations)

TE (Terminal Effector)

TEMRA (Terminal Effector Memory RA)

TFH (T Follicular Helper)

TH (T Helper)

TLRs (Toll-like Receptors)

TNF α (Tumor Necrosis Factor Alpha)

TRM (Tissue Resident Memory)

T-SNE (T-distributed Stochastic Neighbor Embedding)

ULBP (UL16 Binding Protein)

UK (United Kingdom)

USA (United States of America)

UV (UltraViolet)

UVC (UltraViolet Type C)

VEGF (Vascular Endothelial Growth Factor)

WGCNA (Weight Gene Correlation Network Analysis)

xMAP (xMulti Analyte Profiling)

ABSTRACT

Studies in human aging mainly focus on $\alpha\beta$ T cells while $\gamma\delta$ T cells and skin-resident T cells have been neglected despite their role in immunosurveillance. In this thesis, we showed that peripheral V δ 2+ are differentially susceptible to life-long stresses as compared to other $\alpha\beta$ and $\gamma\delta$ T cells. Using aging, cytomegalovirus history as immunological models and via the different functional assays, we show that V δ 2+ T cells are protected from cellular senescence. Next, we also investigated the skin-resident T cells and show that they are also differentially susceptible to life-long stresses as compared to periphery T cells. In conclusion, this shows that not all immune cells in the host undergo similar process of immunosenescence and the underlying biology and mechanisms behind V δ 2+ and skin-resident T cells' protection from cellular senescence with age could be exploited as possible interventions to other cell types or by itself to mitigate the "grey tsunami".

CHAPTER 1: INTRODUCTION OF AGING IN HUMANS AND CELLULAR SENESCENCE

1.1 Aging in Humans

1.1.1 Why is Aging Research important on the Global Scale?

An individual is considered an elderly when he/she is aged >65 and this number is based on retirement ages across the world. However, this is not a scientifically robust parameter as an individual could be aged >65 chronologically but do not show signs of deterioration in health and vice versa.

In the near future, many countries in the world will soon face an impending “grey tsunami”, which is the aging of her population. It is estimated that the amount of people aged >65 in the world will increase from 900 million to 2100 million during 2020-2050 (1-4). This dramatic increase of elderly in the population will have great impact on healthcare policies of many countries as medical expenditures are expected to rise due to higher prevalence of diseases in elderly individuals (5,6).

An aging population is often viewed as a society burden as it does not only increase the country’s spending on issues related to age such as healthcare but also hampers overall economic output and productivity due to lower level of performance in elderly individuals (7). This negative general perception of aging is due to elderly individuals often exhibiting deterioration in many different systems of their body. Dysfunctionality in these systems could then lead to physical frailty, decrease in cognitive capacity and other possible life-threatening health issues such as cancer, cardiovascular disorder, diabetes, neurodegenerative disorders and increased susceptibility to infectious diseases (6,8-20). Thus, in recent years, aging research has been done with much more attention in many countries as they try to mitigate the impact of this impending “grey tsunami”. By deepening our understanding of underlying factors that contribute to age-associated diseases, we could perhaps translate these discoveries to possible interventions. If successful, elderly individuals in the near future will no longer be viewed as a liability as they can continue contributing to society and more importantly, lead a more fruitful life without the burden and limitations of age-associated diseases.

1.1.2 Why is the population greying?

The “greying” of the country’s population is due to many of them having both a post-world-war baby boom and also a low birth rate at the present (1,2). This implies that the population age pyramid of the country will have a dramatic shift in the future as there are fewer young people being born compared to a few decades ago. The majority of the population will be in the age range of >60 in the near future and this phenomenon is even more pronounced in developed countries such as Japan with 25% of the population expected to be older than 75 years old in the near future due to numerous factors (21,22). Another major contributing factor to the aging of the countries’ population is the “extension of life span” in humans. This is due to the discovery of many drugs (i.e. antibiotics, vaccines), advancement in medical interventions (i.e. surgery), technology (i.e. delivery methods of drugs and surgical procedures) and better sanitary conditions in many different countries during the last few decades (23,24). These advancements have helped to improve the quality of life of many by preventing and curing many deadly infections and diseases, which were deemed incurable from the past. However, by extending the life span, this brings another set of issues that we need to face, which are the diseases that have higher incidences in the individual that are aged >65.

1.1.3 Human Aging and Centenarians

Aging per se is not the issue as centenarians (people that live past the age of 100) has shown that it is possible to live to an age of 100 or even further with minimal health complications (25). Studies from the different blue zones (i.e. Okinawa and Sardinia) have shown that this exceptional healthspan is a mixture of genetic, diet, lifestyle choices and other environmental aspects such as social support and a purpose in life (26-34). However, this group of people only forms the minority in the world. As for the majority of the world population, the life expectancy on average, still fall short below 100. The life expectancy average varies between countries with the more developed countries, edging closer to 90 possibly due to better accessibility to healthcare, sanitary conditions and etc (21). Even as we strive to live to an age of 100 like the centenarians, there is debatable evidence whether the limit of average life expectancy of human is around 115 or maybe even more (35). Nonetheless, having a long life-span does not necessarily equate to a better health-span, which is the period of time, one has minimal health burdens that hamper the quality of life.

One of the main factors that contribute to the mortality rate in non-centenarians is often due to the complications and diseases that come with old age. Many studies have shown that old age has an association with many systems functional impairment such as cardiovascular disorders (heart), diabetes (metabolism), Alzheimer's disease (brain), sarcopenia (muscle), reduced vaccine efficacy and increased susceptibility to infections (immune system) (15,16,36-49). Thus, all these diseases have been classified as "age-related diseases". This phenomenon also illustrates that aging could be a multi-systemic disorder as it does not only affect just the heart, the brain, the muscle or the immune system but probably a combination of different organs being dysfunctional at the same time when an individual grow old. To add a layer of complexity in aging studies, not all individuals will exhibit the same dysfunctionality in the same systems at the same age. These factors then contribute to the heterogeneity of individuals, whose samples being analyzed, results in heterogeneous data points as shown in later part of the thesis. Thus, tackling the specific mechanisms for each system before the onset of these age-related morbidities could allow individuals in the future to live like centenarians and not be burden with diseases and disabilities.

1.2 The Mechanisms involved in Cellular Senescence

1.2.1 The Mechanisms involved in Cellular Senescence, SASP and Chronic Inflammation

The aging of a system or an organ cannot be fully comprehended without understanding the fundamentals of cellular senescence as an organ is made up of many individual cells and a system is made up of many organs. Replicative senescence is defined as a stage whereby cells exhibit impaired ability to proliferate. This is also known as the Hayflick's limit as Hayflick discovered in the 1950s that after 50 passages, cells were unable to proliferate anymore and has been termed to be senescent. (50). The hallmarks of cellular senescence can be attributed to many different factors such as telomere attrition, DNA damage, proteasome degradation and etc. (51). It has been proposed that these hallmarks serve as checkpoints to mark cells that are "defective" and therefore shutting down its mechanism to proliferate, preventing the "defects" from propagating to future generation of cells. One example that highlights the importance of cellular senescence is cancer cells, which are cells that harbor detrimental mutations in their genome and should not be allowed to proliferate (52,53). While senescent cells have impaired proliferative capacity, they are not fully dysfunctional as they are capable of secreting certain growth factors and cytokines that helps in wound healing and tissue remodeling. However, the dark side of senescent cells is that they are also able to secrete low levels of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-8 without prior stimulation. This is known as the Senescent Associated Secretory Phenotype (SASP), which was established on fibroblasts (54).

Inflammation is an important mechanism evolved to defend the host by alerting the immune system to foreign insults and recruits immune cells to the site of infection. However, there are two forms of inflammation. One of them is acute inflammation, which dampens down upon resolution (55,56) and the other form of inflammation is the chronic form, which is long-term and a result of a lack of balance of inflammation due to various persistent stressors such as cancer cells, tissue damage and persistent infection such as CMV (Cytomegalovirus) and HIV (Human Immunodeficiency Virus (57-59). Besides persistent infection, the other source of low-grade chronic inflammation is the accumulation of senescent cells with age as they are able to secrete low level of pro-inflammatory cytokines without prior stimulation. Chronic inflammation is detrimental to the host, as it has been shown to be associated with many age-related diseases (60,61). Thus, the removal of senescent cells, which is known as senolysis, and other methods such as nutrition that reduces inflammation level of the host has been proposed as possible interventions to mitigate age-related diseases (62-66).

CHAPTER 2: INTRODUCTION OF THE IMMUNE SYSTEM AND IMMUNOSENESCENCE

2.1 Immune System and Vaccination

2.1.1 Immune System

The immune system is the host natural defense that protects the host from foreign pathogens (i.e. bacteria and viruses). It functions by having different immune cells performing its roles in various settings to help combat the foreign pathogens (67). The immune system in human can generally be classified into two different arms (i.e. innate and adaptive) based on a few general parameters such as speed of response, specificity and memory capabilities (68-71), although the memory aspect has been recently discussed under the spotlight with the new paradigm of trained immunity (72,73).

2.1.2 Innate Immunity

Innate immunity recognizes foreign pathogens by binding to their pathogen-associated molecular patterns (PAMPs). They are also able to recognize damage associated molecular pattern (DAMPs) which are molecules that are able to cause a noninfectious inflammatory response, released by the host cell (74). This is done by using Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-Like receptors (RLRs) that are expressed either on the surface or within the cell. It responds within hours from the time of infection (75-77). The immune cells that are mainly classified under this arm are Neutrophils, Monocytes/Macrophages and Natural Killer (NK) cells.

Recently, a new paradigm has been proposed for innate immunity through the research of plants' immunology (78). This new paradigm of trained immunity research was established on monocytes and shows that the epigenetic modification of the monocyte's changes after an infection for a short duration of time. The epigenetic modification hypomethylates early response genes such as c-myc, JUN, c-fos and also inflammation related genes such as TNF- α and IL-6 (79,80). These modifications allowed the monocytes to express higher level of pro-inflammatory cytokines during the next unrelated infection and therefore this is termed as trained immunity.

2.1.3 Adaptive Immunity

Adaptive immunity differs from innate immunity in that it responds to foreign pathogens in a very specific way as it selectively expands a single clone of T cell or B cell that has the highest affinity binding receptor against the pathogen (81,82). It is also slower in terms of response to a new infection compared to innate immunity, as it requires approximately 7-14 days to mount a detectable response. However, adaptive immunity has a memory component that makes up for its shortcoming in the initial response. Immune memory allows T cells and B cells to mount a very swift and effective response upon the next encounter of the pathogen and last for a time period in the host lifespan. Most vaccines are designed to utilize this feature of the immune system. By using a non-virulent strain of a particular pathogen prior to exposure to the virulent strain, this allow the immune system to register a “memory” and might be able to prevent the onset of severe symptoms that could be detrimental for the host when the actual infection strikes.

2.1.4 Utilizing the Immune System against Infections and Diseases (Vaccination and Immunotherapy)

The discovery of vaccines has helped mankind utilized its immune system and trained it to fight against life-threatening foreign pathogens with a milder form of the pathogen. Edward Jenner first demonstrated this concept when he discovered that milkmaids that have been infected by cowpox virus (a milder version of smallpox in cows) are “immune” to the deadly smallpox virus that was threatening the lives of many (83,84). Vaccines work by exposing the immune system with an attenuated version of the pathogen, which is then “recorded” as a memory. This memory feature then allows the immune system to mount an effective response and clears the pathogen before the onset of more severe symptoms when the host is eventually exposed to the virulent strain in the future (85). Fast-forward to modern days, another healthcare strategy utilizing the immune system is Immunotherapy. Immunotherapy is the use of the different methodology to enhance the immune system in the fight against cancer and has become one of the 4 main treatments against cancer (86-90). These studies illustrate that by understanding the immune system, its dysregulation in different diseases and knowing how to elicit the right immune response have tremendous potential for future therapies against deadly maladies.

2.2 Immunosenescence and Vaccine Efficacy

Despite the tremendous benefits of vaccination in preventing deadly diseases, elderly individuals often exhibit reduced responsiveness to vaccinations (91-93). Coupled with higher susceptibility to infections and cancer, this makes elderly individuals more prone to the different morbidities, which might eventually lead to death. As the immune system is the host natural defense system against foreign pathogen and cancer, these higher incidences of diseases in the elderly could be due to age-related adaptations in the immune system, which is otherwise known as immunosenescence (94-97)

2.2.1 Innate Immunity Immunosenescence

Immunosenescence affects both the innate and adaptive immune system. However, the age-related change of the different immune cells varies. For innate immunosenescence (98,99), neutrophils exhibit impaired signal transduction, which then leads to decreased functional aspects such as phagocytosis, intracellular killing and other responses (100-102). Monocytes exhibit an increased in frequency of the pro-inflammatory non-classical phenotype in the periphery and also have higher basal level production of pro-inflammatory cytokines such as TNF- α and IL-1 α with age (103,104). Macrophages exhibits decreased phagocytic activity, antigen capacity, production of chemokine/cytokines and also factors that aid in wound healing such as fibroblast growth factor-2 and vascular endothelial growth factor with age (105,106). Natural Killer cells exhibit impaired proliferation and higher frequency of pro-inflammatory (CD56^{dim}) phenotype (107-109). Besides the innate immune cells, the environment that affects it also changes as it has been shown that DAMPs accumulate with aging, contributing to low grade chronic inflammation found in the elderly (110-111). Collectively, these illustrate the differential changes in innate immunity and also its environment with age, suggesting that immunosenescence might be due to environment adaptations instead of chronological aging of the cells as neutrophils and monocytes are short lived in the circulation.

2.2.2 B cell immunosenescence

B cells belong to the adaptive arm of the immune system. Its role in the immune system is to produce antibodies that recognize foreign pathogens, and these are usually elicited during vaccination or actual infections. However, these functionalities of B cells are affected in the elderly. It has been shown that there is a reduced number of memory B cells and also reduced somatic hypermutation in the periphery with age (112). In addition, the naïve B cells in the elderly also have a lower threshold in terms of activation as it can be stimulated to secrete

TNF- α and IL-10 with physiological levels of aCD40/IL-4 compared to the young (113-116), where a higher dose is required and this could be one of the factors that contributes to the chronic pro-inflammatory environment often seen in the elderly. Altogether, these highlights possible contributing factors for the reduced vaccine efficacy observed in elderly from a B cell perspective.

2.2.3 $\alpha\beta$ T cells

Classical $\alpha\beta$ T cells can be separated into Helper T cells (i.e. CD4) and Cytotoxic T cells (i.e. CD8). CD4 T Helper cells, as its name implies, helps to secrete cytokines that are important for effective immunological responses involving other immune cells. Besides the secretion of cytokines, CD4 T cells also interact with antigen presenting cells (APC) such as dendritic cells through their surface receptors CD27 to CD70 and CD28 to CD80 on the APC (117). While they are classified as T Helper cells, many types exist, and their identification is dependent on the type of cytokine it secretes. For example, Th1 secrete IFN γ , Th2 secrete IL-4, Th17 secrete IL-17, TFH (T follicular helper) secrete IL-21 and etc. and more of the different subtypes has been discovered in the past few years. (118-123). CD8, on the other hand, kills virus-infected and defective cells by releasing cytotoxic granule such as granzyme and perforin that impairs the membrane of the target cell and induces cellular apoptosis. Despite their differences in functions, both CD4 and CD8 go through a similar differentiation process. This process is well studied, established, can be marked and tracked by the expression of a few surface proteins such as CD27, CD28, CCR7 and CD45RO/RA (124-126). Naïve T cells (CCR7 $^{+}$, CD27 $^{+}$, CD28 $^{+}$, CD45RA $^{+}$, CD45RO $^{-}$) which expresses CCR7 (C-Chemokine Receptor 7) allows the T cells to home into lymph nodes to interact with B cells and APCs using CD27 and CD28 for the generation of antibodies, activation and long-term maintenance (127). Central Memory (CM) T cells (CCR7 $^{+}$, CD27 $^{+}$, CD28 $^{+}$, CD45RA $^{-}$, CD45RO $^{+}$) are able to produce high levels of interleukin 2 and interferon- γ while effector memory (EM) T cells (CCR7 $^{-}$, CD27 $^{-}$, CD28 $^{-}$, CD45RA $^{-}$, CD45RO $^{+}$) are capable of secreting effector molecules such as TNF- α and other cytotoxic molecules (i.e. Perforin and Granzyme B (only in CD8)) but not IL-2. Lastly, terminal effector (TE) cells (CCR7 $^{-}$, CD27 $^{-}$, CD28 $^{-}$, CD45RA $^{+}$, CD45RO $^{-}$), which are more enriched in CD8, have impaired proliferative capacity but able to secrete a wider array of cytokines when stimulated. Besides differentiation markers, senescence associated markers such as KLRG (Killer cell lectin-like receptor subfamily G1) and CD57 and exhaustion markers such as PD-1 (Programmed Cell Death Protein 1) are also used to identify the cellular status of T cells. KLRG1 is an immunoreceptor tyrosine-based inhibitory motif (ITIM) and previous studies have shown that

by preventing the ligation of it, it enhances the proliferation capacity through the increase of AKT, cyclin E, cyclin D and a decrease of cyclin inhibitor p27 (128). On the other hand, CD57 is a glycoepitope and its expression is governed by galactosyltransferase 3- β -glucuronosyltransferase 1 (B3GAT1). The expression of CD57 on T cells has been shown that it has impaired proliferation capacity, suggesting that it is a marker of replicative senescence (129-133). As for identification of exhausted T cells, PD-1 is one of the common molecules that is being utilized. Exhausted T cells are defined by their inability to proliferate and secrete cytokines upon stimulation and this is due to repetitive and chronic stimulation (134-137). Therefore, utilizing CCR7, CD27, CD28, CD45RO/RA, KLRG1, CD57 and PD-1 allows investigators to track the stage and cellular status of T cells in the different disease conditions.

2.2.5 Aging, Persistent Infections and Immunosenescence of $\alpha\beta$ T cells

The main bulk of immunosenescence research data remains in the T cells and this is probably due to the array of surface markers that are established and useful in identifying the different subsets and cellular status of T cells (138). Previous studies around the globe have shown that $\alpha\beta$ T cells are greatly impacted with persistent infections and chronological aging (139-151). One of the main changes with age happens in the thymus, the primary lymphoid organ that is responsible for maturation and development of T cells. It has been shown to start involuting at the age of 40 and this results in lesser naïve T cells being produced (152-155). Not only does this affect the quantity of naïve T cells being produced, it also implies that the repertoire of T cells shrink as one ages. This could be detrimental as it reduces the probability of the elderly individuals' T cells having the right repertoire to fight a new infection in later life if it encounters one (156-158). Having a reduction in the production of new naïve T cells also means that the naïve T cells pool is not constantly renewed and could accumulate age-related defects due to homeostatic proliferation. These defects have detrimental impact on its functions as studies have shown that aged naïve T cells that is able to form an immunological synapse with APCs is half of young naïve T cells, suggesting that there could be an intrinsic defect in signaling functions of aged naïve T cells that can manifest in downstream processes. One of the processes that are affected is the production of IL-2, which is a key cytokine that is required for survival and proliferation of T cells but also have other roles in B cell response and etc (159-161).

The other age-related change is the accumulation of highly differentiated replicative senescent T cells (CD27⁻, CD45RA⁺, CD57⁺). These are cells that have impaired

proliferative capacity and secrete low-grade pro-inflammatory cytokines without prior stimulation. This could then be one of the factors that contribute to the chronic inflammation that is present in elderly individuals that has been associated to many age-related diseases (162). Thus, intervention studies have been focusing on these few aspects to try to restore the “youthfulness” and balance of the T cells in the elderly individuals.

Besides the reduction of naïve T cells and accumulation of differentiated and senescent T cells, another aspect of immunosenescence in $\alpha\beta$ T cells is telomere shortening. Telomere are a region of repetitive nucleotide sequence at the end of the chromosome and it protects the chromosome from deterioration. However, as cells proliferate and differentiate, the telomere length is shortened as the DNA cannot be replicated completely. This effect is however being compensated by the telomerase reverse transcriptase to a certain extent, but the activity of the enzyme decreases with differentiation. This imply that as a host chronologically age, the T cells which are long lived, will also have undergone more cell cycles as compared the younger self as shown in a study in the UK (163). Therefore, based on this aspect, telomere length is often also used a surrogate marker of longevity in many aging studies.

CMV infection is an important factor to consider in the study of $\alpha\beta$ T cells immunosenescence as it has been shown in many studies that it “accelerates” the differentiation of CD4 and especially CD8 T cells. Young donors (age 21-40) that are seropositive for CMV are found to have significantly less frequency of Naïve CD8 and higher frequency of EM phenotype. In some studies that have the opportunity to analyze CMV negative elderly samples, they found that the frequency of naïve CD8 T cells in those samples were similar to the CMV positive young donors but CMV positive elderly still has significantly more differentiated CD8 T cells as compared to CMV negative elderly (164). Also, as CMV is a chronic infection, not only does it affect the phenotype of the T cells, it also affects its repertoire as it has been shown that the dominant repertoire in the CMV positive elderly are mainly recognizing CMV epitopes (164). This implies that the T cells in the CMV positive elderly has less capacity to react against other viruses compared to CMV negative elderly. Together, this shows that CMV seropositivity and chronological age both contributes to the differentiation of $\alpha\beta$ T cells. Knowing its impact, I included CMV as a parameter in this thesis to better stratify the young samples as human data are known to be heterogeneous and this will allow better interpretation of the data. However, seropositivity of the elderly in Singapore is close to 99% and therefore we were unable to obtain CMV negative elderly samples.

2.2.4 Innate T cells

Other than the adaptive $\alpha\beta$ T cells (i.e. CD4 and CD8), the T cell compartment also has an “innate” arm, also being termed as the non-classical (innate-like) T cells (i.e. MAIT, $\gamma\delta$ and iNKT). This is based on the speed of its responses against primary infection and its specificity (165-171). However, as the innate T cells are not as well studied as the adaptive counterparts, the markers that are mentioned above have not yet been functionally validated to have the same implications as classical T cells. One of the objectives of this thesis is to investigate whether the markers used to functionally define adaptive $\alpha\beta$ T cells can also be used to define $\gamma\delta$ T cells.

CHAPTER 3: HUMAN PERIPHERAL $\gamma\delta$ T CELLS IMMUNOSENESCENCE (FOCUS OF THE THESIS PART 1)

3.1.1 $\gamma\delta$ T Cells in Human

Human $\gamma\delta$ T cells are usually classified as non-classical T cells subsets and lies in between the innate and adaptive immune system as they exhibit faster immune response as compared to their classical $\alpha\beta$ counterparts (i.e. CD4 and CD8). $\gamma\delta$ T cells frequency ranges from 5-10% of total T cells in the periphery and approximately 40% of the total T cells in the tissue. However, these frequencies can increase drastically upon infection. The average distributions of $\gamma\delta$ T cells subsets in young human peripheral blood are V δ 1+ (~25%), V δ 2+ (~70%) and DN (Double Negative) $\gamma\delta$ + ($\gamma\delta$ + V δ 1- V δ 2-) (~5%) of total $\gamma\delta$ T cells (172-174). Human $\gamma\delta$ T cells subsets in general possesses the ability to secrete “Th1-like” cytokines such as IFN γ , TNF- α and also cytotoxic molecules such as granzyme, perforin and granulysin. These soluble molecules are induced through the expression of surface molecules such as $\gamma\delta$ TCR, NKG2D, CD27, CD226, CD16 and/or other NK receptors (175).

$\gamma\delta$ T cells are also different from $\alpha\beta$ T cells in that they are not MHC-class restricted and could recognize a range of molecules. For instance, V δ 1+ can recognize CD1d-presented lipids and stress-induced molecules such as MICA, MICB and ULBPs on transformed, infected or cancerous cells (176-182). V δ 2+, the most well-studied human $\gamma\delta$ T cells subsets probably due to its abundance, can respond against phospho-antigens and metabolites (such as isopentenyl pyrophosphate (IPP) of the mevalonate pathway that are elevated in tumor cells and also (E)-4-Hydroxy-3methyl-but-2-enyl pyrophosphate (HMBPP) that are namely produced by mycobacterium tuberculosis, malaria parasites and other bacteria (183,184). V δ 2+ has also been shown in other studies to be able to respond with Antibody dependent cell-mediated cytotoxicity (ADCC) through the expression of CD16, able to phagocytose through the expression of CD36, have antigen presentation capacity to cross present antigens to CD8 T cells and induce DC maturation using TNF- α (185-190).

Within the DN $\gamma\delta$ + T cells, there are other $\gamma\delta$ subsets such as V δ 3 and V δ 5, which are less studied due to their quantity in the periphery and also a lack of reagents to isolate and study them. However, there are some studies that show that V δ 3 are able to respond against CD1d+ targeted cells, induced DC maturation through cytokines and also induce maturation and IgM secretion by B cells (191,192). V δ 5, on the other hand, isolated from a CMV patient was able to react against CMV-infected endothelial cells and epithelial tumors through the binding of endothelial protein C receptor (EPCR), demonstrating its capability to induce cellular toxicity against transformed and infected cells by secreting granzyme B and perforin (193).

Besides the difference in antigen it reacts against, human $\gamma\delta$ T cells have been shown to be able to respond to different cytokines and shift its phenotype and this is known as plasticity. For example, $\gamma\delta$ T17 which is able to secrete IL-17 can be generated with IL-6, IL-1 β and TGF- β , while $\gamma\delta$ T1/17 which can secrete both IL-17 and IFN- γ will require additional IL-23 in neonates.

This plasticity is very relevant in the context of cancer immunotherapy as they have been shown to be capable of both pro and anti-tumor depending on the cytokines it secretes and the tumor microenvironment (194).

Altogether, the human $\gamma\delta$ T cells subsets encompass a wide range of roles and functions in the immune system, which is important in bridging the innate and adaptive immunity (195).

3.2.1 Human $\gamma\delta$ T cells Immunosenescence

Despite human $\gamma\delta$ T cells capabilities against infectious diseases and cancer plus its roles in a wide range of immune response, human $\gamma\delta$ T cells' immunosenescence has not been thoroughly investigated. While there are limited studies done on $\gamma\delta$ T cells immunosenescence, a study in Japan have shown that in the context of aging, $\gamma\delta$ T cells in the periphery decreases with aging but is namely due to a decrease in V δ 2+ but not V δ 1+ (196). Other studies that are conducted elsewhere have shown that with CMV infection and aging, there is an increase "differentiated" of V δ 2- T cells but not for V δ 2+ T cells (197-199). Thus, with the limited knowledge of $\gamma\delta$ T cells' immunosenescence in the literature, there are gaps as $\gamma\delta$ T cells have not been investigated thoroughly into its separate individual subsets (i.e. V δ 1+, V δ 2+, DN $\gamma\delta$ + (V δ 1-, V δ 2-, $\gamma\delta$ +) in terms of other surface markers phenotype, functional capacity (i.e. cytokine/cytotoxic and proliferation) in the context of aging. In terms of human $\gamma\delta$ T cell biology, there are also gaps on whether the surface markers that are used to functionally define $\alpha\beta$ T cells in terms of functional capacity, replicative capacity and telomere length could be applied similarly to the subsets of $\gamma\delta$ T cells.

Thus, one of our objectives in this thesis was to investigate the impact of age on the characteristics of the human $\gamma\delta$ T cell subsets phenotypes, functions, replicative senescence, telomere length, epigenome and their molecular response to stress.

The hypothesis of this study is that human $\gamma\delta$ T cell subsets phenotype will not be differentially affected by the impact of CMV infection and aging, but its cytokine/cytotoxicity and replicative capacity will be affected by aging due to immunosenescence.

However, using CMV history and aging as a model, we discovered that only V δ 2⁺ T cells do not adhere closely to the phenotypes that have been defined for $\alpha\beta$ T cells and are more resilient to cellular aging and environmental stress compared to other $\alpha\beta$ and $\gamma\delta$ T cells as they were able to retain their functional capacity with aging.

TABLE 1

<u>Cell Type</u>	<u>Phenotype</u>
Naïve T cell (i.e. CD4 and CD8)	CCR7 ⁺ , CD27 ⁺ CD28 ⁺ CD45RA ⁺
Central Memory T cells (i.e. CD4 and CD8)	CCR7 ⁺ CD27 ⁺ CD28 ⁺ CD45RA ⁻
Effector Memory T cells (i.e. CD4 and CD8)	CCR7 ⁻ CD27 ⁻ CD28 ⁻ CD45RA ⁺
Terminal Effector T cells (i.e. CD4 and CD8)	CCR7 ⁻ CD27 ⁻ CD28 ⁻ CD45RA ⁺
Exhausted T cells	PD-1 ⁺
Senescent T cells	CD57 ⁺
Naïve B cells	CD19 ⁺ CD27 ⁻ IgD ⁺
Memory B cells	CD19 ⁺ CD27 ⁺ IgD ⁺
Switched Memory B cells	CD19 ⁺ CD27 ⁺ IgD ⁻
Tissue Resident T cells	CD69 ⁺

CHAPTER 4: MATERIALS AND METHODS

4.1 Peripheral $\gamma\delta$ T cells study

4.1.1 Donors Information, Blood and Stool Collection

Participants in the first part of the thesis (n=22, ≥ 60 years old) were recruited in the Singapore Longitudinal Aging Study (SLAS) (Table 1a). The details of the SLAS study design; population sampling and measurements were described in (200). In brief, all residents were identified on a door-to-door basis and eligible people without severe physical or mental disability were invited to participate in the study. Individuals with a history of hospitalization in the past 6 months and high C-Reactive Protein (CRP) levels ($>3\text{mg/L}$) were excluded from the study. The young volunteers (n=24, <40 years old) were recruited from Agency for Science, Technology and Research, Singapore (Singapore Immunology Network). The research was approved by National University of Singapore Institutional Review Board (IRB code NUS-IRB 01-256) and informed consent was obtained from all participants. Blood was collected in Cell Processing Tubes (CPT) (Becton Dickinson (BD), USA) and processed within 2 hours according to manufacturer's instructions. Details of the participants in the study for microbiome and peripheral blood immune composition association, (n=20, >75 years old) (Table 1b) is the same as the SLAS cohort. For human peripheral blood T cell characterization studies, cone blood was obtained from Health Science Authority (HSA) Singapore, approved under NUS-IRB 10-250 and isolated using Ficoll-Paque method (GE Healthcare, USA).

4.1.2 Cell Preparation

Blood was collected in BD CPT vacutainers and processed according to the manufacturer's recommendations. The vacutainers were centrifuged at 1650 rcf for 20 mins at room temperature and stopped with no brakes. Plasma was collected and aliquoted for storage at -80°C . The PBMC layer was collected and washed twice with PBS containing 5% FBS. The PBMC was cryopreserved in 10% DMSO with 90% FBS before storing in liquid nitrogen for later experiments. PBMC were rapidly thawed at 37°C , diluted with RPMI containing 10% FBS and subsequently washed twice with PBS containing 5% FBS before using for the respective experiments.

4.1.3 Cytokine response assay

1×10^6 PBMCs were resuspended in either control medium (RPMI1640 supplemented with 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 10mM HEPES and Penicillin

(100U/ml) Streptomycin (100µg/ml) (Gibco, USA)), with 1µM of (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP; Echelon Biosciences Incorporated, USA) or with 10ng/mL phorbol 12-myristate 13-acetate, (PMA; Sigma-Aldrich, USA) together with 1µM of calcium ionophore ionomycin (Sigma-Aldrich, USA) in 15ml BD falcon tube. Brefeldin A and Monesin (Thermo Scientific, USA) was added in the last 4 hours of stimulation. Stimulation was performed at 37°C, 5% CO₂ for 6 hours to assess the intracellular cytokine production by Vδ1+, Vδ2+, CD4 and CD8 T cells simultaneously.

4.1.4 Proliferation Assay

1µg/ml of CD3 (OKT3, Thermo Scientific, USA) was coated on a 96 well flat bottom plate in 37°C, 5% CO₂ for 3 hours and washed with PBS twice to wash away unbound antibodies. CellTrace™ Violet (Molecular Probes, Invitrogen, USA) was used to label the cells according to manufacturer's instructions. Labeled PBMCs (1x10⁵) were done in duplicates for each donor and re-suspended in either 200ul of control medium (RPMI1640 supplemented with 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 10mM HEPES and Penicillin (100U/ml) Streptomycin (100µg/ml (Gibco, USA)), with recombinant 10ng of IL-2 (R&D System, USA) in CD3 plated wells or with 1µM of (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP; Echelon Biosciences Incorporated, USA). PBMCs were allowed to proliferate for 5 days in 37°C, 5% CO₂ before the duplicates were pooled together for antibodies staining and flow cytometry analysis.

4.1.5 Flow Cytometry

1 x 10⁶ PBMC were stained with LIVE/DEAD Blue or Aqua Fixable Stain Kit (L/D Blue; Molecular Probes, Invitrogen, USA) together with anti-human monoclonal antibodies specific for CD3 (UCHT1), CD4 (RPA-T4), CD8 (SK1), PAN-γδ (11F2), Vδ1 (TS8.2, REA173), Vδ2 (B6), CD27 (M-T271), CD45RA (H100), CD56 (NCAM16.2), CD16 (3G8), CD57 (HCD57), KLRG1 (REA261), CD161 (HP-3G10), Vα7.2 (3C10), CD31 (WM59), CCR9 (L053E8), α4(9F10), β7(FIB504) for 20 minutes in the dark at 4°C in PBS containing 5% FBS, 2mM EDTA (FACS buffer). For CD85j (GHI/75) and CD244 (2-69), cells were single stained with CD85j for 20 mins in the dark at 4°C in FACS Buffer, followed by CD244 for 20 mins in the dark at 4°C before adding the mastermix to the cells for 20 mins in the dark at 4°C. This is to ensure successful staining as shown in Appendix A.

For functional assay, CD107a (eBioH4A3) was added at the start of the stimulation for optimum staining. After stimulation, cells were stained with surface markers before being

fixed and permeabilized for 20 mins in 4°C with BD CytoFix/CytoPerm Fixation and Permeabilization Solution (BD Biosciences, Pharmingen, USA). The cells were then washed twice with 1X Perm/Wash Buffer (BD Biosciences, Pharmingen, USA). PBMCs were then stained in 1x Perm/Wash with anti-human CD154 (5C8), IFN γ (4S.B3), TNF α (MAb11), IL-2 (MQ1-171H12), Granzyme B (GB11), Perforin (B-D48) and IL-17A (EBIO64DEC17) for 30 mins in 4°C and then washed twice before re-suspending in 100 μ l FACS buffer. Samples were acquired in a BD LSRII flow cytometer using automatic compensations.

4.1.6 CYTOF (Cytometry using Time of Flight)

Antibodies conjugation and CyTOF staining.

Frozen samples were thawed and washed in RPMI 10% FBS + DNase (15ug/ml). Cells were stained with Cisplatin (viability marker) 5 μ M in PBS for 5 mins as previously described (201). Cells were then stained with antibody cocktail. After wash, cells were stained in PBS + 0.5% BSA buffer with antibodies at 4°C for 15min. After two washing steps, cells were fixed in fixation FoxP3 buffer (eBioscience – 00-5521-00) for 30 mins at 4°C. After washing in perm buffer, cells were stain with α Eomes-PE for 30 mins at 4°C in perm buffer. Cells were washed and stained with α PE, α T-bet, α Perforin, α GranzymeA, α GranzymeB, for 30 mins at 4°C in perm buffer. After two washing steps, cells were fixed in PBS 2% PFA overnight. Cells were stained for DNA as previously described (206).

Antibody Conjugation.

Purified antibodies lacking carrier proteins were conjugated as the following CD45 (H130, Y89), CD3 (UCHT1, Er168), Pan- $\gamma\delta$ (B1-APC, Yb176), V δ 2 (B6, Ce140), CD127 (AO19D5, Dy163), IL-18R (H44, Ho165), CD57 (HCD57, ln115), CD56 (559043, Gd158), CD16 (3G8, Bi209), CD45RO (UCHL1, Nd148), Perforin (B-D48, Lu175), Granzyme B (CLB-GB11, Dy164), Granzyme A (CB9, Gd157), Tbet (eBio4B10, Gd155), Eomes (WD1928-PE, Yb171), CD27 (LG.7F9, Eu151), CD161 (HP-3G10, Tb159), KLRG-1 (13F2F12, Sm150), Cisplatin (Live/Dead). Antibody conjugation was performed according to the protocol provided by Fluidigm Inc.

Data analysis and t-SNE.

After CyTOF acquisition, which was performed as previously described (201), any zero values were randomized using a uniform distribution of values between zero and minus-one using an R script (as was the default operation of previous CyTOF software). Note also that all other integer values measured by the mass cytometer are randomized in a similar fashion by default.

The signal of each parameter was then normalized based on the EQ beads (Fluidigm) as previously described (202). Cells were manually de-barocoded using FlowJo (Tree Star Inc.). Samples were then used for tSNE analysis similar to that previously described using custom R scripts based on the “flowCore” and “Rtsne” (using CRAN R packages that performs the Barnes-Hut implementation of t-SNE) In R, all data were transformed using the “logicleTransform” function (“flowCore” package) using parameters: w=0.25, t=16409, m=4.5, a=0 to roughly match scaling historically used in FlowJo. For heatmap, Median intensity corresponds to a logical data scale using formula previously describe. The colors in the Heat Map represent the measured means intensity value of a given marker in a given cluster. A four colors scale is used with black–blue indicating low expression values, green–yellow indicating intermediately expressed markers, and red representing highly expressed markers

4.1.7 FLOW-FISH (Fluorescent in-situ hybridization)

We adapted a modified version of the flow cytometry-fluorescent in situ hybridization technique (Flow-FISH) (163) using the heat stable fluorochromes and Brilliant Violet conjugated antibodies. This allowed simultaneous T cell subset in combination with telomere length measurement. Sorted PBMCs were stained with CD27- BV785 (O323), CD45RA - BV605 (H100) and CD57-PB (HCD57). Samples were then washed in PBS, fixed in 1mM BS3 (30mins on ice, Thermo Scientific, USA) and quenched with 50mM Tris in PBS (pH7.2, 20 mins, RT). Cells were then washed twice; first in PBS, and then in hybridization buffer (70% deionized formamide, 28.5 mM Tris HCL pH 7, 1.4% BSA and 0.2M NaCl). Subsequently the samples were re-suspended in hybridization buffer and incubated with 0.75µg/ml of the PNA TelC-Cy5 probe (Panagene, South Korea) and heated for 10 mins at 82°C. Samples were then rapidly cooled on ice and left to hybridize for 1hr at RT in the dark. Lastly, samples were washed twice in post hybridization buffer (70% deionized formamide, 14.25mM Tris HCL pH 7, 0.14% BSA, 0.2M NaCl, 0.14% Tween20) and twice in 2% BSA/PBS before acquisition on BD Fortessa using BD FACS Diva software.

4.1.8 Soluble factor measurement by multiplex (DropArray)

Sorted PBMCs were stimulated with PMA/Ionomycin (10ng/ml) for 4 hrs at 37 oC. PBMCs were then pellet down at 1500rpm at 4 oC. Supernatants was harvested and analyzed using Droparray based on the molecule of interest. Luminex® bead-based multiplex assays are based on Luminex® xMAP® technology and they are designed to simultaneously measure multiple specific protein targets in a single sample.

Customized Human 17-plex kits (R&D Systems, cat# LXSAHM-17), 10-plex (Merck, cat# HCYTOMAG-60K-10) and 3-plex (Merck, cat# HCD8MAG-15K-03) were measured with DropArray™ 96 plate (DA-96) (Curiox Biosystems, cat# 96-CC-BD-05).

17-plex: Human TNF-alpha, IL-6, MMP-3, IL-8/CXCL8, MCP-1/CCL2, VEGF-A, IL-1beta, IFN-gamma, MIP-1a/CCL3, Amphiregulin, IL-1alpha, IL-2, RAGE, GM-CSF, PDGF-AA, VEGF-D, GROalpha

10-plex: Human TNF-alpha, IL-2, IFN-gamma, MIP-1a, IL-10, IL-13, IL-17A, IL-4, IL-5, IL-6

3-plex: Human Granzyme A, Granzyme B, Perforin

Using DA-96, samples or standards were incubated with fluorescent-coded magnetic beads, which had been pre-coated with respective capture antibodies. After an overnight incubation at 4°C with shaking, plates were washed twice with wash buffer. Biotinylated detection antibodies were incubated with the complex for 30 mins (for R&D Systems' protocol) or 1 hour (for Merck's protocol) and subsequently Streptavidin-PE was added and incubated for another 30 mins. Plates were washed twice again, and beads were re-suspended with sheath fluid in PCR plates before acquiring on the FLEXMAP® 3D (Luminex). Data acquisition was done using xPONENT® 4.0 (Luminex) acquisition software and data analysis was done using Bio-Plex Manager™ 6.1.1 (Bio-Rad). Standard curves were generated with a 5-PL (5-parameter logistic) algorithm. Lastly, a report was generated with values for both MFI and concentration data.

4.1.9 Nanostring Experiments

Human Customized Senescence Panel

Direct mRNA expression levels of the samples were measured using the NanoString nCounter gene expression system. 10000 cells (except for 3 sample with ~5500-7700 cells) in 5uL of RLT buffer from Qiagen RNeasy Mini kit (Qiagen, Germany) were hybridized with probes from the nCounter Human Senescence custom panel (Nanostring, USA) at 65°C for 19 hrs according to the nCounter™ Gene Expression Assay Manual. Excess probes were washed away using a two-step magnetic bead-based purification on the nCounter™ Prep Station (GEN1). The nCounter™ Digital Analyzer (GEN1) was used to count individual fluorescent barcodes and quantify target molecules present in each sample. For each assay, a high-density scan (600 fields of view) was performed.

nCounter® Human Inflammation v2

Sorted PBMCs were stimulated with PMA/Ionomycin (10ng/ml) for 4 hrs at 37 °C. PBMCs were then pellet down at 1500rpm at 4 °C. Direct mRNA expression levels of the samples were measured using the NanoString nCounter gene expression system. 6249-10000 cells in 5uL of RLT buffer from Qiagen RNeasy Mini kit (Qiagen, Germany) were hybridized with probes from the nCounter Human Inflammation v2 panel (Nanostring, USA) at 65°C for 19 hours according to the nCounter™ Gene Expression Assay Manual. Excess probes were washed away using a two-step magnetic bead-based purification on the nCounter™ Prep Station (GEN1). The nCounter™ Digital Analyzer (GEN1) was used to count individual fluorescent barcodes and quantify target molecules present in each sample. For each assay, a high-density scan (600 fields of view) was performed.

4.1.10 CMV Serology

Plasma from the participants were thawed and analyzed for the presence of antibodies (IgG) against cytomegalovirus (CMV). This was tested using ELISA kits (Omega Diagnostics, UK) and performed according to the manufacturer's instructions. Young participants that were CMV- (N=12) and CMV+ (N=12), according to the controls, were selected. There was no seronegative for CMV in the elderly individuals in this study (N=12). (Table 1a)

4.1.11 Epigenetic Methylation RRBS Data and Analysis

DNA preparation

Sorted cell population were snap frozen in liquid nitrogen for future use. Frozen samples were taken out and Pure Link Genomic DNA extraction kit (Invitrogen, USA) was used as manufacturer's instructions to extract DNA for epigenetic sequencing.

Reduced representation bisulfite sequencing

RRBS-seq was performed as described (203). In brief, 50 ng of purified DNA was digested with MspI (Fast digest MspI, Thermo Scientific FD0544, USA) for 30 mins at 37°C followed by heat inactivation at 65°C for 5 mins. Library preparation was performed using NEBNext Ultra DNA library prep kit for Illumina (New England BioLabs, E7370L, USA) and ligated with methylated adapters for Illumina at a dilution of 1:10 (New England BioLabs, E7535L, USA). The adapter ligated DNA was subjected to bisulfite conversion with EpiTect fast bisulfite conversion kit (Qiagen, 59824, Germany) using the following cycling conditions: 2

cycles of (95°C; 5mins, 60°C; 10mins, 95°C; 5mins, 60°C; 10mins) and hold at 20°C. Bisulfite converted DNA was PCR amplified for 14-16 cycles using 2.5 U of Pfu Turbo Cx Hotstart DNA polymerase (Agilent Technologies, 600410) and size selected for fragments between 200 bp to 500 bp with Ampure Xp magnetic beads (Agencourt, A63880, USA). The purified DNA was subjected to single end sequencing using the Illumina Hiseq 2000 at 1x 101 bp readlength

DNA methylation data processing

RRBS-seq reads were aligned to the human reference genome, hg19, using Bismark with default parameters. To ensure high data quality, CpGs with $Q < 30$ and read depth of less than 5X were filtered out before calculating the percentage methylation (PM). PM is calculated for each covered C by taking the ratio of reads called methylated C divided by the total number of methylated and unmethylated reads. To achieve an accurate estimate of methylation level, high read cutoff was applied to eliminate PCR effects. CpGs having higher coverage than 99.9% percentile of read counts were removed, using filterByCoverage function in MethylKit package (204). Methylation occurs almost exclusively in the CpG context; hence we focused only on those cytosines in CpG dinucleotides.

Differentially methylated CpGs analysis

DmCpG analysis was performed using R package Methylkit. High coverage bases were filtered to remove potential PCR bias before differential methylation calculation. Read coverages between samples were also normalized to avoid bias introduced by systematically more sequenced samples. Logistic regressions were used to calculate P-values for dmCpGs. P-values were adjusted to Q-values using SLIM method. After q-value calculation, we can select the differentially methylated regions/bases based on q-value and percent methylation difference cutoffs [q-value < 0.01, meth.diff > 20].

WGCNA analysis

Beta values from significant dmCpGs were used to construct methylome modules using weight gene correlation network analysis (WGCNA) [thresholding power 6, minimum module size of 30, mergeCutHeight 0.15]. Eigengene for each module was correlated to the differentiation time point. Modules that showed significant association were visualized using heatmap and boxplots [absolute correlation coefficient, $r > 0.75$, p-value < 0.01].

4.1.12 RNA-Seq

RNA preparation

Total RNA was extracted following the double extraction protocol: RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (TRIzol, Thermo Fisher Scientific, USA) followed by a Qiagen RNeasy Micro clean-up procedure (Qiagen, Germany). All human RNAs were analyzed on Agilent Bioanalyzer for quality assessment with RNA Integrity Number (RIN) range from 6.2 to 9.6 and median RIN 8.9 (Agilent, USA).

RNA-Seq library preparation

cDNA libraries were prepared using 2ng of total RNA and 1ul of a 1:50,000 dilution of ERCC RNA Spike in Controls (Ambion® Thermo Fisher Scientific, USA) using SMARTSeq v2 protocol (205) except for the following modifications: 1. Use of 20μM TSO, 2. Use of 250pg of cDNA with 1/5 reaction of Illumina Nextera XT kit (Illumina, USA). The length distribution of the cDNA libraries was monitored using DNA High Sensitivity Reagent Kit on the Perkin Elmer Labchip (Perkin Elmer, USA). All samples were subjected to an indexed PE sequencing run of 2x51 cycles on an Illumina HiSeq 2000 (16 samples/lane).

RNA-Seq data analysis

The genome assembly and annotation for the RNA-Seq data analysis was downloaded from GENCODE (version 26) (206). The quality of the RNA-Seq data was assessed with FastQC (207). The reads were pseudo-aligned to the transcriptome with kallisto (208), and the transcript expression values were then summarized into gene expression values with tximport (209). The counts were normalized for sequencing depth and gene length using the Transcript per Million (TPM) method (210). The prcomp function from the stats package were used to perform the analyses.

The RNA-Seq data is available as part of a larger GEO repository with accession number GSE107011.

4.1.13 GSEA Analysis

Averaged gene expression data in the form of log₂ RPKM values were used to rank the genes for each of the cell type. This ranked list was then checked for enrichment in the Vδ2+ gene set using a Gene Set Enrichment Analysis (GSEA). GSEA was conducted using the fgsea package in bioconductor running using R version 3.3.1.

4.1.14 DNA Damage Repair (DDR) Assay

1×10^6 PBMCs (re-suspended in RPMI1640 supplemented with 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 10mM HEPES and Penicillin (100U/ml) Streptomycin (100 μ g/ml (Gibco, USA)) were UV-irradiated for 6 hours (6amp, UVC) using Gelman BH Class 2 Series biological safety cabinet with lid on 96 well U-bottom plate. Controls (non-UV irradiated) were placed in 37 °C, 5% CO₂ for 6 hours. PBMCs were then stained with LIVE/DEAD Blue or Aqua Fixable Stain Kit (L/D Blue; Molecular Probes, Invitrogen, USA) together with anti-human monoclonal antibodies specific for CD3 (UCHT1, FITC), CD8 (SK1, V500), V δ 1 (REA173, PE Vio770), V δ 2 (B6, BV711), CD27 (M-T271, BUV395), CD45RA (H100, BV605), CD57 (HCD57, PB) for 20 mins in 4 °C. PBMCs were then washed twice with FACS Buffer. After washing, PBMCs were fixed using BD Cytofix Buffer at 37 °C for 10mins. After washing with FACS Buffer once, 300ul of BD Phosflow Perm Buffer 2 was used to permeabilize the cells for 30mins at 4 °C. After permeabilization, anti-H2AX phospho(Ser139) (2F3, Alexa Fluor 647) were added for 30mins at RT in the dark. PBMCs were then washed twice with FACS Buffer and re-suspended in 100ul of FACS Buffer before acquisition on the flow cytometer BD FACSSymphony.

4.1.15 16S Microbiome Sequencing

Sample Preparation

Stools were collected and frozen in aliquots with glass beads. Stools were then resuspended in Breaking Buffer (2% (v/v) Triton X-100, 1% (v/v) SDS, 100mM NaCl, 10mM Tris-HCl (pH: 8.0), 0.1mM EDTA (pH:8.0). After breaking down the stools, DNA was extracted using Phenol/Chloroform method with RNase A as shown in (211).

Preparation of 16S amplicon libraries

For amplification of the 16s variable regions, PCR was performed using 10ng of gDNA prepared from gut metagenome samples with Long Amp Taq polymerase (New England Biolabs, USA) as described in Jones *et al.* 2016, (212). In brief, first round of PCR enriches for V4 & V5 regions of bacterial 16s rDNA regions and incorporates partial Illumina adapter sequences. The secondary PCR further enriches for variable region sequences while adding complete Illumina adapter tags, barcodes for sequencing and demultiplexing individual samples, respectively. Equimolar concentrations of secondary PCR products were pooled and electrophoresed using 2% agarose gel. This pool of libraries was size selected (~550 bp) by gel purification using Qiaquick Gel Extraction Kit (Qiagen, Germany). Concentrations of gel-purified libraries were estimated using LabChip GX reagents according to the manufacturer's

instructions (PerkinElmer, USA). qPCR was performed (Kapa Biosystems, USA) using the quantified libraries to ascertain the loading concentration. The libraries were sequenced using Illumina MiSeq to generate 250bp paired end reads.

16S Sequencing Analysis

The FASTQ files obtained by the Illumina sequencing by synthesis protocol, from the fragment's library from the 16S amplification, had the Illumina-specific forward and reverse primers removed using Cutadapt; paired-end reads were joined together with the aid of Flash software, and a sliding window quality filter was applied using Trimmomatic.

A Perl pipeline mainly based on QIIME (Quantitative Insights Into Microbial Ecology), designed for the microbial community analysis of DNA sequencing data, was followed. The hypervariable regions in the 16S gene that were amplified, provide species-specific signature sequences that were compared with known sequences in a reference database via the QIIME OTU (Operational Taxonomic Unit, the microbes in the community) picking method against the Silva database version 123 at 99% of redundancy.

R and Spotfire were used respectively to read the QIIME resulting OTU table, calculate relative OTU percentage abundances at genus level, and plot the values in stacked bar plots.

4.1.16 Data Analysis, Statistics and Visualization

For analysis of flow cytometric data, FlowJo version 10.06 (TreeStar, Portland, USA) was used. Statistical analysis was performed using Prism 6 and 7 (Graph Pad Software, Inc La Jolla, USA). For comparisons between two independent groups, the Mann-Whitney U Test was performed. P-values <0.05 were considered significant. For comparisons between 3 or more independent groups, Kruskal-Wallis Test and multiple T tests (corrected with Dunn's Method) was performed. Adjusted P-values <0.05 were considered significant. For correlation analysis, spearman correlation was performed. P-values <0.05 were considered significant. For comparisons between paired samples, Wilcoxon signed-rank test was performed. P-values <0.05 were considered significant. For comparisons between paired samples but with 3 or more groups, Friedman Test and multiple T tests (corrected with Dunn's Method) was performed. Adjusted P-values <0.05 were considered significant. No sample power analysis was done prior to experiment. Number of samples was limited by sample availability, reagents and resources.

For multiplex cytokine data, analysis and presentation of distributions was performed using SPICE version 5.1 and Monte Carlo was performed to compare between 2 SPICE pies in Figure 3. Modfit LT version 3.2 was used to derive the proliferation index by floating method.

The formula for proliferation index

$$\text{Proliferation Index} = \frac{\sum_0^l N_i}{\sum_0^l N_i / 2^i}$$

where l is the generation number (parent generation = 0).

In the absence of proliferation, that is, when all cells are in the parent generation, Eq. (1) gives:

$$\frac{N_0}{N_0/2^0} = 1, \quad \text{defining the lower limit of the PI.}$$

GREAT Analysis (213) was used for Enrichment analysis of epigenetic genes in MeSalmon Module. Microsoft Excel 2016 was used to obtain the radar graphs. RStudio v3.3.2, ggplot2 package and custom R scripts was utilized to obtain the correlation heatmap matrix, PCA Analysis, t-SNE analysis, heatmap of nanostring gene expression, scatter plot and module-trait relationship of epigenetic data. Cytoscape v3.5 was used to generate the association analysis

4.2 Skin Aging of T cells Materials and Methods

4.2.1 Donor Information

Young (n=13, age=25-45) and old (n=22, age=66-87) (Supplementary Table 1c) For peripheral blood and skin comparison, (n=20, ≥ 65 years old) participants were recruited based upon suspected skin carcinoma but skin was taken mostly from a peri-lesional site and some away from the suspected carcinoma. Blood was drawn in EDTA Tubes (Greiner Bio-one) and Peripheral Blood Mononuclear Cells (PBMCs) was isolated by Ficoll-Paque (GE Healthcare) density centrifugation method.

Skin samples were placed in Collagenase Type IV (Worthington Biochemical Corporation, 0.8mg/ml) and DNase I (Sigma, 50ug/ml) in RPMI1640 with 10% FBS (Fetal Bovine Serum) and Pen/Strep (Gibco, 100U/ml) overnight in 37°C to extract the immune cells for downstream experiments. This research was approved by National Healthcare Group (NHG), DSRB 2014/01061.

4.2.2 Flow Cytometry

PBMCs and cells that was extracted from the tissue biopsy were stained with LIVE/DEAD Blue Fixable Stain Kit (L/D Blue; Molecular Probes, Invitrogen, USA) together with anti-human monoclonal antibodies specific for CD3 (UCHT1), CD4 (RPA-T4), CD8 (SK1), PAN- $\gamma\delta$ (11F2), V δ 1 (TS8.2), V δ 2 (B6), CD27 (M-T271), CD45RA (H100), CD57 (HCD57), KLRG1 (REA261), CD69 (FN50), PD1 (MIH4), for 20 minutes in the dark at 4°C in PBS containing 5% FBS, 2mM EDTA (FACS buffer). Samples were acquired in a BD ARIA 5 flow cytometer using automatic compensation.

4.2.3 Data Analysis and Visualisation

For analysis of flow cytometric data, FlowJo version 10.06 (TreeStar, Portland, USA) was used. Statistical analysis was performed using Prism 7 (Graph Pad Software, Inc La Jolla, USA). For comparisons between two independent groups, the Mann-Whitney U Test was performed. P-values <0.05 were considered significant. For correlation analysis, custom R scripts (ggplot2) and spearman correlation was utilized. P-values <0.05 were considered significant. For comparisons between paired samples, Wilcoxon signed-rank test was performed. P-values <0.05 were considered significant.

4.2.4 RNA-seq

Whole Tissue RNA-seq SmartSeqV2

Tissue biopsies were disrupted and homogenised in Trizol using Tomy beads cells disruptor MS100 (Tomy, Japan). Total RNA was extracted following the double extraction protocol: RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (TRIzol, Thermo Fisher Scientific, Waltham, MA, USA) followed by a Qiagen RNeasy Micro clean-up procedure (Qiagen, Hilden, Germany). All human RNAs were analyzed on Agilent Bioanalyzer (Agilent, Santa Clara, CA, USA) for quality assessment with RNA Integrity Number (RIN) range from 7.0 to 8.8. cDNA libraries were prepared using 2ng of total RNA and 1ul of a 1:50,000 dilution of ERCC RNA Spike in Controls (Ambion® Thermo Fisher Scientific, Waltham, MA, USA) using SMARTSeq v2 protocol except for the following modifications: 1. Use of 20µM TSO, 2. Use of 250pg of cDNA with 1/5 reaction of Illumina Nextera XT kit (Illumina, San Diego, CA, USA). The length distribution of the cDNA libraries was monitored using DNA High Sensitivity Reagent Kit on the Perkin Elmer Labchip GX system (Perkin Elmer, Waltham, MA, USA). All samples were subjected to an indexed PE sequencing run of 2x151 cycles on an Illumina HiSeq 4000 (30 samples/lane).

RNA-seq Data analysis

The data was mapped to HG38 using the Gencode V26 annotation and the STAR aligner (214). Gene counts were determined with featurecounts (215). Differential gene detection was performed in edgeR (216) using R v 3.3.1/bioconductor. Hierarchical clustering was performed with the hclust package in R.

4.2.5 Nanostring

Nanostring nCounter® Immunology Panels v2 (Human)

Direct mRNA expression levels of the samples were measured using the NanoString nCounter gene expression system. 3211-10000 cells in 5uL of RLT buffer from Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) were hybridized with probes from the nCounter Human Immunology v2 panel (Nanostring, Seattle, USA) at 65°C for 16 hours according to the nCounter™ Gene Expression Assay Manual. Excess probes were washed away using a two-step magnetic bead-based purification on the nCounter™ Prep Station. The nCounter™ Digital Analyzer (GEN2) was used to count individual fluorescent barcodes and quantify target molecules present in each sample. For each assay, a high-density scan (280 fields of view) was performed.

Nanostring Data Analysis

Nanostring data obtained as RCC files were processed using Python 2.7 and R 3.3.1. The geometrical mean of the positive control probe counts were computed for each lane and a scaling factor computed for each lane being the average of the geometrical means of all lanes divided by the geometrical mean of that particular lane. This scaling factor was then applied to all probe counts for all lanes as a means to normalize for the technical variability of the platform. The house keeping genes *EEF1G*, *OAZ1* and *RPL19* were then used to normalize for any RNA loading differences. This was performed in the same manner as the positive control probes where the scaling factor was computed from the geometrical mean of the house keeping genes. The positive control and housekeeping normalized counts were then logarithmically transformed and used for all subsequent analysis. Differentially expressed genes were then determined using t tests in R 3.3.1. Multiple testing corrections was performed using the method of Benjamini and Hochberg. P values less than 0.05 were deemed to be statistically significant.

RESULTS

CHAPTER 5: HUMAN $\gamma\delta$ T CELLS IMMUNOSENESCENCE

5.1: Human $\gamma\delta$ T Cells in Young and Old Peripheral Blood

5.1.1 Decrease in frequency of V δ 2+ T cells in peripheral blood of the elderly not associated to thymic involution

Previous work by our lab has shown that $\gamma\delta$ T cells in the peripheral blood as a whole exhibit a decrease in terms of frequency in the elderly when compared to the young (217). However, whether this observation affects all the $\gamma\delta$ T cells subsets similarly remains to be investigated. In this thesis, we dissected the $\gamma\delta$ T cells subsets and analyzed them separately. We employed a gating strategy that allows us to analyze the $\gamma\delta$ T cells accurately as it has been shown that some of the V δ 2+ T cells will not be stained positive for pan- $\gamma\delta$ when used with a combination of CD3, V δ 2 and pan- $\gamma\delta$ antibodies (Figure. 1a,b, 218,219, Appendix A). Using this analysis method, we observed a decreased in pan- $\gamma\delta$ in the periphery of the elderly (Figure 1c), which is similar to our previous study and also other studies conducted elsewhere. Going deeper into the individual subsets, we observed that only the V δ 2+ frequency decreases with age significantly but not the other two subsets of $\gamma\delta$ T cells (Figure 1d). We then continued to analyze how does CMV infection and age affects the subsets within the $\gamma\delta$ T cells. We found that CMV infection with an additive of age significantly increased the frequency of V δ 1+/ $\gamma\delta$, whereas V δ 2+/ $\gamma\delta$ decreases (Figure 1e). This illustrates that CMV infection and age can affect the balance and distribution of the $\gamma\delta$ T cells in the periphery of an individual, which could perhaps affect how the host responds to infection and cancer.

Observing a decrease in V δ 2+ in the peripheral blood of the elderly, we hypothesized that the decrease in frequency could be associated to thymic involution. Previous studies have shown that thymus, the organ that T cells develop and mature in, starts involuting after age 40 and thus reducing the production of naïve T cells. As it is difficult to assess the thymus size directly from the patient, we used CD4 RTE's frequency (Recent Thymic Emigrant) as a surrogate marker of thymic output as it has been shown to correlate with the thymus size. However, we did not find any correlation between the frequency of CD4 RTE and V δ 2+ even though both showed a decreased in terms of frequency between young and old individuals (Figure 1h, 1i). This suggests that the decrease of V δ 2+ in the peripheral blood is unlikely due to thymic involution but other unknown factors.

As V δ 2⁺ primary response is against bacterial metabolites and most of them do exhibit the gut-homing phenotype (220,221), we then hypothesized that the microbiome profile of the elderly individual could have an association with the frequency of V δ 2⁺ in the periphery. Our correlation analysis suggest that while most peripheral T cells' phenotype remain unassociated with the abundance of bacteria in the microbiome of the individual, an association was found between the abundance of Parabacteriodes vs $\gamma\delta$ /CD3 (Figure 2a, b,c). This suggests that the abundance of parabacteriodes could have an impact on the frequency of the $\gamma\delta$ in the periphery of the elderly, which could be important for the host to respond against certain bacteria infections or cancer.

FIGURE 1

FIGURE 1. Frequency of $\gamma\delta$ T cells subsets and CD4 RTE in the periphery of the young and elderly. CMV- Young (RED) (n=12), CMV+ Young (BLUE)(n=12) and CMV+ Elderly (GREEN) (n=12) individuals PBMCs were stained and analyzed by flow cytometry. (a) Representative FACS plot of Pan $\gamma\delta$ T cells, (b) $\gamma\delta$ T cells subsets (c) Frequency of $\gamma\delta$ T cells/CD3, (d) Individual subsets of $\gamma\delta$ T cells frequency/CD3, (e) Frequency of $\gamma\delta$ T cells subsets/ $\gamma\delta$ T. Kruskal-Wallis Test and multiple T tests (corrected with Dunn's Method) was performed for (c), (d), (e). Adjusted P-values <0.05 were considered significant. Young (RED) (n=12), Old (BLACK) (n=10) individuals PBMCs were stained and analyzed by flow cytometry. (f) Representative FACS plot of CD4 Naive, (g) CD4 RTE (CD4+, CD27+ CD45RA+ CD31+), (h) Frequency of V δ 2/CD3 and CD4 RTE/CD3 Mann-Whitney U Test was performed for (h). P-values <0.05 were considered significant (Young n=12, Old n=10) i) Spearman Correlation Analysis of V δ 2+ and CD4 RTE (*p<0.05, ** p<0.01)

FIGURE 2

OTU % abundances at Genus level

a)

b)

c)

FIGURE 2. Relationship between Microbiome profile and Periphery immune parameters in elderly.

a) Genus % abundance and diversity of various bacteria using 16s DNA sequencing in elderly stool samples. b) Cytoscape spearman correlation analysis between genus % abundance and various periphery immune parameters. c) Individual spearman correlation graphs of genus % abundance that correlated with periphery immune parameters. For correlation analysis, spearman correlation was performed. P-values <0.05 were considered significant

5.1.2 Peripheral $\gamma\delta$ T cells phenotypic alterations between the young and elderly

With CMV infection and age, the $\alpha\beta$ T cells phenotype shifts from a naïve phenotype (antigen-inexperienced) (CD27⁺ CD45RA⁺) to a highly differentiated phenotype (antigen-experienced) (CD27⁻ CD45RA⁻, CD27⁻ CD45RA⁺). However, whether similar shifts in terms of phenotype occurs in the $\gamma\delta$ T cells subsets remains to be investigated. Using CD27 and CD45RA as markers that are used to define the four subsets of $\alpha\beta$ T cells into Naïve (CD27⁺ CD45RA⁺), CM (CD27⁺ CD45RA⁻), EM (CD27⁻ CD45RA⁻) and TEMRA (CD27⁻ CD45RA⁺) on the $\gamma\delta$ T cells subsets, we observed a similar shift from naïve phenotype to TEMRA with CMV infection and age on the V δ 1⁺ and DN $\gamma\delta$ ⁺ but not on the V δ 2⁺ (Figure 3b-3e). We then also assessed other markers that were known to change with CMV infection and age in CD8 such as CD85j, CD244, CD56 and KLRG1 on the $\gamma\delta$ T cells subsets (222). We observed the same trend as seen with CD27, CD45RA, the significant increase of each marker with CMV infection and age on V δ 1⁺ and DN $\gamma\delta$ ⁺ but not V δ 2⁺ (Figure 4a,b 5a-c). It is worth noting that CD244 and KLRG1 are highly expressed on V δ 2⁺ regardless of CMV infection and age, suggesting they are differentiated with effector functions as these markers are used to define differentiated CD8 T cells. Previous study by Roux *et al.* 2013 has shown that CMV infection expands the V δ 2⁻ $\gamma\delta$ T cells that express CD16. However, whether this expansion occurs in V δ 1⁺, DN $\gamma\delta$ ⁺ or both remains unclear. Analyzing them separately, we showed that it only happens in V δ 1⁺ and not DN $\gamma\delta$ ⁺ T cells. All together, these suggest that CMV infection and age affects the $\gamma\delta$ T cell subsets differently, in terms of phenotype and therefore they should be analyzed separately for future studies with regards to $\gamma\delta$ T cells as they might be affected differently in other diseases.

FIGURE 3

FIGURE 3. Phenotypic Alterations of CD8, V δ 1+, V δ 2+ and DN $\gamma\delta$ + in the periphery of the young and elderly. (CD27, CD45RA)

CMV- Young (RED) n=12, CMV+ Young (BLUE) n=12 and CMV+ Elderly (GREEN) n=12 individuals PBMCs were stained and analyzed by flow cytometry. (a) Representative FACS plots of CD27, CD45RA. (b) Frequency of the different subsets CD27 and CD45RA for (b) CD8, (c) DN $\gamma\delta$ +, (d) V δ 1+, (e) V δ 2+. Kruskal-Wallis Test and multiple T tests (corrected with Dunn's Method) was performed for (b-e). Adjusted P-values <0.05 were considered significant (*p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001)

FIGURE 4

FIGURE 4. Phenotypic Alterations of CD8, V δ 1+, V δ 2+ and DN $\gamma\delta$ + in the periphery of the young and elderly. (CD85j, CD244)

CMV- Young (RED) n=12, CMV+ Young (BLUE) n=12 and CMV+ Elderly (GREEN) n=12 individuals PBMCs were stained and analyzed by flow cytometry. (a) Representative FACS plot of CD85j and Frequency of CD85j+ of CD8 and the $\gamma\delta$ T cells, (b) Representative FACS plot of CD244 and Frequency of CD244+ of CD8 and the $\gamma\delta$ T cells. Kruskal-Wallis Test and multiple T tests (corrected with Dunn's Method) was performed for (a), (b). Adjusted P-values <0.05 were considered significant (*p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001)

FIGURE 5

FIGURE 5. Phenotypic Alterations of CD8, V δ 1+, V δ 2+ and DN $\gamma\delta$ + in the periphery of the young and elderly. (CD56, CD16, KLRG1)

CMV- Young (RED) n=12, CMV+ Young (BLUE) n=12 and CMV+ Elderly (GREEN) n=12 individuals PBMCs were stained and analyzed by flow cytometry. (a) Representative FACS plot of CD56 and Frequency of CD56+ of CD8 and the $\gamma\delta$ T cells, (b) Representative FACS plot of CD16 and Frequency of CD16+ of CD8 and the $\gamma\delta$ T cells, (c) Representative FACS plot of KLRG1 and Frequency of KLRG1+ of CD8 and the $\gamma\delta$ T cells. Kruskal-Wallis Test and multiple T tests (corrected with Dunn's Method) was performed for (a-c). Adjusted P-values <0.05 were considered significant (*p<0.05, ** p<0.01, *** p<0.001, ****p <0.0001).

5.1.3 Peripheral $\gamma\delta$ T cells functional alterations between the young and elderly

Observing the differential impact of the $\gamma\delta$ T cells subsets in their phenotype with CMV infection and age, we continued our investigation to assess whether the $\gamma\delta$ T cells' functional capacity is affected with CMV infection and age. Using CD8 as a reference, we stimulated whole PBMCs with PMA/ionomycin or HMBPP for 6 hours and stained intracellularly to check for the cytokines that were produced. We observed a significant increase in terms of poly-functionality for both cytokines and cytotoxicity in V δ 1+ with CMV infection and age, very much similar to the trend we observe in CD8 T cells. However, the functionality of V δ 2+ remains indifferent for both cytokines and cytotoxicity with CMV infection and age. This is also true even when we used a natural ligand of V δ 2+, which is HMBPP (Figure 6a-d), suggesting that its functional capacity is sustained with CMV infection and age. Having seen a similar trend of V δ 1+ and CD8 with CMV infection and age in both the phenotyping and functional capacity data, we hypothesized that the surface marker expression and its functional relevance of both cell type might be similar. We then correlated the phenotyping and functional capacity data and found a positive correlation for CMV IgG Titre, % of CD27- and TNF α + IFN- γ + cells in CD8 and V δ 1+ but not V δ 2+ (Figure 7a). This suggests that the classical phenotyping markers functional relevance for $\alpha\beta$ T cells might only be applicable for V δ 1+ but not V δ 2+. To further validate the correlation, we sorted CD8, V δ 1+ and DN $\gamma\delta$ + with the two "extreme" population, which is the naïve and the TE, stimulated them with PMA/Ionomycin and analyzed the cytokines present in the supernatants using luminex. We found that the amount of cytokine produced in the 2 "extreme" subsets of the different T cells were very similar (Figure 7b). Thus, this suggests that the classical phenotyping markers for CD8 might be functionally relevant for V δ 1+ and DN $\gamma\delta$ + T cells as it defines them similarly in terms of cytokine profile but not V δ 2+.

FIGURE 6

FIGURE 6. Functional Capacity of CD8, V δ 1+ and V δ 2+ in the periphery of the young and elderly.

SPICE analysis of Cytokine and Cytotoxicity profile of CD8, V δ 1+ and V δ 2+ in the young and elderly. CMV- Young (n=12), CMV+ Young (n=12) and Elderly (n=12) individuals PBMCs were stimulated by 10ng/mL PMA together with 1 μ M of calcium ionophore ionomycin or HMBPP for 6 hours at 37 degrees, stained and analyzed by flow cytometry. (a) PMA/Ionomycin stimulated SPICE Pies of Cytokines for CD8: CMV- Young (Left), CMV+ Young (Middle), CMV+ Old (Right), SPICE Pies of Cytokines for V δ 1+: CMV- Young (Left), CMV+ Young (Middle), CMV+ Old (Right), SPICE Pies of Cytokines for V δ 2+: CMV- Young (Left), CMV+ Young (Middle), CMV+ Old (Right). (b) HMBPP stimulated SPICE Pies of Cytokines for V δ 2+: CMV- Young (Left), CMV+ Young (Middle), CMV+ Old (Right) (c) PMA/Ionomycin stimulated SPICE Pies of Cytotoxicity for CD8: CMV- Young (Left), CMV+ Young (Middle), CMV+ Old (Right). SPICE Pies of Cytotoxicity V δ 1+: CMV- Young (Left), CMV+ Young (Middle), CMV+ Old (Right), SPICE Pies of Cytotoxicity for V δ 2+: CMV- Young (Left), CMV+ Young (Middle), CMV+ Old (Right) (d) HMBPP stimulated SPICE Pies of Cytotoxicity for V δ 2+: CMV- Young (Left), CMV+ Young (Middle), CMV+ Old (Right) Monte Carlo was performed to compared between 2 SPICE pies. P-values <0.05 were considered significant (**** p<0.0001).

FIGURE 7

FIGURE 7. Functional Capacity and correlation with phenotype of CD8, V δ 1+ and V δ 2+ in the young and elderly.

a) Spearman Correlation Heatmap Analysis using different parameters of CD8, V δ 1+, V δ 2+ and DN $\gamma\delta$ +. For correlation analysis, spearman correlation was performed. P-values <0.05 were considered significant. b) Multiplex beads analysis (n=6) of CD27- CD45RA+ and CD27+ CD45RA+ of CD8, V δ 1+ and DN $\gamma\delta$ +. 5×10^3 Sorted cells were stimulated with PMA/Ionomycin (10ng/ml) for 4 hours. Supernatants were collected for Multiplex analysis. Mann-Whitney U Test was performed. P-values <0.05 were considered significant (*p<0.05, **p<0.01).

5.1.4 CD27 delineate expression of molecules for V δ 2⁻ but not V δ 2⁺

To further understand if CD27 expression negative correlates with effector functions with transcription factors in $\gamma\delta$ T cells, we did a CYTOF analysis on $\gamma\delta$ T cells with a range of molecules that include surface markers, intracellular molecules and transcription factors. We observed that the expression of CD27⁺ on the surface was able to segregate V δ 2⁻ T cells into separate clusters using the t-SNE algorithm but not V δ 2⁺ T cells (Figure 8). Collectively, these results reinforce the concept that CD27 expression does not functionally define V δ 2⁺ in the same way as compared to CD8 and V δ 2⁻

FIGURE 8

FIGURE 8. CYTOF analysis on CMV + Young $\gamma\delta$ T cells a) PBMCs were stained with antibodies conjugated with heavy metals and analysed using CyTOF. T-SNE Analysis was done using 10000 cells from total $\gamma\delta$ T cells with 3 CMV+ Young (n=3).

5.1.5 Peripheral $\gamma\delta$ T cells soluble factors secretion capacity between the young and elderly

Observing no difference in the functional capacity for both cytokines and cytotoxicity with CMV infection and age for V δ 2+, we decided to expand the range of soluble factors that are being assessed, as there might be differences in terms of functional capacity in these molecules with CMV infection and age such as those described in Senescence Associated Secretory Phenotype (SASP) fibroblasts. We sorted the cells according to the different populations, stimulated them with PMA/Ionomycin to test their functional capacity for these soluble factors, using the supernatants for analysis. We first did a PCA analysis using the analytes we investigated and found out that the trajectory profile of CD8 and V δ 1+ is similar with CMV infection and age. However, for V δ 2+, they seem to cluster together irrespective of CMV infection and age (Figure 9a). This suggests that in general, V δ 2+ cytokine profile does not change with CMV infection and age unlike CD8 and V δ 1+. Going further with the different detected individual analytes, the trend was similar to what was observed previously, an increase of production of cytokines for V δ 1+ with CMV infection and age but not V δ 2+ (Figure 9b-d). With the same samples that were used with the soluble analytes measured in the multiplex experiment, we spun down the cell pellet and pooled together 4 different donors from each group to investigate the gene expression of genes associated with inflammation using the Human Inflammation V2 Panel designed by nanostring. Using the top 26 genes derived from PC1, we observed that the expression of inflammation genes after stimulation increased for CD8 and V δ 1+ with CMV infection and age. However, for V δ 2+, regardless of CMV infection and age, they clustered together with the elderly individuals' CD8 and V δ 1+. This shows that in terms of gene expression, they possessed an effector-like profile and does not change with CMV infection and age, further reinforcing the concept that V δ 2+ are unaffected not only in terms phenotype but also in term of the functionality (with the molecules we tested) with CMV infection and age.

FIGURE 9

a)

b)

Human Nanostring Inflammation Panel

e) Heat Map

FIGURE 9. Soluble Factors and Inflammation Gene Expression Analysis of CD8, V δ 1+ and V δ 2+ in the young and elderly. 5×10^3 Sorted cells of CMV- Young (n=12), CMV+ Young (n=12) and Elderly (n=12) individuals PBMCs were stimulated by with 50ul of 10ng/mL PMA together with 1 μ M of calcium ionophore ionomycin for 4 hours at 37 degrees. Supernatants were then harvested and analyzed by multiplex beads assay and represented in pg/ml. (a) PCA analysis profiling of CD8, V δ 1+ and V δ 2+ in CMV- Young, CMV+ Young and CMV+ Old. Concentration of (b) Analytes for V δ 1+ and V δ 2+, (c) Granzyme B and (d) Perforin of activated cells in all 3 groups. For the Radar Graphs, the significance of the comparison between two groups is reported if significant, where blue denotes comparison between CMV- Young and CMV+ Young, green denotes comparison between CMV+ Young and CMV+ Old, red denotes comparison between CMV- Young and CMV+ Old. Kruskal-Walis Test and multiple T tests (corrected with Dunn's Method) was performed for (b), (c), (d). Adjusted P-values <0.05 were considered significant) e) Heatmap clustering and Gene expression analysis of Inflammation Panel from Nanostring. 10000 cells of V δ 1+ and V δ 2+ were stimulated with PMA/Ionomycin, pooled from 4 donors of the respective group (CMV- Young, CMV+ Young and CMV+ Old), were sorted and lysed in RNA Later and stored in - 80 before using it for nanostring experiment. Top 26 genes were selected from PC1 as shown in Heatmap. (*p<0.05, *** p<0.005).

5.1.6 Replicative senescence of $\gamma\delta$ T cells in young and elderly

Having observed that phenotypically and functionally that V δ 2+ does not change with CMV infection and age, we proceeded to test the proliferative capacity of the $\gamma\delta$ T cells subsets. A marker often used in the study of T cell immunosenescence is CD57 as $\alpha\beta$ T cell expressing this marker have impaired proliferation capacity. We were then interested in investigating whether CD57 expression has the same implication for the $\gamma\delta$ T cells subsets as it is for the $\alpha\beta$ T cells. We first assessed the frequency of CD57+ in the different $\gamma\delta$ T subsets with CMV infection and age. Not surprising, in CD3+ pan- $\gamma\delta$ -, V δ 1+ and DN $\gamma\delta$ + T there was a significant increase of CD57+ with CMV infection and age but not in V δ 2+, which is similar to the other markers we have assessed previously (Fig 10b,c). Seeing no difference in terms of CD57+ frequency for V δ 2+, we continued to test the proliferative capacity of the V δ 2+ T cells in the different group with HMBPP (Figure 10d). However, we also observed no differences, implying that the proliferative capacity of V δ 2+ is also preserved with CMV infection and age. However, whether the expression of CD57+ has the same functional implication as $\alpha\beta$ T cells on the $\gamma\delta$ T cells remained to be investigated. With this in mind, we stimulated whole PBMCs with CD3/IL2 over 5 days and assess the proliferation index of the different subsets, using CD4 and CD8 as control and analyzing V δ 1+, V δ 2+ in the CD57+/- populations. We found out that there is a significant difference in proliferation capacity between CD57+ and CD57- populations, regardless of the cell type that was being interrogated (Figure 10e,f). This is even true when we stimulated V δ 2+ T cells with its natural ligand HMBPP in the different groups (Figure 10g). Therefore, this shows that the expression of CD57 expression on V δ 2+ T cells has the same functional implication as $\alpha\beta$ T cells (which is replicative senescence) unlike CD27/45RA mentioned previously which was used to define the naïve/effector subsets for $\alpha\beta$ T cells and V δ 2- but not applicable to V δ 2+ T cells. Together, this result along with previous ones have shown that V δ 2+ T cells does not follow the “ $\alpha\beta$ T cells immunosenescence path” in terms of phenotype, functional and proliferative capacity with CMV infection and age.

FIGURE 10

FIGURE 10. Frequency of CD57+ Pan $\gamma\delta$ -, V δ 1+, V δ 2+ and DN $\gamma\delta$ + in young and elderly and proliferation index of CD57-, CD57+ CD4, CD8

V δ 1+ and V δ 2+. For phenotyping, CMV- Young (RED) (n=12), CMV+ Young (BLUE) (n=12) and Elderly (n=12) (GREEN) individuals PBMCs were stained and analyzed by flow cytometry. For proliferation, PBMCs was labeled with CellTrace™ and were stimulated with the respective stimulants for 5 days to induce proliferation. After 5 days, cells were stained and analyzed by flow cytometry. Proliferation Index was derived from using ModFIT LT Version 3.2 a) Representative FACS plot of CD57+. b) Frequency of CD57+ of the pan $\gamma\delta$ -, c) $\gamma\delta$ T cells subsets. (d) Proliferation Index of V δ 2+ in CMV- Young (RED), CMV+ Young (BLUE), CMV+ Old (Green) with HMBPP 1uM. Kruskal-Wallis Test and multiple T tests (corrected with Dunn's Method) was performed for (b-d). Adjusted P-values <0.05 were considered significant e) Proliferation Index of CD4 CD57- (RED) CD57+ (BLACK) and CD8 CD57- (RED). f) CD57+ (BLACK) V δ 1+ CD57- (RED) CD57+ (BLACK) and V δ 2 CD57- (RED) CD57+ (BLACK) after CD3/IL-2 stimulation for 5 days (n=12). (g) Proliferation Index of CD57- (RED) and CD57+ (BLACK) V δ 2+ in the respective group with the HMBPP. Mann-Whitney U Test was performed for (e-g). P-values <0.05 were considered significant. (* p<0.05, *** p<0.005, **** p<0.001) (* p<0.05, *** p<0.001 **** p<0.0001)

5.2: Characterization of Human $\gamma\delta$ T Cells

5.2.1 Telomere length characterization of $\gamma\delta$ T cells

Telomeres are the caps at the end of each strand of DNA that protects the chromosomes from deletion after cell replication. Telomere erosion, which is a hallmark of cellular aging, occurs when the cells proliferate faster than what the telomerase can repair. This continual erosion of telomere often results in the cell reaching the replicative senescence stage whereby it is unable to replicate further. Using this as a basis, we then asked the question whether V δ 2⁺ T cells follows the characterization of $\alpha\beta$ T cells in terms of CD27/CD45RA, CD57 telomere length and might have a longer telomere length in comparison with other $\alpha\beta$ and $\gamma\delta$ T cells to explain its ability to resist cellular senescence. To answer this question, we employed a FLOW-FISH assay.

We observed that V δ 1⁺ and DN $\gamma\delta$ ⁺ T exhibit the same trend as CD4 and CD8 in terms of CD27/45RA phenotypic characterization and telomere length but not for V δ 2⁺. However, the same trend was observed on all cell types with CD57. This further reinforces that CD27/45RA also does not define V δ 2⁺ in terms of telomere length in the same way as it is for $\alpha\beta$ T cells. However, CD57 expression have the same implication for CD4, CD8, V δ 1⁺ and V δ 2⁺ in terms of not only proliferative capacity but also telomere length as CD57⁻ cells across the board exhibit longer telomere length compared to CD57⁺ (Figure 11c-h). One factor that controls the telomere length of the cell is hTerC and it is known that as T cell differentiate, they lose the ability of this enzyme, which results in telomere attrition. Using CD8 Naïve and TE as a control, we then analyzed this molecule in CMV⁻ Young, CMV⁺ Young and CMV⁺ Old in V δ 1⁺ and V δ 2⁺. We observed that with CMV infection and age, the hTerC gene expression is decreased but there is no change for V δ 2⁺ (Figure 11i). We then investigated if other genes that have been associated with cellular senescence using a customized panel in collaboration with nanostring (Appendix C). We observed that V δ 2⁺ in general, expressed higher levels of KLR genes which might have direct implications with its cytotoxic capacity but not higher level of CD57, reconfirming the phenotype we observed previously. V δ 2⁺ also do not express much higher level of senescence associated genes such as SIRT1 and p16 with age, further suggesting that they are resistant to cellular senescence with age. In terms of clustering with CD8 Naïve and TE, V δ 2⁺ regardless of CMV infection and age, cluster together and towards the CD8 Naïve (Figure 11j), implying that V δ 2⁺ expression of senescence associated genes is more similar to naive CD8 cells. All together, these results might suggest why V δ 2⁺ is resistant to cellular aging albeit with CMV infection

and aging even though it does not have a longer telomere length compared to other $\alpha\beta$ and $\gamma\delta$ T cells.

FIGURE 11

j)

Senescence
GENES
HEATMAP

Vδ2+

FIGURE 11. Telomere Length of CD27, CD45RA and CD57 subsets of CD4, CD8 and $\gamma\delta$ subsets. a) Representative plots of the various sorted populations after FLOW-FISH process. Sorted cells from apheresis cone (donor's age unknown) were combined with unstained cells to top up the number needed for FLOW-FISH protocol. b) Representation of histogram (semi-overlay) of the CD27/CD45RA subsets with TelC Cy5 Probe. c-g) Telomere Length of the various population and their CD27/CD45RA, CD57 subsets (n=9) Wilcoxon signed-rank test was performed. P-values <0.05 were considered significant for CD57-/+ subsets. Friedman Test and multiple T tests (corrected with Dunn's Method) was performed for (c-g). Adjusted P-values <0.05 were considered significant for CD27/45RA subsets for (c-g) (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $P < 0.0001$) h) Representative histogram plots of the various sorted populations. <25% and >25% of the histogram were taken and their CD27/CD45RA, CD57 profile is shown. i) Normalized Count of hTerC gene expression from the various populations (n=3) Gene expression Heatmap analysis of Customized Senescence Associated genes. j) 10000 cells of V δ 1 and V δ 2 from CMV- Young (n=3), CMV+ Young (n=3), CMV+ Old (n=3) and CD8 Naïve, TEMRA populations (CMV+ Young, n=3) were sorted and lysed in RNA Later and stored in -80 before using it for nanostring experiment. Senescence-related genes were selected as shown in the Heatmap

5.2.2 RRBS Methylation Epigenetic Profiles of CD4, CD8 and $\gamma\delta$ T cells

Epigenetic is the modification of the genome that could affect the gene transcription activity. Methylation of the genome has been shown to repress the gene's transcription activity and epigenetic alterations have also been deemed as one of the hallmarks of cellular aging. As the telomere assay was unable to explain V δ 2+ resistance to cellular senescence, we decided to explore the epigenetic aspect. Using RRBS sequencing on FACS-sorted CD4, CD8, V δ 1+, V δ 2+ and DN $\gamma\delta$ +. This approach was inspired by the “epigenetic clock” that was invented by Steve Horvath (223) as he was able to measure individuals' tissue and cells biological age using DNA methylation. Thus, our aim was to understand whether this approach could be used for T cells as their differentiation history is sort of representative to their proliferative history. Overall, we observed a general decrease of methylation in CD4 and CD8 as it differentiates into the later stages (Figure 12a). This is similar to what other recent studies have shown (224,225). Going deeper into the various sections of the gene, we observed the main decrease in methylation is within the promoter region (Figure 12b). This suggests that as CD4 and CD8 differentiate, more genes are being expressed due to a decreased in methylation % as methylation of a gene usually refers to the gene having lower transcription activity (226,227). To establish the genes that changes linearly in terms of methylation as CD4 and CD8 differentiates, we used the WCGNA analysis method and managed to identify 2 modules of genes that were either hyper or hypo methylated as CD4 and CD8 differentiates (Figure 12c). As it is challenging to have the numbers required for RRBS with the different phenotype of the $\gamma\delta$ subsets, we used the identified CD4 and CD8 gene modules and the bulk data of $\gamma\delta$ subsets along with the phenotype to observe how they will behave in the unbiased PCA analysis. We observed that V δ 1+ follows similarly to CD4 and CD8 in the PCA as it moves across PC1 when the donor has more “differentiated” cells. Although DN $\gamma\delta$ moves in the different direction (across PC2), there is still heterogeneity in the donors. However, in the case of V δ 2+, they cluster together tightly irrespective of donors, nearer to the CM and EM of CD4 and CD8, suggesting that their epigenetic profile are more similar to CM/EM and less heterogeneity between donors (Figure 12d).

FIGURE 12

a)

b)

c)

CD4

Module-trait relationships

CD8

Module-trait relationships

Blue/Turquoise Modules

FIGURE 12: Epigenetic profiles of CD4, CD8 phenotypic subsets and the various $\gamma\delta$ subsets. a) Overall average methylation % of the respective cell type. b) Average methylation % of the respective cell type in the different regions of the gene. c) Module Trait correlation matrix of the CD4, CD8 differentiation stages. Beta values of gene methylation rate of genes in blue and turquoise modules in CD4, CD8. PCA analysis using genes from blue and turquoise module in CD4, CD8. d) FACS plot of the phenotype of the individual $\gamma\delta$ cell type from the different donors, representative FACS plot of sorted CD4, CD8 Naïve and Memory Subsets. Heatmap clustering of genes from blue and turquoise modules from both CD4 and CD8 with the various $\gamma\delta$ subsets. PCA analysis using genes of blue and turquoise module from CD4 and CD8 with the various $\gamma\delta$ subsets. Samples were from apheresis cone (donor's information is unknown)

5.3: Vδ2+ Anti-Aging Mechanisms

5.3.1 Unique Epigenetic Signature of Vδ2+

We then delve deeper into the dataset and investigated whether there could be a module of genes that were unique to Vδ2+ as this could lead us to some mechanisms to explain why Vδ2+ seems to be resistant to cellular aging albeit with all the stress encountered during aging. Using the same WCGNA approach, we found the salmon module, which is a cluster of genes (Appendix E) that were hypo-methylated only in Vδ2+ (Figure 13a-d). We then ran this set of genes using GREAT platform to enrich and identify pathways that are unique to Vδ2+ and found that the pathways these genes are involved in are associated with anti-aging pathways that have been shown in other studies such as NAD⁺ metabolism and proteasome degradation (228) (Figure 13e). Thus, these pathways could explain why Vδ2+ are resistant to cellular aging.

FIGURE 13

Module–trait relationships

V52+ Cell Type Specific (Salmon Module)

c)

d)

e)

FIGURE 13: Unique Epigenetic signature of V δ 2+ T cells. a) Module Trait correlation matrix b) Heatmap clustering of genes from MeSalmon module for the respective cell type. c) PCA analysis of genes from the MeSalmon module with the respective cell type, d) beta values of the genes from the MeSalmon module for the respective cell type. e) MSigDB Pathway Analysis using GREAT from genes of Salmon Module (FDR <0.05 and Enrichment set at 1.3-fold) (n=3)

Samples were from apheresis cone (donor's information is unknown)

5.3.2 Epi-Transcriptome analysis of $\gamma\delta$ T cells

Going further, we investigated the transcriptome of V δ 2⁻, V δ 2⁺ and CD8 subsets using RNA-seq. We observed that at the transcriptome level, V δ 2⁺ expression profile is closer to CD8 EM in the PCA analysis, further reinforcing that they might be effector-like cells regardless of their phenotype as shown in previous results (Figure 14a).

As epigenetic modifications could have an effect on the transcriptomic level, we then analyzed these two datasets together using Gene Set Enrichment Analysis (GSEA) and overlaid the hypo-methylated genes found in the salmon modules with the RNA-seq data. We observed that the enrichment is significant only in V δ 2⁺, which suggest that most of the hypo-methylated genes were also the same genes that were highly expressed in V δ 2⁺ as compared to CD8 subsets and V δ 2⁻ (Figure 14b).

5.3.3 DNA Damage repair response differs for V δ 2⁺ compared to CD8 and V δ 1⁺ with differentiation

Interestingly, one of the genes that were striking was *MAD2L2*. *MAD2L2* has been shown to be able to control DNA repairs at telomeres (229) and they are found to be in higher expression in $\gamma\delta$ populations compared to the CD8 subsets (Figure 14c). This suggests that $\gamma\delta$ populations might have a higher capacity to maintain genomic integrity at the telomere end compared to CD8 subsets. However, the amount of genetic material at the telomere contains only a small portion of DNA and is not the major bulk of the genetic material in the cell.

Therefore, we continued our investigation to assess CD8 and the different $\gamma\delta$ T cells' ability to maintain/repair the major genetic material with stress as the ability to maintain genomic material integrity is a way to mitigate cellular senescence. DNA damage was induced by UV irradiation for 6 hours and the amount of H2A.X phosphorylation at Ser139 was assessed using MFI. Phosphorylation of H2A.X at Ser139 was used as a surrogate marker for DNA damage repair (DDR) capacity as it has been found to promote DNA repair and maintain genomic stability. We observed that when CD8 and V δ 1⁺ lose the expression of CD27, there is a significant decrease in the MFI of the phosphorylated H2A.X after UV damage.

However, this is not the case for V δ 2 as there was only a decrease in the CD27⁻ CD45RA⁺ subset (Figure 14g-i). This show that as CD8 and V δ 1⁺ differentiates to gain effector function, they gradually lose the capacity to repair its DNA, which could then contribute to cellular senescence as genomic instability is one of the hallmarks of cellular aging. However, in the case of V δ 2⁺, they are able to exhibit effector function without compromising on its

DNA repair mechanism. This could then explain why they might be resistant to cellular aging with stress. Collectively, these data suggest that the reason why V δ 2⁺ is resistant to cellular aging could be due a synergistic effect of more efficient DNA repair mechanism, higher expression of MAD2L2, which maintains telomere stability and other pathways such as NAD⁺ metabolism and proteasome degradation, all of which, are pathways and hallmarks of preventing cellular aging.

FIGURE 14

d)

e)

f)

h)

g)

i)

FIGURE 14. DNA Damage repair response differs for V δ 2+ compared to CD8 and V δ 1+ with differentiation. a) PCA analysis of RNA-seq transcriptome of CD8 (naïve, memory subsets), V δ 2- and V δ 2+ (n=4). b) GSEA analysis of genes in MeSalmon module with RNA-seq data. c) Gene expression of *MAD2L2* in the different cell types. d) Representative histogram of UV-induced expression of p-H2AX (Ser139). e) Geometric MFI of p-H2AX with and without UV-induction. f) Representative histogram of p-H2AX in the various subsets. Geometric MFI of p-H2AX of the subsets in g) CD8, h) V δ 1+ and i) V δ 2+. Wilcoxon signed-rank test was performed for (e), CD57-/+ subsets for (g-i). P-values <0.05 were considered significant for CD57-/+ subsets. Friedman Test and multiple T tests (corrected with Dunn's Method) was performed for CD27/45RA subsets (g-i). Adjusted P-values <0.05 were considered significant for CD27/45RA subsets for (c-g) (* = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001). Samples were from young donors (n=4 for (a) – (c) and n=9 for (d)-(i)

CHAPTER 6: SKIN-RESIDENT T CELLS IMMUNOSENESCENCE

(FOCUS OF THE THESIS PART 2)

6.1.1 Tissue Resident T cells

Other than the periphery, T cells also reside in the tissues to provide protection for the host against foreign pathogens at various locations (230-233). In recent years, tissue-resident memory (Trm) T cells has gained great interest (234) as it has been shown to play an important role in enhancing and providing local immunity in situations such as during respiratory virus infection in the lung, herpes simplex virus infection in non-lymphoid tissues and leishmania infection in the skin. (235-237). They are the first responders to many infections as they do not recirculate around the body and are functionally, phenotypically and molecularly different from the peripheral T cells (238-242). Researchers are targeting them in many translational applications such as cancer immunotherapy and vaccine design since most of the disease perturbations happen in the tissues and organs rather than in the periphery (243,244). Understanding them in the different disease conditions in the tissues and organs will allow scientists to have a more targeted approach, which could then enhance the overall immune response.

6.1.2 Skin-Resident T cells Immunosenescence

As there are higher incidences of skin-related diseases and skin cancer in the elderly, tissue immunity in them could have been compromised with age (245-247). However, aging studies thus far have been focusing on the peripheral T cells while less is done on the T cells that reside in the skin despite the relevance of skin-immunity in the different skin-related diseases and skin cancer.

The second objective of this thesis is to investigate the phenotype and expression of immunological-associated genes of skin-resident memory T cells, compare them to peripheral T cells within the same elderly individual and also compare between the age groups (i.e. young and old). The initial hypothesis of this study is that the skin-resident T cells will exhibit similar phenotype changes (i.e. accumulation of highly differentiated/senescent phenotype) as observed in the periphery with aging. Thus, it could contribute to the low-grade chronic inflammation of the skin in the elderly.

However, in this study, we observed that the skin-resident memory T cells adopt a strikingly different phenotype and immunological gene expression profile compared to the periphery in the same individual. In addition, we also compared the phenotype and the expression of

immunological-associated genes of skin-resident memory T cells in the two age groups and found that there were no differences between the young and old skin-resident memory T cells' in terms of phenotype except for PD-1 expression in CD4 and V δ 1+.

Collectively, this suggests that the skin-resident T cells are affected differently with age, compared to prior knowledge of what we observed with peripheral T cells.

6.2 Phenotypic and Transcriptomic analysis of elderly skin-resident and peripheral T cells

6.2.1 Skin-Resident and peripheral T cells adopt different phenotype in elderly individual.

Collagenase incubation of the cells could digest away certain CD markers (248). Thus, we first took PBMCs and incubated them with our digestion medium to ensure that the markers we were investigating are not drastically affected by it (Appendix B). After ensuring that the CD markers remain detectable after digestion, we compared the phenotype of $\alpha\beta$ (i.e. CD4+ and CD8+) and $\gamma\delta$ (V δ 1+, V δ 2+, DN $\gamma\delta$ +) T cells in both the skin and blood of elderly individuals. We observed that the T cells in the skin have a significantly higher expression of CD69 compared to the blood (Figure 15b). This is not surprising, as other studies have shown that CD69 is a marker of tissue resident T cells (249). Although it is expected, this result gave us the confidence that the majority of the T cells in the skin biopsy were tissue-resident with very minor contamination from the blood. Next, we observed that the CD27/CD45RA phenotype of the T cells is significantly different between the skin and the blood of the same elderly individual, with higher enrichment of “memory” subsets (CD45RA-) for the T cells’ subsets in the skin (Figure 15d-h). This suggests that the T cells that populate the skin are mostly “antigen-experienced” rather than naive. Moving on, we compared the expression of exhaustion (PD-1) and senescence-related markers (KLRG1 and CD57) in the T cells between blood and skin. We observed that the T cells in the skin are more “exhausted” and less senescent as there are higher expression of PD-1 and lower expression of KLRG1 and CD57 in the skin when compared to the blood (Figure 16a-c). Collectively, this suggests that the T cells, be it $\alpha\beta$ or $\gamma\delta$, the phenotype in the skin and the blood is significantly different and not reflective of each other, even in the same elderly individual.

FIGURE 15

Figure 15: Blood and Skin-Resident T cells' CD27/CD45RA phenotype in elderly individuals. Elderly (n=20) individuals PBMCs and skin-resident cells were extracted and stained before being analyzed by flow cytometry (a) Representative FACS plot of Blood and Skin-Resident T cells of CD4/CD8/V δ 1/V δ 2/DN $\gamma\delta$ +. b) Representative FACS Plot of Blood and Skin-Resident T cells and Frequency of blood and skin-resident T cells for CD69. c) Representative FACS Plot of Blood and Skin-Resident T cells and Frequency of blood and skin-resident T cells for CD27/CD45RA of the different T cells. Wilcoxon signed-rank test was performed. P-values <0.05 were considered significant for b, (d-h) (*p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001)

FIGURE 16

● BLOOD
■ SKIN

Figure 16: Blood and Skin-Resident T cells' Senescence and Exhaustion associated phenotype in elderly individuals. Elderly (n=20) individuals PBMCs and skin-resident cells were extracted and stained before being analyzed by flow cytometry (a) Representative FACS Plot of Blood and Skin-Resident T cells and Frequency of blood and skin-resident T cells for CD57. b) Representative FACS Plot of Blood and Skin-Resident T cells and Frequency of blood and skin-resident T cells for KLRG-1. c) Representative FACS Plot of Blood and Skin-Resident T cells and Frequency of blood and skin-resident T cells for PD-1. Wilcoxon signed-rank test was performed. P-values <0.05 were considered significant for (a-c) (*p<0.05, ** p<0.01, ****p<0.0001)

6.2.2 CD4 and CD8 phenotype are associated in both skin and blood

To understand if the environment where the T cells reside could have similar impact on the phenotype of the different T cells, we investigated whether the different cell type and their respective phenotype are associated with each other (i.e. frequency of CD8 Naïve T cells and frequency of CD4 Naïve T cells and etc). We observed that in the blood, the CD8 and CD4 ($\alpha\beta$) have significant correlation with each other respective phenotype and not with the $\gamma\delta$. This suggests that the circulatory environment of the blood does not have similar impact on both $\alpha\beta$ and $\gamma\delta$ T cells' phenotype. However, in the skin, besides the same significant association of phenotype between CD8 and CD4 T cells, we also observed that the frequency of central memory (CM) CD8 also correlated with the different subsets of $\gamma\delta$ CM phenotype (Figure 17). This result suggests that the environment in the skin might have some influence on the T cells to maintain the "CM phenotype" in all T cell subpopulations. Thus, it suggests that perhaps environment do play a role to a certain extent in shaping the phenotype of T cells.

FIGURE 17

BLOOD

a)

Naive = CD27+
CD45RA+

CM = CD27+
CD45RA-

EM = CD27-
CD45RA-

TEMRA = CD27-
CD45RA+

SKIN

b)

FIGURE 17: Spearman correlation analysis in elderly individuals of CD8 respective phenotypes with CD4 and $\gamma\delta^+$ phenotype. a) Blood. b) Skin. For correlation analysis, spearman correlation was performed. P-values <0.05 were considered significant. (* P<0.05, **P<0.01, ***P<0.001, ****P<0.0001) (n=20)

6.2.3 Differential immunological gene expression in skin-resident and peripheral T cells in elderly individuals

To understand if the skin-resident and peripheral T cells of an elderly individual could also be different in terms of gene expression, we compared the immunological associated gene profile of skin-resident and peripheral T cell of the same elderly individual and found them to be strikingly different with 84 DEGs being detected. Using these 80 DEGs (Appendix F), both heatmap clustering and PCA analysis was able to separate the sample group. (Figure 18a,b). To further our understanding of which pathways these genes might be involved in, we ran an IPA analysis to do enrichment pathway analysis. We observed that many of the pathways that the DEGs are enriched in are involved in signaling pathways (Figure 18c). This suggests that the signaling pathways that are being activated in skin-resident and peripheral T cells are different. Collectively, this shows that the skin-resident and peripheral T cells are phenotypic and transcriptomic different in an elderly individual.

FIGURE 18

Figure 18: DEGs and IPA analysis of Immunological genes of sorted CD4 and CD8 from elderly individuals' peripheral blood and skin. a) Heatmap clustering and dendogram of 80 DEGs using sorted CD4 and CD8 T cells from elderly individuals isolated from peripheral blood and skin. (n=4) b) PCA analysis using 80 DEGs (Appendix F) using sorted CD4 and CD8 T cells from elderly individuals isolated from peripheral blood and skin. (n=4) c) IPA Analysis of 80 DEGs using sorted CD4 and CD8 cells from elderly individuals isolated from peripheral blood and skin. (n=4)

6.3. Skin-resident T cells in young and elderly

6.3.1 PD-1 expression is decreased in skin-resident memory CD4 and V δ 1+ T cells with age.

We first investigated if the frequency of the $\gamma\delta$ T cells subsets are affected with age as seen in the periphery. However, we did not find any changes with age in all 3 subsets of $\gamma\delta$ T cells (Figure 19). Moving on, we analyzed the phenotype of skin-resident T cells in both young and old to investigate the possible phenotypic differences such as those seen in the periphery. We utilized classical markers that are known to modulate with age in the periphery T cells (i.e. CD27, CD45RA, KLRG1, CD57 and PD-1) as shown by previous studies. However, we did not observe a change in phenotype in most of these markers when we compared young and old skin-resident T cells. The only significant difference we observed was a decreased in expression level of PD-1 in the skin-resident total CD4 and V δ 1+ (Figure 20h) T cells of the old when compared to the young. This suggests that the skin-resident T cells might be less susceptible to differentiation and senescence with age as compared to the periphery.

FIGURE 19

Figure 19: Frequency of $\gamma\delta$ T cells subsets in young and elderly. a) Young (n=13, BLACK) and Elderly (n=19, RED) individuals' skin-resident cells were extracted and stained before being analyzed by flow cytometry. Frequency of a) DN $\gamma\delta^+$ /CD3, b) DN $\gamma\delta^+$ / $\gamma\delta$, c) V δ 1/CD3, d) V δ 1/ $\gamma\delta$, e) V δ 2/CD3, f) V δ 2/ $\gamma\delta$. Mann-Whitney U Test was performed for (a-f). P-values <0.05 were considered significant (*p<0.05, ** p<0.01)

FIGURE 20

a) **CD4**

b) **CD8**

c) **DN $\gamma\delta^+$**

d) **Vδ1**

e) **Vδ2**

f) **CD57**

g) **KLRG1**

h) PD-1

Figure 20: Skin-resident T cells' phenotype in young and elderly individuals. a) Young (n=13, BLACK) and Elderly (n=22, RED) individuals skin-resident cells were extracted and stained before being analyzed by flow cytometry. Frequency of CD27/CD45RA for a) CD4, b) CD8, c) DN $\gamma\delta$ +, d) V δ 1, e) V δ 2. Frequency of the different T cells was done on total T cells for f) CD57+, g) KLRG1+, h) PD-1+. Mann-Whitney U Test was performed for (a-h). P-values <0.05 were considered significant (*p<0.05, ** p<0.01)

6.3.2 No difference between young and old whole tissue and CD4, CD8 skin-resident T cells gene expression

Observing not much difference in terms of T cells' phenotype in the two age groups, we took a wider approach to investigate other possible changes in young and old skin that could suggest the higher incidences of skin-associated diseases and cancer in the elderly. However, unsupervised clustering of global gene expression profile in tissue and targeted CD4, CD8 skin-resident immunological gene profile could not separate the different age groups and conditions, suggesting that there are no age differences (Fig 21a,b). This shows that the skin represents an environment that does not really have much change with age in the T cell compartment.

FIGURE 21

a)

b)

Figure 21: Gene expression analysis of young and old individuals in whole tissue and sorted CD4 and CD8 T cells isolated from skin. a) Whole tissue transcriptomics of young and old individuals using RNA-seq. Clinically inflamed skin (positive control), Away from excision site (negative control), Young (age 25-40, n=5), Middle (age 43-62, n= 5), Old (age 66-84, n=4). b) Immunological-associated gene expression of CD4 and CD8 T cells isolated from skin of young and old individuals using nCounter Immunology Panel Human V2 from Nanostring. (n=4)

CHAPTER 7: DISCUSSION

In this thesis, we thoroughly investigated the impact of aging and associated confounder (CMV infection) on the differential capacities of various $\gamma\delta$ T cell populations towards differentiation and senescence. While much is known on $\gamma\delta$ T cells, emerging data suggest a dichotomy between V δ 1+ and V δ 2+ T cells, especially regarding their belonging to the innate or adaptive arm of immunity.

We assessed the classical markers used to functionally define classical T cells on the $\gamma\delta$ T cells, namely; CD27, CD45RA, CD57 and also other molecules that are known to be modulated with CMV infection and age in CD8. Besides the phenotypic analysis, we coupled this investigation with the evaluation of their functional capacity such as cytokine/cytotoxic secretion, proliferation, telomere length, epigenetic profile and also DNA damage response in relation to aging with its (CD27/CD45RA or CD57) phenotype.

The results showed that V δ 2- subsets (V δ 1+ and DN $\gamma\delta$ +) but not V δ 2+ are adapting their phenotype and functional capacity similarly to CD8, with CMV and age. The V δ 2+ adaptation to life-long stimulation is unique and interesting in terms of functional capacity, telomere length, epigenetic methylome profile and DNA damage response capacity as they do not have changes with age.

The data in this study correlates very well with two recent human $\gamma\delta$ T cells studies by Ryan *et al.* (250) and Davey *et al.* (251). Ryan *et al.* showed that the “profile” of V δ 2+ is stable in each individual and not affected by age, gender and HDI (Human Development Index). They also showed that V δ 2+ do already have the effector-like functions even in the “earlier” (CD27+, CD28+) stage as what we have similarly shown in (252). In the other study, Davey *et al.* showed that the TCR repertoire of the “Naïve” (CD27+ CD45RA+) are more diverse compared to the “TE” (CD27- CD45RA+) for V δ 1+ but not V δ 2+. This suggests that V δ 2+ might be more “innate-like” while V δ 1+ is more adaptive-like based on repertoire analysis. A recent twin study by Mangino *et al.* also showed that V δ 1+ immune traits are more influenced by the environment while V δ 2+ immune traits are more influenced by heritability (253). Together with our datasets, it suggests that V δ 1+ are more moldable (“adaptive-like”) by stressors encountered during life while V δ 2+ are more resilient and have an “innate-like” behavior that perhaps is influenced by heritability.

As shown in this thesis, V δ 2+ are unique leukocytes as they do not reach the senescence stage with stressors (i.e. CMV and age) unlike other innate-like cells such as V δ 1+ and NK cells whereby there is an increased frequency of late-stage phenotype that exhibits higher capacity to secrete effector molecules and lower proliferative capacity.

Some possible explanations to why V δ 2⁺ are more resilient against cellular senescence could lie in their unique epigenetic and transcriptomic signatures. The genes that are hypomethylated and highly transcribed are enriched in pathways mitigating cellular senescence such as NAD⁺ metabolism, proteasome degradation and biological oxidation as shown by the GREAT analysis. Maintenance of genomic material is another essential component in mitigating cellular senescence. We demonstrated that V δ 2⁺ DNA damage repair capacity is unlike V δ 1⁺ and CD8, whereby the DDR capacity of V δ 1⁺ and CD8 decreases upon losing CD27 expression. This shows that in order for the V δ 1⁺ and CD8 to gain effector functions, they lose their DDR capacity and therefore increasing its chances of compromising the genomic material integrity, leading to cellular senescence. Together with the higher expression of *MAD2L2* in $\gamma\delta$ T cells, this suggest that the ability of the cell to maintain its functional capacity without compromising on its ability to maintain the integrity of its genomic material at both the core and telomere could be essential in preventing the cells from reaching the senescence stage with stressor as shown in V δ 2⁺.

As for the biological relevance of surface marker expression, only some markers employed in classical T cells have the same implication for V δ 2⁺ (for e.g. CD57) while most of them do not apply. This has been also shown with KLRG1 in another study (254). These findings could suggest that V δ 2⁺ could have a different ontogeny when compared to other $\gamma\delta$ T cells subsets (255) and therefore assumptions should not be made that the markers used to define $\alpha\beta$ T cells can be applied onto V δ 2⁺.

The observed decreased of V δ 2 in the elderly in the periphery is unlikely due to thymic involution that occurs with chronological aging as we did not observe a correlation between the frequency of V δ 2 and CD4 RTE (a surrogate marker for thymic involution, CD4⁺ CD27⁺ CD45RA⁺ CD31⁺). However, we did observe that the abundance of the bacteria *Parabacteriodes* in the gut correlates with the frequency of $\gamma\delta$ /CD3 in the periphery in the elderly, suggesting that the abundance of *Parabacteriodes* in the gut could explain the variation of $\gamma\delta$ /CD3 frequency observed in the elderly but overall has a minor importance in V δ 2⁺ homeostasis in aging.

However, the other possible explanation for the reduction of V δ 2⁺ in the peripheral could be due to low-grade inflammation often seen in elderly, which is also known as inflammaging (256). This possible explanation is hypothesized from other studies that are inflammation related diseases such as psoriasis (257) as V δ 2⁺ has been observed to have a reduction in the

periphery but increased in tissue sites in those cases. Therefore, immunosenescence might have differential impact on different T cells, with some T cells being senescent and some T cells having a reduction in frequency in the periphery.

Having said that, the fact that V δ 2+ does not exhibit cellular senescence with age makes it an interesting immune cell to exploit in many different fields such as cancer immunotherapy and vaccination. Understanding how V δ 2+ T cells response with age after immunization could allow scientists in the future to design a vaccine utilizing V δ 2+ T cells. However, many of the vaccine and some cancer immunotherapy studies thus far have been looking at Pan $\gamma\delta$ T cells as a whole, making it hard to understand the contribution of V δ 2+ T cells in vaccination response as the relative frequency of V δ 1+ T cells increases with age.

In terms of cancer immunotherapy, V δ 2+ ability to react to cancer cells and resistance to senescence with age suggest that they might be the “last line” of defense for the host in its ability to battle against cancerous cells. Seeing their potential, future studies should work towards in exploiting these cells for both cancer and vaccination which could then increase healthspan for mankind. However, we first have to start delineating V δ 2+ T cells from Pan $\gamma\delta$ T cells in the future studies and analysis to better understand their contribution in the different context.

In conclusion of the first part of the thesis, we showed that a strong dichotomy exists between the human $\gamma\delta$ T cells subsets, adding knowledge to concurrent reports using aging and susceptibility to senescence as models to exemplify the particular role of V δ 2+ in human biology. Future work would enable to identify whether this potential of being resilient to stressors in V δ 2+ could be promoted in other cell type and lead to better response to infections and in the field of cancer immunotherapy.

Utilizing the knowledge that we obtained from the first objective of the thesis, we then moved on to the second objective of this thesis, which is the immunosenescence of T cells in the human skin. We investigated the young and old skin-resident T cells and observed no differences in terms of phenotype in the skin-resident memory T cells using the markers that were known to change with age in the periphery such as CD27/CD45RA, CD57 and KLRG1. Similar observations were also made in another study conducted in the United Kingdom (UK) (258). This suggests that the skin-resident T cells are not as susceptible to cellular senescence as compared to the peripheral T cells with age. In another comprehensive study, the authors investigated the T cell's phenotype in both the lymphoid and non-lymphoid tissues. They

observed that there were only changes in T cells' phenotype in lymphoid but not non-lymphoid tissues with age (259-261). Collectively, this suggests that the environment the tissue-resident T cells reside in could have an impact on its phenotype with age, which could be due to thymic involution that results in less naïve T cells being produced. The main difference between our study and the UK study is the expression of PD-1 on T cells with age. While they observed an increase of PD-1 expression T cells with age, we observed the opposite. One possible factor is the location of the country on the globe and the ethnicity of the donors as UK and Singapore citizens could have different amount of sun exposure and lifestyle, which could have impact on the PD-1 expression. PD-1 is an inhibitory receptor protein that T cells expressed to prevent hyper activation of T cells. Dysregulation of PD-1 signaling pathways has been attributed in autoimmune diseases, which then has been associated with skin-cancer. Therefore, this might explain the higher incidences of skin cancer and skin-associated autoimmune diseases in the elderly. However, more studies in the different countries across the globe needs to be done to have a better understanding of how ethnicity and sun exposure could affect the PD-1 expression of the skin-resident T cells with aging.

In conclusion of the second part of the thesis, this study indicates that the skin-resident memory and peripheral T cells adopt very different phenotypes and immunological gene profiles in the same elderly individual. There is also no difference between young and old skin-resident memory T cells, using the classical markers such as CD27, CD45RA, CD57, KLRG1, no difference in term of basal gene expression in young and old skin and also no difference in CD4 and CD8 immunological gene expression in young and old.

The only difference in phenotype between young and old skin-resident T cells is the lowered expression of PD-1 of CD4 and V δ 1+ in the elderly, which suggests that age-associated skin diseases could be due to the dysregulation of inhibitory receptor PD-1, which affects T cell activation and not due to accumulation of senescent T cells with age or dysregulated basal level of inflammation genes.

CHAPTER 8: CONCLUSION

Overall in this thesis, we showed that the peripheral $\gamma\delta$ T cells are differentially affected by persistent infection (CMV) and age. While $V\delta 2^-$ T cells ($V\delta 1$ and DN $\gamma\delta^+$) are affected in terms of phenotype and functionality with CMV infection and age, $V\delta 2^+$ T cells remains indifferent. We then went on to characterize $V\delta 2^+$ T cells using different methods, including CYTOF, telomere length, epigenetic methylome profiling and also DNA Damage repair capacity to better understand why $V\delta 2^+$ T cells seems resistant to cellular senescence. We discovered that $V\delta 2^+$ T cells are able to exhibit effector-like functions without a compromise on its DNA Damage repair capacity unlike the $V\delta 1^+$ T cells and CD8 T cells. This observation together with the enrichment pathway analysis on the epigenetic data that are unique for $V\delta 2^+$ T cells might explain why $V\delta 2^+$ T cells are resistant to cellular senescence with CMV infection and age. This new finding also reinforces the concept that $V\delta 2^+$ T cells have a more “innate-like” behavior while $V\delta 2^-$ T cells have a more “adaptive-like” behavior, very much like the $\alpha\beta$ T cells. Moving on, we continued our investigation of $\gamma\delta$ T cells with aging in the human skin by applying the same parameters that we did with the periphery $\gamma\delta$ T cells to investigate the tissue-resident $\gamma\delta$ T cells with aging. However, we found that there is no accumulation of highly differentiated and senescent $\gamma\delta$ T cells (all three subsets) with age. This was also the same in CD4 and CD8 T cells. The only parameter that was different was the frequency of PD-1, which is lower in the elderly for CD4 and $V\delta 1^+$. This suggests that the tissue-resident $\gamma\delta$ and $\alpha\beta$ T cells phenotype are not affected with age the same way as the periphery T cells. This is possibly due to the environment it resides in or it simply follows a different trajectory in the expression of markers as it differentiates in the skin tissue. Collectively, the data in this thesis shows that there is a strong dichotomy between $V\delta 2^+$ T cells and $V\delta 2^-$ T cells, the markers that are functionally relevant for $\gamma\delta$ T cells and how aging affects the peripheral and tissue resident $\gamma\delta$ T cells subsets differently. This is of great value for future $\gamma\delta$ T cells and also aging studies as future interventions against aging or vaccine designs for the elderly can be geared towards utilizing peripheral $V\delta 2^+$ or/and skin-resident T cells to boost immune response as they show no signs of cellular senescence with aging.

SUMMARY

CD27 CD45RA

CD57 CD45RA

Vδ1

CD27 CD45RA

CD57 CD45RA

Vδ2

CD27 CD45RA

CD57 CD45RA

DN γδ+

CHARACTERIZATION OF γδ+

FUNCTION	CD27+	CD57+
CYTOKINE POTENTIAL	NO	HIGH
CYTOTOXIC POTENTIAL	NO	HIGH
PROLIFERATION CAPACITY	HIGH	LOW
TELOMERE LENGTH	LONG	SHORT
DNA DAMAGE REPAIR CAPACITY	HIGH	LOW

FUNCTION	CD27+	CD57+
CYTOKINE POTENTIAL	HIGH	HIGH
CYTOTOXIC POTENTIAL	MID	HIGH
PROLIFERATION CAPACITY	HIGH	LOW
TELOMERE LENGTH	MID	SHORT
DNA DAMAGE REPAIR CAPACITY	HIGH	LOW

FUNCTION	CD27+	CD57+
CYTOKINE POTENTIAL	NO	HIGH
CYTOTOXIC POTENTIAL	NO	HIGH
PROLIFERATION CAPACITY	HIGH	LOW
TELOMERE LENGTH	LONG	SHORT
DNA DAMAGE REPAIR CAPACITY	HIGH	LOW

SUMMARY (BLOOD)

SUMMARY

Possible Mechanisms

- Better DNA Damage Repair Capacity at Telomere End and Core Genome
 - Better NAD⁺ Metabolism
 - Better Proteasome Degradation
 - Reduction of Biological Oxidation

SUMMARY (SKIN)

YOUNG

OLD

Decreased
in PD1
expression

No Change

YOUNG

OLD

Decreased
in PD1
expression

No Change

No Change

AUTHOR'S PUBLICATIONS

Xu.W *et al.* Mapping of γ/δ T cells reveals V δ 2+ T cells resistance to senescence.

EbioMedicine. December 2018. doi.org/10.1016/j.ebiom.2018.11.053

Xu W. and Larbi A. Immunity and inflammation: From Jekyll to Hyde. *Exp Gerontol*. 2017 Nov 26. pii: S0531-5565(17)30515-6. doi: 10.1016/j.exger.2017.11.018.

Xu W. and A. Larbi. Markers of T Cell Senescence in Humans. *Int. J. Mol. Sci.* **2017**, *18*(8), 1742; doi:10.3390/ijms18081742

Appanna R, Kg S, Xu MH, Toh YX, Velumani S, Carbajo D, Lee CY, Zuest R, Balakrishnan T, Xu W., Lee B, Poidinger M, Zolezzi F, Leo YS, Thein TL, Wang CI, Fink K. Plasmablasts During Acute Dengue Infection Represent a Small Subset of a Broader Virus-specific Memory B Cell Pool. *EBioMedicine*. 2016 Oct;12:178-188. doi: 10.1016/j.ebiom.2016.09.003

Tan CT, Wistuba-Hamprecht K, Xu W., Nyunt MS, Vasudev A, Lee BT, Pawelec G, Puan KJ, Rotzschke O, Ng TP, Larbi A. V δ 2+ and α/β T cells show divergent trajectories during human aging. *Oncotarget*. 2016 Jul 19;7(29):44906-44918. doi: 10.18632/oncotarget.10096.

POSTERS, AWARDS AND ORAL PRESENTATIONS

Poster Presentations

Biology of Aging Conference, 2015 (Singapore, Singapore)

- Phenotypic and Functional Capacity Differences between the Young and the Elderly in the Non-Classical T lymphocytes subsets

$\gamma\delta$ T Cells Conference 2016 (United Kingdom, London)

- When Human $\gamma\delta$ T cells decide not to follow the Rules: from Differentiation to Senescence

PhD Student Retreat 2016 (Singapore, Singapore)

- When Human $\gamma\delta$ T cells decide not to follow the Rules: from Differentiation to Senescence

Biology of Aging Conference 2, 2017 (Singapore, Singapore)

- V δ 2+ “Anti-Aging” Secrets (Pathways)

SgSI Conference 2018 (Singapore, Singapore)

- Mapping of γ/δ T cells Reveals V δ 2+ T cells Resistance to Senescence

Oral Presentations

CYTOASIA 2017 (Singapore, Singapore)

- V δ 2+ T cells: The Outlaws of T cells’ Immunosenescence in Human Aging as revealed by multi-parametric FLOW-Analysis

$\gamma\delta$ T Cells Conference 2018 (France, Bordeaux)

- Mapping of γ/δ T cells Reveals V δ 2+ T cells Resistance to Senescence

TABLE 2

a)

Group	n	Age	Gender	Ethnic	CMV IgG Titre
CMV- Young	12	22-38	6 Female 6 Male	Chinese	<3.0 IU/ml
CMV+ Young	12	24-31	6 Female 6 Male	11 Chinese (1 Malay)	3.5-24.18 IU/ml
CMV+ Old	12	60-83	6 Female 6 Male	Chinese	4.71-30.1 7 IU/ml

TABLE 2a. Donor Information

b)

Group	n	Age	Gender	Ethnic
Young	12	25 - 30	6 Female 6 Male	Chinese
Old	10	61 - 80	6 Female 4 Male	Chinese

TABLE 2b. Donor Information of blood samples for FIGURE 1f-i

c)

Group	n	Age	Gender	Ethnic
Old	20	79 - 89	10 Female 10 Male	Chinese

TABLE 2c. Donor Information of blood and stool samples for FIGURE 2

TABLE 3

a)

GROUP	n	Age	Gender	Race
Elderly	20	60-84	12 Female 8 Male	Chinese

TABLE 3a. Donor Information of skin and blood samples for FIGURE 15 and 16

b)

GROUP	n	Age	Gender	Race
YOUNG	13	25-45	7 Male, 6 Female	Chinese
OLD	22	66-87	9 Male, 13 Female	Chinese

TABLE 3b. Donor Information of skin samples for FIGURE 19

APPENDIX

APPENDIX A

APPENDIX A. FACS Plot of Human $\gamma\delta$ T cell Staining with different fluorophore and clones

APPENDIX B

APPENDIX B. PBMCs CD Marker Expression with and without overnight Collagenase Digestion. Red Histogram = collagenase digestion overnight, blue histogram = rested overnight without collagenase digestion.

APPENDIX C: Nanostring Panel (Senescence associated gene)

<u>Gene Name</u>	<u>Molecule Name</u>	<u>Gene Name</u>	<u>Molecule Name</u>
YWHAZ	14-3-3	IL2RG	IL2RG
ABCB1	ABCB1	IL3	IL3
ACTN1	ACTN1	IL32	IL32
PARP1	ADPRT	IL4	IL4
AFG3L1P	AFG3L1	IL4R	IL4R
AHR	AHR	IL5	IL5
AKT1	AKT1	IL6	IL6
AKT2	AKT2	IL6R	IL6R
AKT3	AKT3	IL6ST	IL6ST
AKR1A1	Aldo	IL7	IL7
PRDX6	AOP2	IL7R	IL7R
CLU	APOJ	IL8	IL8
ARHGDIB	ARHGDIB	IL9	IL9
ATF2	ATF2 / CREB2 / CREBP1	ILF3	ILF3
ATG10	ATG10	IRF4	IRF4
ATG12	ATG12	IRF7	IRF7
ATG13	ATG13	IRF8	IRF8
ATG14	ATG14	ITGA4	ITGA4
ATG16L1	ATG16L	ITGA5	ITGA5
ATG2A	ATG2	ITGA6	ITGA6
ATG3	ATG3	JAG1	Jagged1
ATG4D	ATG4-D	JAKMIP3	JAKMIP3
ATG5	ATG5	JKAMP	JNK1
ATG7	ATG7	MAPK9	JNK2
ATG9A	ATG9L1	JUN	JUN
ATM	ATM	MKI67	Ki67
ATR	ATR	TCF25	KIAA1049
AXL	AXL	KIR3DS1	KIR_Activating_Subgroup_1
B2M	B2M	KIR2DS1	KIR_Activating_Subgroup_2
BAD	Bad	KIR2DL1	KIR_Inhibiting_Subgroup_1
BAG6	BAT3 (bag6)	KIR2DL3	KIR_Inhibiting_Subgroup_2
BAX	Bax	KIR2DS4	KIR2DS4
BCKDK	BCKDK	KIR3DL3	KIR3DL3
BCL2	Bcl2	KLRB1	KLRB1
BCL3	BCL3	KLRC1	KLRC1
BCL6	BCL6	KLRD1	KLRD1
BCL2L1	BclX	KLRG1	KLRG1
BECN1	Beclin-1	KLRK1	KLRK1
BID	Bid	KIF2C	KNSL6
BIN1	BIN1	XRCC6	Ku
PRDM1	Blimp1 (PRDM1)	MRPL22	L22
BMX	BMX	LARP1	La
HNRNPC	C1/C2	LAG3	LAG3
ABL1	c-Abl	LMNB1	Lamin B1
CANX	CANX	LAPTM4B	LAPTM4B

CASP2	CASP2	LAT	LAT
CASP3	CASP3	MAP1LC3A	LC3
CASP7	CASP7	LCK	LCK
CASP8	CASP8	LIN7B	LIN7B
CASP9	CASP9	STK11	LKB1/ STK11
CAV1	CAV1	LOC10013359	LOC100133591
CCDC69	CCDC69	LRRC8C	LRRC8C
CCL11	CCL11	LTB	LTB
CCL13	CCL13	MAF	MAF
CCL15	CCL15	MAPK11	MAPK11
CCL16	CCL16	MAPK12	MAPK12
CCL18	CCL18	MAPK13	MAPK13
CCL19	CCL19	MAPK14	MAPK14
CCL2	CCL2	MDH1	MDH
CCL20	CCL20	DBNDD1	MGC3101
CCL22	CCL22	MICA	MICA
CCL23	CCL23	MICB	MICB
CCL24	CCL24	MMP28	MMP28
CCL26	CCL26	MPST	MPST
CCL3	CCL3	MPZL2	MPZL2
CCL4	CCL4	MSRA	MSRA
CCL5	CCL5	MSRB2	MSRB
CCL7	CCL7	MT1G	MT1G
CCL8	CCL8	MT1IP	MTE
CCNA1	CCNA1	MTOR	mTOR
CCNB1	CCNB1	NCR1	NCR1
CCND1	CCND1	NFATC1	NFATC1
CCND2	CCND2	NFATC2	NFATC2
CCND3	CCND3	NFATC3	NFATC3
CCNE1	CCNE1	NKAP	NF-kB
CCNF	CCNF	NLRP3	NLRP3
CCNH	CCNH	NME1	NME1
CCR10	CCR10	NOTCH1	Notch1
CCR2	CCR2	NOTCH3	Notch3
CCR5	CCR5	OSGIN1	OKL38
CCR6	CCR6	CDKN2A	p16
CCR7	CCR7	CDKN1B	p27
CCR8	CCR8	CDK5R1	p35
ACKR4	CCRL1	TP53	p53
CCRL2	CCRL2	CDKN1C	p57
CD160	CD160	ARHGEF6	PAK
CD244	CD244	PARP8	PARP8
CD247	CD247	PCNA	PCNA
CD27	CD27	PDCD1	PD1
CD28	CD28	PDE4A	PDE4A
ENTPD1	CD39 (ENTPD1)	PDE9A	PDE9A

CD3E	CD3E	PDK1	PDK1
CD3EAP	CD3EAP	PIK3CA	PI3K
CD4	CD4	PINK1	PINK1
PTPRC	CD45RA	PINX1	PINX1
PTPRC	CD45RB	PPP1R14A	PKC
PTPRC	CD45RO	PKM	PKM2
B3GAT1	CD57	PLA2G1B	PLA2
CD8A	CD8	PLAU	PLAU
CD96	CD96	PLEK	PLEK
CDC42BPA	CDC42BPA	POT1	POT1
CDC42BPB	CDC42BPB	POTEF	POTEF
CDC42BPG	CDC42BPG	PPP2R5B	PP2A
CDK10	CDK10	PRF1	PRF1
CDK2	CDK2	PRKAA1	PRKAA1
CDK4	CDK4	PRKCA	PRKCA
CDKN1A	CDKN1A	PRKCB	PRKCB
CENPF	CENPF	PRKCD	PRKCD
CKB	CKB	PRKCE	PRKCE
CLEC2D	CLEC2D	PRKCG	PRKCG
CMTM8	CMTM8	PRKCH	PRKCH
MYCBP	C-Myc	PRKCI	PRKCI
CPNE7	CPNE7	PRKCQ	PRKCQ
UBE2V1	CROC1A	PRKCZ	PRKCZ
CTDSP2	CTDSP2	PSMD11	PSMD11
CTLA4	CTLA4	PTGES2	PTGES2
CTSB	CTSB	RAP1GAP	Rap1
CTSD	CTSD	RB1	Rb1
CTSL	CTSL	RELA	RELA
CX3CL1	CX3CL1	RELB	RELB
CX3CR1	CX3CR1	RGS13	RGS13
CXCL1	CXCL1	RGS16	RGS16
CXCL10	CXCL10	RIF1	Rif1
CXCL11	CXCL11	RORC	RORc
CXCL12	CXCL12	RRAD	RRAD
CXCL13	CXCL13	RPS9	S9
CXCL2	CXCL2	SH2D1A	SAP/ SH2D1A
CXCR1	CXCR1	SGK1	SGK1
CXCR2	CXCR2	SGK2	SGK2
CXCR3	CXCR3	SGPP2	SGPP2
CXCR4	CXCR4	SIRT1	SIRT1
CXCR6	CXCR6	SKI	SKI
CYCS	cyc	SLAMF1	SLAMF1
DAG1	DAG1	CD48	SLAMF2
DLK1	delta-like 1	LY9	SLAMF3
DLK2	Delta-like 4	CD84	SLAMF5
DIXDC1	DIXDC1	SLAMF6	SLAMF6

DLG1	DLG1 / SAP97	SLAMF7	SLAMF7
TFDP1	DP1	SLAMF8	SLAMF8
DPP4	DPP4	SLAMF9	SLAMF9
DKC1	Dyskerina/ NAP57	SLC2A14	SLC2A14
E2F2	E2F2	SLC9A2	SLC9A2
EEF2K	EEF2K	LCP2	SLP76/ LCP2
EEF2	EF-2	SOD2	SOD2
EGFR	EGFR	SORL1	SORL1
EGR1	EGR1	SP1	SP-1
EGR3	EGR3	SPHK1	SPHK1
ELF4	Elf4	SPRY2	SPRY2
EOMES	Eomes	ST8SIA1	ST8SIA1
SMG5	Est1	TAB1	TAB1
ETFB	ETFB	TAB2	TAB2
ETS1	ETS1	TAB3	TAB3
FADD	FADD	MAP3K7	TAK1/ MAP3K7
FANCA	FANCA	TNKS	Tankirase
FAS	Fas	TBX21	Tbet (TBX21)
FGFBP2	FGFBP2	TBXA2R	TBXA2R
FHIT	FHIT	TEP1	TEP 1
RB1CC1	FIP2000	TGIF1	TGFb
EBLN2	FLJ10213	TGFB1	TGFB1
FNDC9	FNDC9	TGFBR2	TGFb-RII
FOXO3	FOXO3	TIAM2	TIAM2
FOXP3	FOXP3	HAVCR2	Tim-3(HAVCR2)
FOXP4	FOXP4	TINF2	TIN2
G6PD	G6PD	TLCD1	TLC1
DDIT3	GADD153	TLR1	TLR1
LGALS9	Galectin-9 (LGALS9)	TLR2	TLR2
GAPDH	GAPD	TLR3	TLR3
GAS8	GAS11	TLR4	TLR4
GABARAPL2	GATE-16	TLR5	TLR5
GIPR	GIPR	TLR6	TLR6
SCYL1	GKLP	TLR7	TLR7
GLB1	GLB1	TLR8	TLR8
SLC2A1	GLUT1 / SLC2A1	TLR9	TLR9
CSF2	GM-CSF (CSF2)	TNF	TNF
GNLY	GNLY	TNFAIP3	TNFAIP3
GSK3A	GSK3A	TNFRSF9	TNFRSF9
GSK3B	GSK3B	TNFRSF18	TNFRSF18 (GITR)
GSN	GSN	TOX2	TOX2
GSTP1	GSTPi	TP53RK	TP53RK
GZMA	GZMA	TRAF1	TRAF1
GZMB	GZMB	TRAF6	TRAF6
GZMH	GZMH	TERF1	TRF1
HERC6	HERC6	TERF2	TRF2

GAR1	hGAR1	TUBB4A	TUBB4
HIPK2	HIPK2	ULBP1	ULBP1
HDAC9	Histone deacetylase (HDAC)	ULBP2	ULBP2
HK1	HK1	ULBP3	ULBP3
HMOX1	HMOX	RAET1E	ULBP4
NHP2	HNHP2	RAET1G	ULBP5
NOP10	hNOP10	RAET1L	ULBP6
HNRNPA1	HNRNPA1	ULK1	ULK1
HNRNPL	HNRNPL	ULK2	ULK2
HNRNPLL	HNRNPLL	WIP1	WIP1 1-4
HSP90AA1	HSP90	WNT7A	WNT7A
STAU1	hStau	WRN	wrn
TERC	hTERC	WT1	WT1
TERT	hTERT	XCL1	XCL1
ICOS	ICOS	ZAP70	ZAP70
IFITM1	IFITM1	ZBTB42	ZBTB42
IFNA1	IFNA1	ZNF841	ZNF841
IFNA2	IFNA2	IL1R1	IL1R1
IFNAR1	IFNAR1	IL1R2	IL1R2
IFNAR2	IFNAR2	IL1RAP	IL1RAP
IFNB1	IFNB1	IL1RL1	IL1RL1
IFNG	IFNG	IL1RL2	IL1RL2
IFNGR1	IFNGR1	IL1RN	IL1RN
IL10	IL10	IL2	IL2
IL10RA	IL10RA	IL20	IL20
IL11	IL11	IL21	IL21
IL11RA	IL11RA	IL21R	IL21R
IL12A	IL12A	IL22	IL22
IL12B	IL12B	IL22RA2	IL22RA2
IL12RB1	IL12RB1	IL23A	IL23A
IL13	IL13	IL23R	IL23R
IL13RA1	IL13RA1	IL26	IL26
IL15	IL15	IL27	IL27
IL15RA	IL15RA	IFNL2	IL28A
IL16	IL16	IFNL3	IL28B
IL17A	IL17A	IFNL1	IL29
IL17B	IL17B	IL2RA	IL2RA
IL17F	IL17F	IL2RB	IL2RB
IL18	IL18	IL1A	IL1A
IL18R1	IL18R1	IL1B	IL1B
IL18RAP	IL18RAP		
IL19	IL19

BLUE LASER CONFIGURATION

ULTRA VIOLET LASER CONFIGURATION

RED LASER CONFIGURATION

YELLOW GREEN LASER CONFIGURATION

VIOLET LASER CONFIGURATION

**Violet 2 Laser
(405nm)**

VIOLET 2 LASER CONFIGURATION

APPENDIX E

SYMBOL	MODULE	KME	PVALUE
MAST4	salmon	0.793308078	3.67E-08
EPHB3	salmon	0.66655991	2.28E-05
ZBTB16	salmon	0.860254595	1.40E-10
EPHB3	salmon	0.862235515	1.13E-10
ME1	salmon	0.659149976	3.03E-05
RBPJL	salmon	0.52340262	0.001774069
KCNQ2	salmon	0.531145939	0.00147062
IRAK2	salmon	0.89396391	2.47E-12
SCART1	salmon	0.678493978	1.43E-05
NPDC1	salmon	0.658235905	3.13E-05
RGCC	salmon	0.622668512	0.000108999
DIP2A	salmon	0.717387154	2.63E-06
SMIM10L2A	salmon	0.329623703	0.061031298
WDR37	salmon	0.841718994	8.40E-10
NRROS	salmon	0.714550752	3.00E-06
ZBTB16	salmon	0.877634238	2.02E-11
NTSR1	salmon	0.433706109	0.011683806
PTMS	salmon	0.598958389	0.000230772
WDR37	salmon	0.852739954	2.98E-10
CUL3	salmon	0.499495549	0.003081779
MAD2L2	salmon	0.699272202	5.97E-06
ZBTB16	salmon	0.927784542	8.14E-15
ACSF3	salmon	0.793645738	3.59E-08
TP73	salmon	0.576478765	0.000445939
HK1	salmon	0.802363511	1.96E-08
EPHB3	salmon	0.83549743	1.46E-09
CDC7	salmon	0.662568309	2.66E-05
PTMS	salmon	0.60825	0.000173225
SCART1	salmon	0.702924259	5.08E-06
ERN1	salmon	0.740194494	8.50E-07
HCAR2	salmon	0.616222587	0.000134457
ITCH	salmon	0.908814396	2.64E-13
EPHB3	salmon	0.87519286	2.70E-11
CYB561	salmon	0.883269553	1.01E-11
ZBTB16	salmon	0.940927096	3.96E-16
KCNE4	salmon	0.489446764	0.003842387
JADE1	salmon	0.807786142	1.33E-08
ERN1	salmon	0.715598821	2.86E-06
MAD2L2	salmon	0.727577897	1.61E-06
LINC00327	salmon	0.87742993	2.07E-11
GJA8	salmon	0.35469215	0.042829053
EPHB3	salmon	0.873455162	3.30E-11
CCR6	salmon	0.789657642	4.69E-08

SLC25A44	salmon	0.730532382	1.39E-06
ZBTB16	salmon	0.853987631	2.63E-10
EPHB3	salmon	0.813616629	8.60E-09
GRAMD1A	salmon	0.824488177	3.67E-09
ACSF3	salmon	0.680661241	1.31E-05
ZBTB16	salmon	0.936496018	1.18E-15
DIP2A	salmon	0.676811291	1.53E-05
CCR6	salmon	0.801614301	2.07E-08
TBC1D31	salmon	0.725134466	1.81E-06
MAST4	salmon	0.79470039	3.34E-08
COL6A2	salmon	0.61714246	0.000130526
NRROS	salmon	0.747271939	5.85E-07
EPHB3	salmon	0.818456387	5.93E-09

APPENDIX E: HYPOMETHYLATED GENES IN VD2+

APPENDIX F

CD44
CXCR4
CDKN1A
CXCR6
CASP3
CD6
CCR8
CTSS
CD83
CD28
CD48
CD45RO
CCR7
BCL3
BCL6
CCDN3
GPI
FYN
HLA-A
HLA-DQB1
ICAM3
IL4R
IL2RB
IKZF3
IRF4
ITGA6
ITGAE
IRAK4
ICOSLG
IRAK1
ITGA4
BCL2L11
BATF
ATG16L1
CISH
MIF
STAT1
STAT3
STAT5A
STAT5B
S1PR1
SLAMF1
SOCS1
RELA

RELB
SELL
TNF
TCF7
TRAF1
TRAF4
TNFAIP3
TNFRSF1B
TNFRSF9
TNFRSF14
TABP
ZEB1
PTPN6
PTGER4
PRDM1
SIGIRR
SKI
XBP1
CD70
ZAP70
PDCD1
PSMB10
LEF1
LCP2
LAIR1
MCL1
NFATC3
MAPKAPK2
NLRP3
LGALS3
ETS1
JAK1
NKFB2
NOD2
DUSP4
SH2D1A

APPENDIX F :DEGS FOR FIGURE 18

REFERENCES

1. Christensen, K., Doblhammer, G., Rau, R., Vaupel, J.W. Ageing populations: the challenges ahead. *Lancet* **374(9696)** 1196-1208 (2009).
2. He, W., Goodkind, D. & Kowal, P. An Aging World : 2015 International Population Reports. *Aging (Albany, NY)*. **165** (2016).
3. Kanasi, E., Ayilavarapu, S. & Jones, J. The aging population: demographics and the biology of aging. *Periodontol. 2000* **72**, 13–18 (2016).
4. Sander, M. *et al.* The challenges of human population ageing. *Age Ageing* **44**, 185–187 (2015).
5. Prasad, S., Sung, B., Aggarwal, B.B. Age-Associated Chronic Diseases Require Age-Old Medicine: Role of Chronic Inflammation. *Prev Med.* May **54**(Suppl): S29–S37 (2012) .
6. Perry, D. P. Introduction to aging, cancer, and age-related diseases. *Ann. N. Y. Acad. Sci.* **1197**, (2010).
7. National Research Council. *Aging and the Macroeconomy: Long-Term Implications of an Older Population*. (2012).
8. Fulop, T. *et al.* Aging, frailty and age-related diseases. *Biogerontology* **11**, 547–563 (2010).
9. Ng, T. P., Feng, L., Nyunt, M. S. Z., Larbi, A. & Yap, K. B. Frailty in Older Persons: Multisystem Risk Factors and the Frailty Risk Index (FRI). *J. Am. Med. Dir. Assoc.* **15**, 635–642 (2014).
10. Konar, A., Singh, P. & Thakur, M. K. Age-associated Cognitive Decline: Insights into Molecular Switches and Recovery Avenues. *Aging Dis.* **7**, 121 (2016).

11. Harada, C. N., Love, M. C. N. & Triebel, K. Normal Cognitive Aging. *Clin Geriatr Med.* **29**, 737–752 (2013).
12. White, M. C. *et al.* Age and cancer risk: A potentially modifiable relationship. *Am. J. Prev. Med.* **46**, 1–16 (2014).
13. Berger, N. A. *et al.* Cancer in the elderly. *Trans. Am. Clin. Climatol. Assoc.* **117**, 147–55; discussion 155–6 (2006).
14. de Magalhães, J. P. How ageing processes influence cancer. *Nat. Rev. Cancer* **13**, 357–65 (2013).
15. Dhingra, R. & Vasan, R. S. Age as a Cardiovascular Risk Factor. *Med. Clin. North Am.* **96**, 87–91 (2012).
16. Caspersen, C. J., Thomas, G. D., Boseman, L. A., Beckles, G. L. A. & Albright, A. L. Aging, Diabetes, and the Public Health System in the United States. *Am. J. Public Health* **102**, 1482–1497 (2012).
17. Kirkman, M. S. *et al.* Diabetes in older adults. *Diabetes Care* **35**, 2650–2664 (2012).
18. Goldstein, D. R. Aging, imbalanced inflammation and viral infection. *Virulence* **1**, 295–298 (2010).
19. Van Duin, D. Diagnostic challenges and opportunities in older adults with infectious diseases. *Clin. Infect. Dis.* **54**, 973–978 (2012).
20. Kline, K. A. & Bowdish, D. M. E. Infection in an aging population. *Curr. Opin. Microbiol.* **29**, 63–67 (2016).
21. Lloyd-Sherlock, P. Population ageing in developed and developing regions: implications for health policy. *Soc. Sci. Med.* **51**, 887–895 (2000).

22. Oliver. A.J.. *et al.* Japan's aging population. Implications for healthcare. *Pharmacoeconomics*. (1997)
23. Klenk, J., Keil, U., Jaensch, A., Christiansen, M. C. & Nagel, G. Changes in life expectancy 1950-2010: Contributions from age- and disease-specific mortality in selected countries. *Popul. Health Metr.* **14**, 1–11 (2016).
24. Crimmins, E. M. Lifespan and healthspan: Past, present, and promise. *Gerontologist* **55**, 901–911 (2015).
25. Willcox, B.J., Willcox, D.C., Ferrucci, L. Secrets of Healthy Aging and Longevity From Exceptional Survivors Around the Globe: Lessons From Octogenarians to Supercentenarians. *J Gerontol A Biol Sci Med Sci*. November ; **63**(11): 1181–1185 (2008)
26. Poulain, M., Pes, G. & Salaris, L. A population where men live as long as women: Villagrande strisaili, sardinia. *J. Aging Res.* (2011).
27. Vasto, S., Rizzo, C. & Caruso, C. Centenarians and diet: What they eat in the Western part of Sicily. *Immun. Ageing* **9**, 1–6 (2012).
28. Arai, Y., Sasaki, T. & Hirose, N. Demographic, phenotypic, and genetic characteristics of centenarians in Okinawa and Honshu, Japan: Part 2 Honshu, Japan. *Mech. Ageing Dev.* **165**, 80–85 (2017).
29. Poulain, M. *et al.* Identification of a geographic area characterized by extreme longevity in the Sardinia island: The AKEA study. *Exp. Gerontol.* **39**, 1423–1429 (2004).
30. Miyagi, S., Iwama, N., Kawabata, T. & Hasegawa, K. Longevity and Diet in Okinawa, Japan: The Past, Present and Future. *Asia Pacific J. Public Heal.* **15**, S3–S9 (2003).
31. Horiuchi, S. Major Causes of the Rapid Longevity Extension in Postwar Japan. *Japanese J. Popul.* **9**, 162–171 (2011).

32. Suzuki, M., Willcox, D. C., Rosenbaum, M. W. & Willcox, B. J. Oxidative stress and longevity in Okinawa: An investigation of blood lipid peroxidation and tocopherol in okinawan centenarians. *Curr. Gerontol. Geriatr. Res.* **2010**, (2010).
33. Pala, M. *et al.* Population- and individual-specific regulatory variation in Sardinia. *Nat. Genet.* **49**, 700–707 (2017).
34. Chiara, M., Id, F., Hitchcott, P. K., Mulas, I. & Ruiiu, M. Behavioral sciences Resilience in Elders of the Sardinian Blue Zone : An Explorative Study. 1–17 (2018).
35. Dong, X., Milholland, B., Vijg, J.. Evidence for a limit to human lifespan. *Nature*. Oct 13;**538(7624)**:257-259 (2016)
36. Raji, C. A., Lopez, O. L., Kuller, L. H., Carmichael, O. T. & Becker, J. T. Age, Alzheimer disease, and brain structure. *Neurology* **73**, 1899–1905 (2009).
37. Steenman, M. & Lande, G. Cardiac aging and heart disease in humans. *Biophys. Rev.* **9**, 131–137 (2017).
38. Lang, P. O. *et al.* Effectiveness of influenza vaccine in aging and older adults: Comprehensive analysis of the evidence. *Clin. Interv. Aging* **7**, 55–64 (2012).
39. Wang, I. K. *et al.* Effectiveness of influenza vaccination in elderly diabetic patients: A retrospective cohort study. *Vaccine* **31**, 718–724 (2013).
40. McElhaney, J. E. Influenza vaccine responses in older adults. *Ageing Res. Rev.* **10**, 379–388 (2011).
41. van Essen, G. A. *et al.* Influenza symptoms and their impact on elderly adults: Randomised trial of AS03-adjuvanted or non-adjuvanted inactivated trivalent seasonal influenza vaccines. *Influenza Other Respi. Viruses* **8**, 452–462 (2014).
42. McElhaney, J.E. Influenza Vaccine Responses in Older Adults. *Ageing Res Rev.* July ; **10(3)**: 379–388 (2011)

43. Fujita, S., Volpi, E. Nutrition and sarcopenia of ageing. *Nutr Res Rev.* June ; **17(1)**: 69–76 (2004)
44. Kim, T. N. & Choi, K. M. Sarcopenia: definition, epidemiology, and pathophysiology. *J. bone Metab.* **20**, 1–10 (2013).
45. Ali, S., Garcia, J.M.. Sarcopenia, Cachexia and Aging: Diagnosis, Mechanisms and Therapeutic Options. *Gerontology.* **60(4)**: 294–305 (2014)
46. Guerreiro, R. & Bras, J. The age factor in Alzheimer’s disease. *Genome Med.* **7**, 1–3 (2015).
47. Yazdanyar, A., Newman, A.B.. The Burden of Cardiovascular Disease in the Elderly: Morbidity, Mortality, and Costs. *Clin Geriatr Med.* November ; **25(4)**: 563–vii (2009)
48. Derhovanessian, E. & Pawelec, G. Vaccination in the elderly. *Microb. Biotechnol.* **5**, 226–232 (2012).
49. Weinberger, B.. Vaccines for the elderly: current use and future challenges. *Immunity & Ageing* **15:3** (2018)
50. Shay, J.W., Wright, W.E.. Hayflick , his limit , and cellular ageing. *Nat MolCellBio.* **1**, 72–76 (2000).
51. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The hallmarks of aging. *Cell* **153**, (2013).
52. Chen, Z. *et al.* Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* **436**, 725–730 (2005).
53. Braig, M. *et al.* Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* **436**, 660–665 (2005).

54. Coppé, J.-P., Desprez, P.-Y., Krtolica, A. & Campisi, J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu. Rev. Pathol.* **5**, 99–118 (2010).
55. Ryan, G. & Majno, G. Acute inflammation. A review. *Am. J. Pathol.* **86**, 185–274 (1977).
56. Kumar, R., Clermont, G., Vodovotz, Y. & Chow, C. C. The dynamics of acute inflammation. *J. Theor. Biol.* **230**, 145–155 (2004).
57. Lawrence, T. & Gilroy, D. W. Chronic inflammation: A failure of resolution? *Int. J. Exp. Pathol.* **88**, 85–94 (2007).
58. Weltevrede, M., Eilers, R., de Melker, H. E. & van Baarle, D. Cytomegalovirus persistence and T-cell immunosenescence in people aged fifty and older: A systematic review. *Exp. Gerontol.* **77**, 87–95 (2016).
59. Deeks, S.G.. HIV Infection, Inflammation, Immunosenescence, and Aging. *Annu Rev Med.*; **62**: 141–155. (2011)
60. Childs, B.G., Durik, M., Baker, D.J., Deursen, J.M.V. Cellular senescence in aging and age-related disease: from mechanisms to therapy. *Nat Med.* December ; **21(12)**: 1424–1435 (2015)
61. Ruhland, M. K., Coussens, L. M. & Stewart, S. A. Senescence and cancer: An evolving inflammatory paradox. *Biochim. Biophys. Acta - Rev. Cancer* **1865**, 14–22 (2016).
62. Zhu, Y. *et al.* New agents that target senescent cells: The flavone, fisetin, and the BCL-XL inhibitors, A1331852 and A1155463. *Aging (Albany. NY)*. **9**, 1–9 (2017).
63. Baar, M. P. *et al.* Targeted Apoptosis of Senescent Cells Restores Tissue Homeostasis in Response to Chemotoxicity and Aging. *Cell* **169**, 132–147.e16 (2017).

64. Baar, M. P. *et al.* Targeted Apoptosis of Senescent Cells Restores Tissue Homeostasis in Response to Chemotoxicity and Aging. *Cell* **169**, 132–147.e16 (2017).
65. Tyrovolas, S. *et al.* Factors associated with components of arterial pressure among older individuals (the multinational medis study): The role of the Mediterranean diet and alcohol consumption. *J Clin Hypertension (Greenwich)* 2014
66. Pedersen, B.K. Anti-inflammation- just another word for anti-ageing? *J Physiol* 587.23 (2009) p 5515
67. Chaplin, D. D. Overview of immune response. *J Allergy Clin Immunol.* **125**, 41 (2010).
68. Stuart E. T., and David H. B.. CHAPTER 2 Innate Immunity. *Immunology* **125**, 1–4 (2006).
69. Mogensen, T. H. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin. Microbiol. Rev.* **22**, 240–273 (2009).
70. Bonilla, F. A. & Oettgen, H. C. Adaptive immunity. *J. Allergy Clin. Immunol.* **125**, S33–S40 (2010).
71. Liu, Y., Luo, J. & Bennett, C. Adaptive Immunity. Based on the dual recognition responses of $\alpha\beta$ T cells. **1**, *Landes Bioscience*. 62–66 (2010).
72. Netea, M. G., Quintin, J. & Van Der Meer, J. W. M. Trained immunity: A memory for innate host defense. *Cell Host Microbe* **9**, 355–361 (2011).
73. Netea, M.G. *et al.* Trained immunity: a program of innate immune memory in health and disease. *Science*. April 22; **352**(6284) (2016)
74. Roh, J.S. and Sohn, D.H.. Damage-Associated Molecular Patterns in Inflammatory Diseases. *Immune Netw.* 2018 Aug;18 (4)e27.

75. Kawai, T. & Akira, S. The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int. Immunol.* **21(4)**, 317-337 (2009).
76. Takeda, K. & Akira, S. Toll-like receptors in innate immunity. *Int. Immunol.* **17**, 1–14 (2005).
77. Medzhitov, R. TLR-mediated innate immune recognition. *Semin. Immunol.* **19**, 1–2 (2007).
78. Reimer-Michalski, E. M. & Conrath, U. Innate immune memory in plants. *Semin. Immunol.* **28**, 319–327 (2016).
79. van der Heijden, C. D. *et al.* Epigenetics and trained immunity. *Antioxid. Redox Signal.* **00**, ars.2017.7310 (2017).
80. Saeed, S. *et al.* Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science*. September **345**:6204 (2014)
81. Flajnik, M.F. and Kasahara, M.. Origin and evolution of the adaptive immune system: genetic events and selective pressures. *Nat Rev Genet.* January ; **11(1)**: 47–59. (2010)
82. MacLeod, M. K. L., Kappler, J. W. & Marrack, P. Memory CD4 T cells: Generation, reactivation and re-assignment. *Immunology* **130**, 10–15 (2010).
83. Riedel, S. Edward Jenner and the History of Smallpox and Vaccination. *Baylor Univ. Med. Cent. Proc.* **18**, 21–25 (2005).
84. Smith, K. A. Edward Jenner and the small pox vaccine. *Front. Immunol.* **2**, 1–6 (2011).
85. Pulendran, B. & Ahmed, R. Immunological mechanisms of vaccination. *Nat. Immunol.* **12**, 509–517 (2011).

86. Dillman, R. O. Cancer Immunotherapy. *Cancer Biother. Radiopharm.* **26**, 1–64 (2011).
87. Wraith, D. C. The future of immunotherapy: A 20-year perspective. *Front. Immunol.* **8**, (2017).
88. Davis, I. D. An overview of cancer immunotherapy. *Immunol. Cell Biol.* **78**, 179–195 (2000).
89. Farkona, S., Diamandis, E. P. & Blasutig, I. M. Cancer immunotherapy: The beginning of the end of cancer? *BMC Med.* **14**, 1–18 (2016).
90. Jiang, T. & Zhou, C. The past, present and future of immunotherapy against tumor. *Transl. lung cancer Res.* **4**, 253–64 (2015).
91. Pinti, M. *et al.* Aging of the immune system: Focus on inflammation and vaccination. *Eur. J. Immunol.* **46**, 2286–2301 (2016).
92. Goronzy, J. J. & Weyand, C. M. Understanding immunosenescence to improve responses to vaccines. *Nat. Immunol.* **14**, 428–436 (2013).
93. Lord, J. M. The effect of aging of the immune system on vaccination responses. *Hum. Vaccines Immunother.* **9**, 1364–1367 (2013).
94. Pawelec, G. Age and immunity: What is “immunosenescence”? *Exp. Gerontol.* **105**, 4–9 (2018).
95. Aw, D., Silva, A. B. & Palmer, D. B. Immunosenescence: Emerging challenges for an ageing population. *Immunology* **120**, 435–446 (2007).
96. Gruver, A.L., Hudson, L.L., Sempowski, G.D. Immunosenescence of ageing. *J Pathol*; **211**: 144–156 (2007)
97. Nikolich-Zugich, J. The Aging Immune System: Challenges for the 21st Century. *Semin Immunol.* October ; 24(5): 301–302 (2012)

98. Mahbub, S., Brubaker, A. L. & Kovacs, E. J. Aging of the Innate Immune System: An Update. *Curr. Immunol. Rev.* **7**, 104–115 (2011).
99. Panda, A. *et al.* Human innate Immunosenescence: causes and consequences for immunity in old age. *Trends Immunol.* July ; **30(7)**: 325–333. (2009)
100. Butcher, S. K., Wang, K., Lascelles, D. & Lord, J. M. Neutrophil ageing and immunosenescence. *NeuroImmune Biol.* **4**, 41–55 (2005).
101. Drew, W., Wilson, D. V. & Sapey, E. Inflammation and neutrophil immunosenescence in health and disease: Targeted treatments to improve clinical outcomes in the elderly. *Exp. Gerontol.* **105**, 70–77 (2018).
102. Chen, M.M *et al.* Age-Related Differences in the Neutrophil Response to Pulmonary Pseudomonas Infection. *Exp Gerontol.* June ; **0**: 42–46. (2014)
103. Linton, P. J. & Thoman, M. L. Immunosenescence in monocytes, macrophages, and dendritic cells: Lessons learned from the lung and heart. *Immunol. Lett.* **162**, 290–297 (2014).
104. Ong, S. M. *et al.* The pro-inflammatory phenotype of the human non-classical monocyte subset is attributed to senescence article. *Cell Death Dis.* **9**, 1–12 (2018).
105. Linehan, E. & Fitzgerald, D. Ageing and the immune system: focus on macrophages. *Eur. J. Microbiol. Immunol.* **5**, 14–24 (2015).
106. Stout, R. D. & Suttles, J. Immunosenescence and macrophage functional plasticity: Dysregulation of macrophage function by age-associated microenvironmental changes. *Immunol. Rev.* **205**, 60–71 (2005).
107. Gayoso, I. *et al.* Immunosenescence of human natural killer cells. *J. Innate Immun.* **3**, 337–343 (2011).

108. Tarazona, R. *et al.* Immunosenescence: limitations of natural killer cell-based cancer immunotherapy. *Cancer Immunol. Immunother.* **66**, 233–245 (2017).
109. Camous, X., Pera, A., Solana, R. & Larbi, A. NK cells in healthy aging and age-associated diseases. *J. Biomed. Biotechnol.* **2012**, (2012).
110. Feldman, N., Rotter-Maskowitz, A. and Okun, E.. DAMPs as mediators of sterile inflammation in aging-related pathologies. *Ageing Research Review* 24 (2015) 29–39
111. Huang, J. *et al.* DAMPs, Ageing, and Cancer: The “DAMP Hypothesis” *Ageing Res Rev.* 2015 November ; 24(0 0): 3–16.
112. Daniela, F. and Blomberg, B.B. Effects of aging on B cell function. *Curr Opin Immunol.* 2009 August ; 21(4): 425–430
113. Buffa, S. *et al.* B cell immunosenescence: Different features of naive and memory B cells in elderly. *Biogerontology* **12**, 473–483 (2011).
114. Bulati, M. *et al.* B cells and immunosenescence: A focus on IgG+IgD-CD27-(DN) B cells in aged humans. *Ageing Res. Rev.* **10**, 274–284 (2011).
115. Colonna-Romano, G. *et al.* B Cell Immunosenescence in the Elderly and in Centenarians. *Rej Res.* **Volume 11**, Number 2, (2008)
116. Cancro, M.P. *et al.* B cells and aging: molecules and mechanisms. *Trends Immunol.* July; **30(7)**: 313–318 (2009)
117. Robson, N. C., Beacock-Sharp, H., Donachie, A. M. & Mowat, A. M. The role of antigen-presenting cells and interleukin-12 in the priming of antigen-specific CD4+T cells by immune stimulating complexes. *Immunology* **110**, 95–104 (2003).
118. Luckheeram, R. V., Zhou, R., Verma, A. D. & Xia, B. CD4+T cells: Differentiation and functions. *Clin. Dev. Immunol.* **2012**, (2012).

119. Rothoeft, T., Gonschorek, A., Bartz, H., Anhenn, O. & Schauer, U. Antigen dose, type of antigen-presenting cell and time of differentiation contribute to the T helper 1/T helper 2 polarization of naive T cells. *Immunology* **110**, 430–439 (2003).
120. Golubovskaya, V. & Wu, L. Different subsets of T cells, memory, effector functions, and CAR-T immunotherapy. *Cancers (Basel)*. **8**, (2016).
121. Zhu, J., Yamane, H. & Paul, W. Differentiation of effector CD4 T cell populations. *Annu Rev Immunol.* **28**, 445–489 (2010).
122. Wan, Y. Y. & Flavell, R. a. How Diverse — CD 4 Effector T Cells and their Functions. *J. Mol. Cell Biol.* **1**, 20–36 (2009).
123. Wan, Y. Y. Multi-tasking of helper T cells. *Immunology* **130**, 166–171 (2010).
124. Xu, W. & Larbi, A. Markers of T cell senescence in humans. *Int. J. Mol. Sci.* **18**, 1–13 (2017).
125. Larbi, A. & Fulop, T. From “truly naïve” to “exhausted senescent” T cells: When markers predict functionality. *Cytom. Part A* **85**, 25–35 (2014).
126. Kared, H., Camous, X. & Larbi, A. T cells and their cytokines in persistent stimulation of the immune system. *Curr. Opin. Immunol.* **29**, 79–85 (2014).
127. Itano, A. A. & Jenkins, M. K. Antigen presentation to naive CD4 T cells in the lymph node. *Nat Immunol* **4**, 733–739 (2003).
128. Henson, S. M. & Akbar, A. N. KLRG1-more than a marker for T cell senescence. *Age (Omaha)*. **31**, 285–291 (2009).
129. Kared, H., Martelli, S., Ng, T. P., Pender, S. L. F. & Larbi, A. CD57 in human natural killer cells and T-lymphocytes. *Cancer Immunol. Immunother.* **65**, 441–452 (2016).

130. Focosi, D., Bestagno, M., Burrone, O. & Petrini, M. CD57+ T lymphocytes and functional immune deficiency. *J. Leukoc. Biol.* **87**, 107–116 (2010).
131. Onyema, O. O. *et al.* Cellular aging and senescence characteristics of human T-lymphocytes. *Biogerontology* **13**, 169–181 (2012).
132. Brenchley, J. M. *et al.* Expression of CD57 denotes replicative senescence and antigen-induced apoptotic death of CD8. **101**, 2711–2720 (2003).
133. Strioga, M., Pasukoniene, V. & Characiejus, D. CD8+CD28- and CD8+CD57+ T cells and their role in health and disease. *Immunology* **134**, 17–32 (2011).
134. Goods, B. A. *et al.* Functional differences between PD-1+ and PD-1- CD4+ effector T cells in healthy donors and patients with glioblastoma multiforme. *PLoS One* **12**, e0181538 (2017).
135. Dong, Y., Sun, Q. & Zhang, X. PD-1 and its ligands are important immune checkpoints in cancer. *Oncotarget* **8**, 2171–2186 (2015).
136. Simon, S. & Labarriere, N. PD-1 expression on tumor-specific T cells: Friend or foe for immunotherapy? *Oncoimmunology* **7**, 1–7 (2017).
137. Riley, J.L. PD-1 signaling in primary T cells. *Immunol Rev.* May ; **229(1)**: 114–125 (2009)
138. Wingender, G. & Kronenberg, M. OMIP-030: Characterization of human T cell subsets via surface markers. *Cytom. Part A* **87**, 1067–1069 (2015).
139. Wertheimer, a. M. *et al.* Aging and Cytomegalovirus Infection Differentially and Jointly Affect Distinct Circulating T Cell Subsets in Humans. *J. Immunol.* **192**, 2143–2155 (2014).
140. Maue, A. C. & Haynes, L. CD4+ T cells and immunosenescence - A mini-review. *Gerontology* **55**, 491–495 (2009).

141. Miles, D. J. C. *et al.* Cytomegalovirus Infection in Gambian Infants Leads to Profound CD8 T-Cell Differentiation. *J. Virol.* **81**, 5766–5776 (2007).
142. Miles, D. J. C. *et al.* Cytomegalovirus infection induces T-cell differentiation without impairing antigen-specific responses in Gambian infants. *Immunology* **124**, 388–400 (2008).
143. Pita-Lopez, M. L. *et al.* Effect of ageing on CMV-specific CD8 T cells from CMV seropositive healthy donors. *Immun. Ageing* **6**, 11 (2009).
144. Sokoya, T., Steel, H. C., Nieuwoudt, M. & Rossouw, T. M. HIV as a Cause of Immune Activation and Immunosenescence. *Mediators Inflamm.* **2017**, (2017).
145. Koch, S., Solana, R., Rosa, O. Dela & Pawelec, G. Human cytomegalovirus infection and T cell immunosenescence: A mini review. *Mech. Ageing Dev.* **127**, 538–543 (2006).
146. Tu, W. & Rao, S. Mechanisms underlying T cell immunosenescence: Aging and cytomegalovirus infection. *Front. Microbiol.* **7**, 1–12 (2016).
147. Molina-Pinelo, S. *et al.* Premature immunosenescence in HIV-infected patients on highly active antiretroviral therapy with low-level CD4 T cell repopulation. *J. Antimicrob. Chemother.* **64**, 579–588 (2009).
148. Dock, J. N. & Effros, R. B. Role of CD8 T Cell Replicative Senescence in Human Aging and in HIV-mediated Immunosenescence. *Aging Dis.* **2**, 382–397 (2011).
149. Elwenspoek, M. M. C. *et al.* T cell immunosenescence after early life adversity: Association with cytomegalovirus infection. *Front. Immunol.* **8**, 1–12 (2017).
150. Chou, J. P. & Effros, R. B. T cell replicative senescence in human aging. *Curr. Pharm. Des.* **19**, 1680–98 (2013).

151. Vallejo, A. N., Weyand, C. M. & Goronzy, J. J. T-cell senescence: A culprit of immune abnormalities in chronic inflammation and persistent infection. *Trends Mol. Med.* **10**, 119–124 (2004).
152. Aw, D. & Palmer, D. B. The origin and implication of thymic involution. *Aging Dis.* **2**, 437–43 (2011).
153. Palmer, D. B. The effect of age on thymic function. *Front. Immunol.* **4**, 1–6 (2013).
154. Gui, J., Mustachio, L. M., Su, D.-M. & Craig, R. W. Thymus Size and Age-related Thymic Involution: Early Programming, Sexual Dimorphism, Progenitors and Stroma. *Aging Dis.* **3**, 280–90 (2012).
155. Junge, S. *et al.* Correlation between recent thymic emigrants and CD31+(PECAM-1) CD4+T cells in normal individuals during aging and in lymphopenic children. *Eur. J. Immunol.* **37**, 3270–3280 (2007).
156. Vallejo, A. N. Age-dependent alterations of the T cell repertoire and functional diversity of T cells of the aged. *Immunol. Res.* **36**, 221–228 (2006).
157. Yager, E. J. *et al.* Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *J. Exp. Med.* **205**, 711–723 (2008).
158. Yoshida, K. *et al.* Aging-related changes in human T-cell repertoire over 20 years delineated by deep sequencing of peripheral T-cell receptors. *Exp. Gerontol.* **96**, 29–37 (2017).
159. Ferrando-Martínez, S. *et al.* Age-related deregulation of naive T cell homeostasis in elderly humans. *Age (Omaha)*. **33**, 197–207 (2011).
160. Goronzy, J.J. *et al.* Naïve T cell maintenance and function in human aging. *J Immunol.* May 1; **194**(9): 4073–4080 (2015)

161. Nikolich-Zugich, J. T cell aging: naive but not young. *J. Exp. Med.* **201**, 837–840 (2005).
162. Bektas, A., Schurman, S. H., Sen, R. & Ferrucci, L. Human T cell immunosenescence and inflammation in aging. *J. Leukoc. Biol.* **102**, jlb.3RI0716-335R (2017).
163. Riddell, N. E. *et al.* Multifunctional cytomegalovirus (CMV)-specific CD8⁺ T cells are not restricted by telomere-related senescence in young or old adults. *Immunology* **144**, 549–560 (2015).
164. Almanzar, G. *et al.* Long-term cytomegalovirus infections leads to significant changes in the composition of the CD8⁺ T-Cell Repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons. *JOURNAL OF VIROLOGY*, Mar. 2005, p. 3675–3683
165. Juno, J. A., Keynan, Y. & Fowke, K. R. Invariant NKT Cells: Regulation and Function during Viral Infection. *PLoS Pathog.* **8**, (2012).
166. Kjer-Nielsen, L. *et al.* An overview on the identification of MAIT cell antigens. *Immunol. Cell Biol.* (2018).
167. Gold, M.C. *et al.* MR1-restricted mucosal associated invariant T (MAIT) cells in the immune response to *Mycobacterium tuberculosis*. *Immunol Rev.* March ; **264(1)**: 154–166 (2015)
168. Seyda, M. *et al.* T cells going innate. *Trends Immunol.* August ; **37(8)**: 546–556. (2016)
169. Godfrey, D.I. *et al.* The burgeoning family of unconventional T cells. *Nat. Immunol.* **16** , 1114–1123 (2015)
170. Liuzzi, A. R., McLaren, J. E., Price, D. a & Eberl, M. Early innate responses to pathogens: pattern recognition by unconventional human T-cells. *Curr. Opin. Immunol.* **36**, 31–37 (2015).

171. Born, W. K., Reardon, C. L. & O'Brien, R. L. The function of $\gamma\delta$ T cells in innate immunity. *Curr. Opin. Immunol.* **18**, 31–38 (2006)
172. Hayday, A. C. $\gamma\delta$ Cells: A Right Time and a Right Place for a Conserved Third Way of Protection. *Annu. Rev. Immunol.* **18**, 975–1026 (2000).
173. Vantourout, P. & Hayday, A. Six-of-the-best: Unique contributions of $\gamma\delta$ T cells to immunology. *Nat. Rev. Immunol.* **13**, 88–100 (2013).
174. Zheng, J., Liu, Y., Lau, Y. L. & Tu, W. $\gamma\delta$ -T cells: An unpolished sword in human anti-infection immunity. *Cell. Mol. Immunol.* **10**, 50–57 (2013).
175. Lawand, M. *et al.* Key Features of Gamma-Delta T cell Subsets in Human Diseases and their immunotherapeutic implications. *Frontiers in Immunology*. June Volume 8 Article 761 (2017)
176. Adams, E. J., Gu, S. & Luoma, A. M. Human gamma delta T cells: evolution and ligand recognition. **296**, 31–40 (2016).
177. Witherden, D. A. & Havran, W. L. EPCR: A stress trigger for $\gamma\delta$ T cells. *Nat. Immunol.* **13**, 812–814 (2012).
178. Hayday, A. C. $\gamma\delta$ T Cells and the Lymphoid Stress-Surveillance Response. *Immunity* **31**, 184–196 (2009).
179. Modlin, R. L., Brenner, M. B., Bloom, B. R. & Morita, C. T. Nonpeptide ligands for human. **91**, 8175–8179 (1994).
180. Chien, Y., Jores, R. & Crowley, M. P. RECOGNITION BY γ / δ T CELLS. (1996).
181. Groh, V., Steinle, A., Bauer, S. & Spies, T. Recognition of stress-induced MHC molecules by intestinal epithelial $\gamma\delta$ T cells. *Science (80-.)*. **279**, 1737–1740 (1998).

182. Marlin, R. *et al.* Sensing of cell stress by human $\gamma\delta$ TCR-dependent recognition of annexin A2. *Proc. Natl. Acad. Sci.* **114**, 3163–3168 (2017).
183. Gober, H.-J. *et al.* Human T Cell Receptor $\gamma\delta$ Cells Recognize Endogenous Mevalonate Metabolites in Tumor Cells. *J. Exp. Med.* **197**, 163–168 (2003).
184. Puan, K.-J. *et al.* Preferential recognition of a microbial metabolite by human V γ V δ 2 T cells. *Int. Immunol.* **19**, 657–673 (2007).
185. Couzi, L. *et al.* Antibody-dependent anti-cytomegalovirus activity of human $\gamma\delta$ T cells expressing CD16 (Fc γ RIIIa). *Blood* **119**, 1418–1427 (2012).
186. Moser, B. & Brandes, M. $\gamma\delta$ T cells: An alternative type of professional APC. *Trends Immunol.* **27**, 112–118 (2006).
187. Wu, Y. *et al.* Human $\gamma\delta$ T Cells: A Lymphoid Lineage Cell Capable of Professional Phagocytosis. *J. Immunol.* **183**, 5622–5629 (2009).
188. Zhu, Y. *et al.* Human $\gamma\delta$ T Cells Augment Antigen Presentation in *Listeria* Monocytogenes Infection. *Mol. Med.* **22**, 1 (2016).
189. Himoudi, N. *et al.* Human $\gamma\delta$ T Lymphocytes Are Licensed for Professional Antigen Presentation by Interaction with Opsonized Target Cells. *J. Immunol.* **188**, 1708–1716 (2012).
190. Brandes, M. Willimann, K., Moser, B. Professional Antigen-Presentation Function by Human $\gamma\delta$ T cells. *Science*. July, **309**. (2005)
191. Dennis, M. K. *et al.* CD1d restriction and Th1/Th2/Th17 cytokine secretion by human V δ 3 T cells. *J. Immunol.* **188**, 358–366 (2012).
192. Petrasca, A., Melo, A. M., Breen, E. P. & Doherty, D. G. Human V δ 3+ $\gamma\delta$ T cells induce maturation and IgM secretion by B cells. *Immunol. Lett.* **196**, 126–134 (2018).

193. Willcox, C. R. *et al.* Cytomegalovirus and tumor stress surveillance by binding of a human $\gamma\delta$ T cell antigen receptor to endothelial protein C receptor. *Nat. Immunol.* **13**, 872–9 (2012).
194. Zhao, Y., Niu, C. and Cui, J. Gamma-delta T cells: friend or foe in cancer development? *J Transl Med* (2018) 16:3
195. Godfrey, D. I., Le Nours, J., Andrews, D. M., Uldrich, A. P. & Rossjohn, J. Unconventional T Cell Targets for Cancer Immunotherapy. *Immunity* **48**, 453–473 (2018).
196. Michishita, Y. *et al.* Age-associated alteration of $\gamma\delta$ T-cell repertoire and different profiles of activation-induced death of V δ 1 and V δ 2 T cells. *Int J Hematol* (2011) 94:230–240
197. Wistuba-Hamprecht, K., Frasca, D., Blomberg, B., Pawelec, G. & Derhovanessian, E. Age-associated alterations in gammadelta T-cells are present predominantly in individuals infected with Cytomegalovirus. *Immun Ageing* **10**, 26 (2013).
198. Roux, A. *et al.* Differential Impact of Age and Cytomegalovirus Infection on the γ/δ T Cell Compartment. *J. Immunol.* **191**, 1300–1306 (2013).
199. Wistuba-Hamprecht, K., Haehnel, K., Janssen, N., Demuth, I. & Pawelec, G. Peripheral blood T-cell signatures from high-resolution immune phenotyping of $\gamma\delta$ and $\alpha\beta$ T-cells in younger and older subjects in the Berlin Aging Study II. *Immun. Ageing* 1–12 (2015).
200. Ng, T. P. *et al.* Markers of T-cell senescence and physical frailty: insights from Singapore Longitudinal Ageing Studies. *npj Aging Mech. Dis.* **1**, 15005 (2015).
201. Simoni, Y. *et al.* Erratum: Human Innate Lymphoid Cell Subsets Possess Tissue-Type Based Heterogeneity in Phenotype and Frequency *Immunity* **48**, 1060 (2018).

202. Finck, R. *et al.* Normalization of mass cytometry data with bead standards. *Cytom. Part A* **83 A**, 483–494 (2013).
203. Gu, H. *et al.* Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. *Nat. Protoc.* **6**, 468–481 (2011).
204. Akalin, A. *et al.* MethyKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biol.* **13**, (2012).
205. Picelli, S. *et al.* Full-length RNA-seq from single cells using Smart-seq2. *Nat. Protoc.* **9**, 171–181 (2014).
206. Harrow, J. *et al.* GENCODE: The Reference Human Genome Annotation for The ENCODE Project. *Genome Res.* **22**, 1760–1774 (2012).
207. Andrews S. (2010). FastQC: a quality control tool for high throughput sequence data. Available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>
208. Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq quantification. *Nat. Biotechnol.* **34**, 525–527 (2016).
209. Sonesson, C., Love, M. I. & Robinson, M. D. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Research* **4**, 1521 (2016).
210. Li, B., Ruotti, V., Stewart, R. M., Thomson, J. A. & Dewey, C. N. RNA-Seq gene expression estimation with read mapping uncertainty. *Bioinformatics* **26**, 493–500 (2009).
211. Pavelka, N. *et al.* Aneuploidy confers quantitative proteome changes and phenotypic variation in budding yeast. *Nature*. November 11; **468(7321)**: 321–325 (2010)

212. Jones, L. *et al.* A subpopulation of high IL-21-producing CD4⁺T cells in Peyer's Patches is induced by the microbiota and regulates germinal centers. *Sci. Rep.* **6**, 1–16 (2016).
213. McLean, C.Y., *et al.* GREAT improves functional interpretation of cis-regulatory regions. *Nat Biotechnol.* May; **28(5)**: 495-501 (2010)
214. Dobin, A. *et al.* STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).
215. Liao, Y., Smyth, G. K. & Shi, W. FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–930 (2014).
216. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140 (2009).
217. Vasudev, A. *et al.* γ/δ T cell subsets in human aging using the classical α/β T cell model. *J. Leukoc. Biol.* **96**, 647–655 (2014).
218. Wistuba-Hamprecht, K., Pawelec, G. & Derhovanessian, E. OMIP-020: Phenotypic characterization of human γ/δ T-cells by multicolor flow cytometry. *Cytom. Part A* **85**, 522–524 (2014).
219. Moncunill, G. *et al.* OMIP-024: Pan-leukocyte immunophenotypic characterization of PBMC subsets in human samples. *Cytometry A*. December; **85(12)**: 995-998 (2014)
220. Do, J. *et al.* $\gamma\delta$ T Cells Coexpressing Gut Homing $\alpha 4\beta 7$ and αE Integrins Define a Novel Subset Promoting Intestinal Inflammation. *J. Immunol.* **198**, 908–915 (2017).

221. Mann, E. R. *et al.* Skin- and gut-homing molecules on human circulating $\gamma\delta$ T cells and their dysregulation in inflammatory bowel disease. *Clin. Exp. Immunol.* **170**, 122–130 (2012).
222. Tarazona, R. *et al.* Increased expression of NK cell markers on T lymphocytes in aging and chronic activation of the immune system reflects the accumulation of effector/senescent T cells. *Mech. Ageing Dev.* **121**, 77–88 (2001).
223. Horvath, S. DNA methylation age of human tissues and cell types. *Genome Biol* **14**, R115 (2013).
224. Durek, P. *et al.* Epigenomic Profiling of Human CD4⁺T Cells Supports a Linear Differentiation Model and Highlights Molecular Regulators of Memory Development. *Immunity* **45**, 1148–1161 (2016).
225. Moskowitz, D.M. *et al.* Epigenomics of human CD8 T cell differentiation and aging. *Sci Immunol.* February; **2(8)** (2017)
226. Jin, B., Li, Y. & Robertson, K. D. DNA methylation: Superior or subordinate in the epigenetic hierarchy? *Genes and Cancer* **2**, 607–617 (2011).
227. Hamilton, J. P. Epigenetics: Principles and practice. *Dig. Dis.* **29**, 130–135 (2011).
228. Verdin, E. NAD⁺ in aging, metabolism, and neurodegeneration. *Science (80-.).* **350**, (2015).
229. Boersma, V. *et al.* MAD2L2 controls DNA repair at telomeres and DNA breaks by inhibiting 5' end resection. *Nature.* 521,537-540 (2015)
230. Gebhardt, T. & Mackay, L. K. Local immunity by tissue-resident CD8⁺ memory T cells. *Front. Immunol.* **3**, 1–12 (2012).
231. Sathaliyawala, T. *et al.* Distribution and Compartmentalization of Human Circulating and Tissue-Resident Memory T Cell Subsets. *Immunity* **38**, 187–197 (2013).

232. Schenkel, J. M. & Masopust, D. Tissue-resident memory T cells. *Immunity* **41**, 886–897 (2014).
233. Gebhardt, T., Palendira, U., Tschärke, D. C. & Bedoui, S. Tissue-resident memory T cells in tissue homeostasis, persistent infection, and cancer surveillance. *Immunol. Rev.* **283**, 54–76 (2018).
234. Mueller, S. N. & Mackay, L. K. Tissue-resident memory T cells: local specialists in immune defence. *Nat. Rev. Immunol.* **16**, 1–11 (2015).
235. Teijaro, J. R. *et al.* Cutting Edge: Tissue-Retentive Lung Memory CD4 T Cells Mediate Optimal Protection to Respiratory Virus Infection. *J. Immunol.* **187**, 5510–5514 (2011).
236. Glennie, N. D. *et al.* Skin-resident memory CD4⁺ T cells enhance protection against *Leishmania major* infection. *J. Exp. Med.* **212**, 1405–1414 (2015).
237. Rosato, P. C., Beura, L. K. & Masopust, D. Tissue resident memory T cells and viral immunity. *Curr. Opin. Virol.* **22**, 44–50 (2017).
238. Ariotti, S., Haanen, J. B. & Schumacher, T. N. *Behavior and Function of Tissue-Resident Memory T cells. Advances in Immunology* **114**, (Elsevier Inc., 2012).
239. Thome, J.J.C. and Farber, D.L. Emerging concepts in tissue-resident T cells: lessons from humans. *Trends Immunol.* July ; **36(7)**: 428–435. (2015)
240. Kumar, B.V. *et al.* Human tissue-resident memory T cells are defined by core transcriptional and functional signatures in lymphoid and mucosal sites. *Cell Rep.* September 19; **20(12)**: 2921–2934 (2017)
241. Mackay, L. K. *et al.* Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. *Proc. Natl. Acad. Sci.* **109**, 7037–7042 (2012).

- 242. Topham, D. J. & Reilly, E. C. Tissue-Resident Memory CD8⁺ T Cells: From Phenotype to Function. *Front. Immunol.* **9**, (2018).
- 243. Shin, H. & Iwasaki, A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature* **491**, 463–467 (2012).
- 244. Clark, R. A. Resident memory T cells in human health and disease. *Sci. Transl. Med.* **7**, 269rv1-269rv1 (2015).
- 245. Blume-Peytavi, U. *et al.* Age-associated skin conditions and diseases: Current perspectives and future options. *Gerontologist* **56**, S230–S242 (2016).
- 246. Garcovich, S. *et al.* Skin Cancer Epidemics in the Elderly as An Emerging Issue in Geriatric Oncology. *Aging Dis.* **8**, 643–661 (2017).
- 247. Lanoy, E. & Engels, E. A. Skin cancers associated with autoimmune conditions among elderly adults. *Br. J. Cancer* **103**, 112–114 (2010).
- 248. Autengruber, A., Gereke, M., Hansen, G., Hennig, C. & Bruder, D. Impact of enzymatic tissue disintegration on the level of surface molecule expression and immune cell function. *Eur. J. Microbiol. Immunol.* **2**, 112–120 (2012).
- 249. Carbone, F. R., Mackay, L. K., Heath, W. R. & Gebhardt, T. Distinct resident and recirculating memory T cell subsets in non-lymphoid tissues. *Curr. Opin. Immunol.* **25**, 329–333 (2013).
- 250. Ryan, P. L. *et al.* Heterogeneous yet stable V δ 2⁽⁺⁾ T-cell profiles define distinct cytotoxic effector potentials in healthy human individuals. *Proc. Natl. Acad. Sci.* **2**, 201611098 (2016).
- 251. Davey, M. S. *et al.* Clonal selection in the human V δ 1 T cell repertoire indicates $\gamma\delta$ TCR-dependent adaptive immune surveillance. *Nat. Commun.* **8**, 14760 (2017).
- 252. Tze, C. *et al.* V δ 2⁺ and α/β T cells show divergent trajectories during human aging. *Oncotarget*, **7** (2016)

253. Mangino, M., Roederer, M., Beddall, M. H., Nestle, F. O. & Spector, T. D. Innate and adaptive immune traits are differentially affected by genetic and environmental factors. *Nat. Commun.* **8**, 1–7 (2017).
254. Eberl, M., Engel, R., Aberle, S., Fisch, P. & Jomaa, H. Human V γ 9 / V δ 2 effector memory T cells express the killer cell lectin-like receptor G1 (KLRG1) sent an unconventional lymphocyte population that cells comprise the majority of peripheral human $\gamma\delta$ **77**, 16–19 (2005).
255. De Rosa, S. C. *et al.* Ontogeny of $\gamma\delta$ T Cells in Humans. *J. Immunol.* **172**, 1637–1645 (2004).
256. Goldberg, E.L. and Dixit, V.D.. Drivers of age-related inflammation and strategies for healthspan extension. *Immunol Rev.* 2015 May: 265(1):63-74 (2015)
257. Laggner, U. *et al.* Identification of a novel proinflammatory human skin-homing V γ 9V δ 2 T cell subset with a potential role in psoriasis. *J Immunol.* 2011 Sep 1:187(5):2783-93 (2011)
258. Vukmanovic-Stejic, M. *et al.* The Characterization of Varicella Zoster Virus Specific T Cells In Skin and Blood During Ageing Europe PMC Funders Group. *J Invest Dermatol* **135**, 1752–1762 (2015).
259. Thome, J.J.C. *et al.* Spatial map of human T cell compartmentalization and maintenance over decades of life. *Cell.* November 6l 159(4): 814-828 (2014)
260. Jankovic, V., Messaoudi, I. & Nikolich-Zugich, J. Phenotypic and functional T-cell aging in rhesus macaques (*Macaca mulatta*): differential behavior of CD8 and CD4 subsets. *Blood* **102**, 3244–3251 (2003).
261. Thome, J. J. C. *et al.* Long-term maintenance of human nai ve T cells through in situ homeostasis in lymphoid tissue sites. *Sci. Immunol.* **1**, eaah6506-eaah6506 (2016).